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CELLULAR AND MOLECULAR BIOLOGY

Lutzomyia longipalpis: an update on this sand fly vector

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Abstract: *Lutzomyia longipalpis* is the most important vector of *Leishmania infantum*, the etiological agent of visceral leishmaniasis (VL) in the New World. It is a permissive vector susceptible to infection with several *Leishmania* species. One of the advantages that favors the study of this sand fly is the possibility of colonization in the laboratory. For this reason, several researchers around the world use this species as a model for different subjects including biology, insecticides testing, host-parasite interaction, physiology, genetics, proteomics, molecular biology, and saliva among others. In 2003, we published our first review (Soares & Turco 2003) on this vector covering several aspects of *Lu. longipalpis*. This current review summarizes what has been published between 2003-2020. During this period, modern approaches were incorporated following the development of more advanced and sensitive techniques to assess this sand fly.

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

INTRODUCTION

Lutzomyia longipalpis sensu lato Lutz & Neiva, 1912 is considered the main vector of *Leishmania infantum* Nicole, 1908 in the American continent (Lainson & Rangel 2005). This species is widely distributed, occurring in diverse ecological niches, such as dry habitats, humid forests but especially in urban and rural areas, where it has successfully established and spread itself (Ximenes et al. 2000, Souza et al. 2009b, Brazil 2013, Rodrigues et al. 2014, Dvorak et al. 2018).

Several components are involved in the urbanization and dispersion of *Lu. longipalpis* including climatic, environmental and sociocultural factors. This topic has been deeply reviewed by Salomón et al. (2015). Furthermore, the occurrence and the likely geographical distribution of this sand fly in Brazil has been predicted and modeled using geographic information systems and remote sensing (Andrade-Filho et al. 2017).

A great deal of information about *Lu. longipalpis* has already been reviewed by Soares & Turco (2003), therefore, here we discuss updates throughout the last decades on this sand fly vector, focusing on the information generated from 2003 to early 2020.

Lutzomyia Longipalpis SPECIES COMPLEX AND SEX PHEROMONES

Understanding the evolutionary history of *Lu. longipalpis,* as well as how geographical barriers and, more recently, anthropogenic environmental changes and activities have contributed to the evolution of sibling species continues to remain a challenge. Combined analyses using molecular markers and behavioral traits such as love songs and pheromones strongly suggest that *Lu. longipalpis* is a complex species, with distinct population structures as well as reproductively isolated populations (Arrivillaga et al. 2003,

2009, Hodgkinson et al. 2003, Bottecchia et al. 2004, Watts et al. 2005, Balbino et al. 2006, Bauzer et al. 2007, Araki et al. 2009). Details on the current status of the *Lu. longipalpis* species complex have been reviewed by Souza et al. (2017), especially regarding the historical overview, behavioral traits, courtship song and genetic characteristics of the group. Therefore, factors involved in mating have undoubtedly played (and continue to play) a significant role in maintaining reproductive isolation among the different sibling species.

The most current data on genetic diversity using several molecular markers of *Lu. longipalpis* indicate the presence of two clades: the first one is composed by Brazilian and Argentinian haplogroups and the second clade includes populations from Central America and northern South America (Guatemala, Honduras, Costa Rica, Colombia and Venezuela) (Pech-May et al. 2018). However, even belonging to the same clade, Argentinian and Brazilian populations present distinct genetic polymorphisms (Araki et al. 2009), resulting in separated populations (sub-clades) using a more refined analysis. A complex population structure of *Lu. longipalpis* from Brazil has been presented on a geographical scale by Casaril et al. (2019). The presence of geographical barriers may also contribute to divergence and the speciation process that seems to be occurring within the species complex. Genetic studies of *Lu. longipalpis* provide information about the heterogeneity of vector capacity/competence and vector susceptibility to insecticides as will be discussed later.

Based on the geographical distribution and pheromone types, it is predicted that (S)-9-methylgermacrene-B (9MGB) is the ancestral chemotype in *Lu. longipalpis* across South America, followed by subsequent speciation to either diterpenes (1S,3S,7R)-3-methyl-α-himachalene (3MαH) or cembrene (CEMB-1 or CEMB-2). To date, populations that produce diterpenes has been found only in Brazilian populations. All pheromone typed species in South and Central America, excluding Brazil, were 9MGB (Spiegel et al. 2016). Within the sibling species of *Lu. longipalpis* complex, *Lutzomyia pseudolongipalpis* from Venezuela produces 3MαH (Hamilton et al. 2005, Watts et al. 2005) and *Lutzomyia cruzi* from Corumbá, Mato Grosso do Sul state, Brazil, produces 9MGB (Vigoder et al. 2010). There is no information about the pheromone type produced by *Lutzomyia gaminarai*, a species endemic in the southern region of Brazil, occurring in the States of Paraná and Rio Grande do Sul (Galati 2018).

Several aspects on *Lu. longipalpis* complex still remains an open field to the investigators. Although most studies have focused on the genetic structure of the sibling species, description of pheromones and love songs, the female of *Lu. gaminarai* has not yet been formally described. Moreover, until today it is not clear how many species or incipient species within the *Lu. longipalpis*-complex exist in Brazil, or even which species is the original type, since the specimens used to describe this sand fly (Lutz & Neiva 1912) no longer exist. Furthermore, few specimens have been collected in Benjamin Constant, Minas Gerais State, Brazil, the type locality of *Lu. longipalpis* (Brazil et al. 2006), becoming one of the biggest bottlenecks on sand fly study, given the difficulty to establish a new species-type for the complex and consequently the description of sibling species.

Within the Brazilian populations of *Lu. longipalpis*, Araki et al. (2009) have proposed to segregate the species complex into two groups: the first one, more homogeneous, representing a single species in which males produce burst-type copulation songs and CEMB-1 pheromones; the other group, more heterogeneous, probably

represents incipient species that produce different combinations between pulse-type songs (five patterns of pulse-type) and pheromones such as 9MGB, 3MαH, CEMB-1 and CEMB-2, totaling at least six sibling species (Vigoder et al. 2015). Genetic evidence suggests that introgressive hybridization has been a crucial phenomenon of the recent speciation process that occurs within the *Lu. longipalpis* complex (Araki et al. 2013). Microsatellite data have shown limited genetic flow and introgression between *Lu. longipalpis* and *Lu. cruzi* in which the divergence level was similar to that observed among Brazilian populations of *Lu. longipalpis* (Vigoder et al. 2010, Lins et al. 2012, Santos et al. 2013). However, data from 12S rDNA sequencing did not differentiate *Lu. longipalpis* from *Lu. cruzi* (Corumbá), suggesting that the speciation process is recent or still occurring (Ribolla et al. 2016). Nevertheless, more genetic data is needed to confirm the occurrence of the recent speciation process between *Lu. longipalpis* and *Lu. cruzi*. Moreover, introgression patterns in the genome seem to have a relevant effect on transmission dynamics of *Leishmania* parasites. Therefore, exploring these aspects on *Lu. longipalpis* complex may be a good way to understand the vectorial capacity of the sibling species (Araki et al. 2013). Distinct genetic composition of populations from Espírito Santo, Brazil, seems to affect their susceptibility to *Leishmania* or even the capability to transmit the pathogen in an anthroponotic environment by the low adaptability of *Lu. longipalpis* to this environment (Rocha et al. 2011). Although a lot of papers have focused on establishing the genetic and pheromone variations in the *Lu. longipalpis* species complex, there is still a gap in how those variations affect interaction with *Le. infantum*. It would be extremely important to address the vectorial competence of a given *Lu. longipalpis* population. Although it is a permissive vector, intra-populations variability may result in a lack of interaction between the parasite and the vector. This was the case of allopatric populations of *Nyssomyia umbratilis* collected in the south and north of Negro River in the Amazon (Soares et al. 2018). In this paper, using the *in vitro* system, the authors observed that the south population was refractory to interaction with *Le. guyanensis*. However, we do not know if such a phenomenon would occur in *Lu. longipalpis* and this would be a very interesting direction of further molecular and biochemical studies.

The sympatric populations from Sobral, State of Ceará, Brazil, have been deeply studied, focusing on the genetic, evolutionary and epidemiologic significance of the one-pair-of-spots (S1) and two-pairs-of-spots (S2) male phenotypes of *Lu. longipalpis*. Lins et al. (2008) have identified a clear difference between these populations using the paralytic (*para*) gene as well as an association of the *para* and the resistance to pyrethroid insecticides. Further studies on genetic polymorphisms in period gene (*per*) have also suggested the presence of two sibling species in Sobral (Costa-Júnior et al. 2015). This data added crucial information about these reproductive isolated populations, suggesting the importance of premating barriers in *Lu. longipalpis* sibling species speciation (Maingon et al. 2003). Additionally, genetic divergence in the cacophony gene (*cac*) showed that S2 population is more related to Natal population (both produce burst type and CEMB-1) whereas S1 (pulse type 3 and 9MGB) was closer to Jacobina (pulse type 1 and 3MαH) and Lapinha (pulse type 2 and 9MGB). The genetic diversity observed in S1 and S2 may also reflect distinct physiological and behavioral aspects for both populations. However, until today, there is a lack of information on host-parasite interaction comparing sympatric populations

as S1 and S2. The genetic divergence between these populations may affect the interaction with *Le. infantum*. Although few variations have been observed, both males and females from the S2 population seem to initiate their crepuscular activity a little earlier than S1 (Rivas et al. 2008). However, more studies are needed to confirm distinct patterns of hourly activity as well as other differences in biological behavior between these populations. Besides their circadian rhythms, also the pheromones and patches were shown to affect bionomic aspects of *Lu. longipalpis*. Populations that produce homosesquiterpene (C16), such as sand flies from Jacobina (3MαH), Lapinha and Sobral one spot (1S) (both 9MGB) seems to be more easily adapted to the colonization conditions than the population whose males produces diterpenes (CEMB-1) such as the sand flies from Natal and Sobral two spots (2S) (Souza et al. 2009a). Since colonization is an important aspect that hinders sand fly studies, a better knowledge of those pheromones could also help to choose a more productive colony.

Finally, although most of the studies focused on patches occurred in Sobral, those phenotypes were also detected in other states. For example, S2 male phenotype was found in Jaíba (Minas Gerais State), Estrela de Alagoas (Alagoas State), Raposa and Codó (Maranhão State) (Araki et al. 2009). Consistent with the studies in Sobral, Silva et al. (2011) have shown genetic polymorphisms between Raposa and Codó sympatric populations, suggesting a clear segregation related to spot phenotypes (Lins et al. 2008, Costa-Júnior et al. 2015).

In conclusion, a large number of papers published before 2003 have addressed the pheromones and the genetic aspects of the *Lu. longipalpis* complex. Since 2003 those numbers have decreased, probably due to the acceptance of the species complex idea. How such variations affect the interaction with *Le. infantum* is an open field still needed to be explored by the investigators.

Lutzomyia Longipalpis CONTROL

Although nowadays it is still difficult to control the sand flies vector populations, important tools have been arising to improve the strategies, especially for VL control. The first problem is the difficulty to find the larval stages in the environment (Casanova 2001, Sangiorgi et al. 2012). In a field evaluation using an adulticide-larvicide mixture (100 mg of permethrin and 2 mg/m2 of pyriproxyfen), a significant decrease in the number of *Lu. longipalpis* was reported for at least two weeks (Juan et al. 2016). However, further studies are needed to evaluate the persistence of the residual effect of pyriproxyfen in controlling *Lu. longipalpis* larvae. For this reason, most of the studies have focused on the adult stages. Volatile compounds based on male pheromones and kairomones have demonstrated a good efficacy if used combined with automatic light traps improving catch rates, especially for *Lu. longipalpis*. Furthermore, synthetic pheromones can feasibly improve the efficacy of sand fly control programs when used alongside insecticides. This combined strategy attracts and kills both sexes, preventing host-seeking females from transmitting *Le. infantum* and males from establishing alternative aggregation sites elsewhere (Bray et al. 2009). A decrease in the number of sand flies attracted usually occurs as a consequence of insecticide treatments, however, the application of synthetic pheromones into insecticide-sprayed experimental sheds seems to prevent and reverse it, improving the catch rates of *Lu. longipalpis* (Bray et al. 2010). The number of pheromone-lures seems to have an influence

on the effectiveness of this strategy to attract sand flies. Bell et al. (2018) have shown that increasing the number of lures results in an upward trend in the number of sand flies that are caught in the field, especially males. Kairomones have been extensively used to attract hematophagous insects, such as mosquitoes and tse tse flies, however, there are few studies focusing on sand fly attraction. The compounds 1-octanol, a volatile component of bovine and human breath, and 1-nonanol, a volatile from cattle urine, elicited the highest attractiveness response in *Lu. longipalpis* adults in a dose-dependent manner (Magalhães-Junior et al. 2014). However, these alcohols have been identified at small levels in human breath or skin odors, which may justify the lack of interest in their potential role as an attractant for sand flies (Magalhães-Junior et al. 2014).

Although the chemical attraction has been the newest tool in this field, sand fly control programmes still often rely on spraying potential resting sites (intra or peridomiciliary sites) with residual insecticides, especially pyrethroids as lambda-cyhalothrin (Feliciangeli et al. 2003, Camargo-Neves et al. 2007a), deltamethrin (Santini et al. 2010), alpha cypermethrin (Pessoa et al. 2015) and permethrin (Alexander et al. 2009), with varying effectiveness. However, spraying also requires training to be conducted effectively, in order to ensure that the correct concentration of insecticide is applied, minimizing exposure to sub-lethal amounts which might promote the onset of resistance to several compounds. Denlinger et al. (2015) have quantified the insecticide susceptibility in laboratory-reared *Lu. longipalpis* to ten insecticides, comprising four chemical classes: pyrethroid, organophosphate, carbamate and organochlorine. The organophosphate insecticides caused delayed mortality in the sand fly population, while carbamate caused mortality faster. Both insecticides classes have similar modes of action, and, despite the differences in killing rates for carbamates and organophosphates, *Lu. longipalpis* are most susceptible to bendiocarb and propoxur carbamates as well as to the organophosphate fenitrothion (Denlinger et al. 2015). Furthermore, the doses for each insecticide have been determined using the CDC bottle bioassay to assess *Lu. longipalpis* resistance, providing starting points to test on field populations (Denlinger et al. 2016).

The use of insecticide-impregnated nets has also been used as a complementary tool for sand fly control, especially *Lu. longipalpis*. The entomological efficacy of 25% deltamethrin EC insecticide-treated bednets has been evaluated by Courtenay et al. (2007), in a crossover field study in Amazon Brazil (Marajó Island, State of Pará). Compared with untreated nets, the insecticide ones increased the barrier effect of the nets by 39%, reduced human landing rates by 80% and increased the 24 hours mortality rate (Courtenay et al. 2007). The lambda-cyhalothrin seems to have a short residual effect, whose efficacy declined to 74% after six months. On the other hand, permethrin-impregnated nets maintained its effectiveness close to 100% lethality 24 hours post exposure for at least a year under laboratory conditions (Bray & Hamilton 2013). However, those conditions may not be possible in the field. Trials using a variety of indoor and outdoor surfaces are needed to confirm the effectiveness of this netting-treatment protocol in the field, especially close to animal shelters.

A distinguished feature in *Lu. longipalpis* populations is their ability to respond differently to the action of pyrethroids and organophosphates. For example, Montes Claros sand flies were most susceptible to malathion, fenithorion and deltamethrin, while those from

Lapinha were most susceptible to cialotrhin, malathion and permethrin in laboratory conditions (Alexander et al. 2009). Moreover, a significant reduction in the susceptibility to the insecticides reinforced the importance of developing tools for detecting resistance (Alexander et al. 2009). Since the efficacy of insecticides differ within *Lu. longipalpis* populations, the combined use of insecticides may be a better strategy for the sand fly control. In this context, the repellent efficacy of a spot-on topical combination of fipronil and permethrin has been evaluated in dogs (Cutolo et al. 2018). A significant repellent effect against *Lu. longipalpis* as soon as it was applied on the dogs and high protection rates for 28 days has been shown. However, due to the short anti-feeding effect, regular application in dogs may hinder its protective effect in VL-endemic areas (Cutolo et al. 2018). Likewise, the 4% deltamethrin-impregnated canine collar (ICC) has not presented a long-lasting effect compared with spot-on topical repellents; however, the ICC is currently being considered as a relevant tool for VL control (Albuquerque e Silva et al. 2018). The ICC tends to reduce the prevalence of canine VL, in two basic ways: 1) reducing the blood feeding by the vector and, 2) reducing the vector population, mediated by repellent and insecticidal action of deltamethrin (Coura et al. 2019). The use of ICC reduced the number of *Lu. longipalpis* captured in an interventional area in Montes Claros, State of Minas Gerais (14% of reduction) and Fortaleza, State of Ceará (60% of reduction). Moreover, a 40% decrease in canine VL prevalence has been reported in both municipalities (Albuquerque e Silva et al. 2018). The anti-feeding effect of ICC has also been reported in Europe for *Phlebotomus perniciosus*, the vector of *Le. infantum* (Maroli et al. 2001, Manzillo et al. 2006, Ferroglio et al. 2008). Until today, there are few studies in Brazil that evaluate the efficacy of this strategy in the field. Longer follow-up studies on how ICC affects vector population and its impact on VL cases are needed. Considering the importance of protecting dogs from sand fly bites, it would be interesting to evaluate the potential role of mass use of ICC as a strategy to reduce canine visceral leishmaniasis incidence. However, the short-lasting effect, the need to frequently replace the ICC, and local symptoms in dogs, are still problems to be solved.

Although insecticide-based control measures are available for sand flies, there is still an urgent need for novel and alternative methods that do not affect or are less harmful to the environment. In this context, biological control could represent an important initiative for future studies. The combined use of chemical insecticides and selective pathogens may increase the efficiency of insect control. In this way, a possible alternative to current strategies may be the biological control of the vector using the entomopathogenic fungi *Beauveria bassiana*. Amóra et al. (2009) report that *Lu. longipalpis* eggs infected with this fungus reduced the hatching to 59%, suggesting a pathogenic potential on both larvae and adults. Moreover, *Metarhizium anisopliae* var. *acridum*, another entomopathogenic fungal, was harmful to sand flies in the adult stage (Amóra et al. 2010). Even in the laboratory, the studies on entomopathogenic fungi are very scarce. This reinforces the need for more studies on the impact-cost of such organisms while applying them in the field for controlling sand flies.

Several studies have investigated the use of plants to control vector-borne diseases. Plants from the Meliaceae family (*Azadirachta indica*) have been deeply studied due to their effects against many insects, especially those of agricultural importance. However, few studies have focused on sand flies. Few ovicidal and

larvicidal effects have been reported even in high concentration of *A. indica* oil when *Lu. longipalpis* eggs and larvae were treated in laboratory conditions (Maciel et al. 2010). On the other hand, the triterpenoid azadirachtin seems to block the metamorphosis when added to larval food of *Lu. longipalpis* (Andrade-Coelho et al. 2006). Studies have also showed that *A. indica* and *Melia azedarach* fruit and leaves *in natura* significantly increased larval mortality in comparison to untreated insects (Andrade-Coelho et al. 2009). Azadirachtin also seems to affect *Lu. longipalpis* oviposition and may increase the mortality in adults, indicating that azadirachtin may be a potent sterilizer that could be used against the development of *Lu. longipalpis* populations (Andrade-Coelho et al. 2014).

In conclusion, there are few field studies that have evaluated the impact of biological controls against sand fly vectors. Although distinct classes of insecticides are available, sand fly resistance has been reported in Brazil and other endemic countries (Surendran et al. 2005, Lins et al. 2008, Hassan et al. 2012). Thus, while studies on sand fly control are extremely relevant, other strategies than chemical control are necessary.

FOOD SOURCE IDENTIFICATION

During the past decades, several studies have been performed to identify the blood source of engorged females of potential and proven vectors such as *Lu. longipalpis*. Initially, the precipitin test was the most common technique to identify blood meal (Dias et al. 2003, Camargo-Neves et al. 2007b, Missawa et al. 2008) and ELISA (Marassá et al. 2006, Afonso et al. 2012). However, those techniques have some limitations, such as the need to know the previous local fauna and consequently obtain the specific antisera. Further, molecular methods (PCR and DNA sequencing) gradually replaced those techniques, improving blood meal identification by using *CytB* as universal primers (Sant'Anna et al. 2008, Soares et al. 2014, Carvalho et al. 2017b).

Lutzomyia longipalpis has broad-range feeding habits due to their adaptation to different habitats in both intradomiciliary and peridomiciliary sites. Several authors have reported that this vector fed on dogs, cats, pigs, cattles, horses, chickens and synanthropic vertebrates (rats and opossums). With the exception of chicken, most of the aforementioned hosts are potential reservoirs of *Leishmania* (Dias et al. 2003, Marassá et al. 2006, Camargo-Neves et al. 2007b, Missawa et al. 2008, Sant'Anna et al. 2008, Afonso et al. 2012, Soares et al. 2014, Carvalho et al. 2017b). Although chickens are refractory to *Leishmania* infection, Sant'Anna et al. (2010) have shown that, this vertebrate provides valuable blood sources to support the *Lu. longipalpis* population in peridomestic sites. The quality of chicken blood supports the development of transmissible *Leishmania* infections in *Lu. longipalpis* (Sant'Anna et al. 2010).

Besides blood, both females and males feed on plant-derived sugar meals as a source of energy. Sugary solutions such as nectar or honeydew (secreted by plant-sucking homopteran insects) and phloem sap are ingested by sand flies by probing plant tissues with their mouthparts. Many studies have addressed *Lu. longipalpis* plants preference. DNAs from Anacardiaceae, Meliaceae and Fabaceae families have been detected in the sand flies (Lima et al. 2016). More recently, the source of sand fly plant meals based on next generation sequencing (NGS) of chloroplast DNA gene ribulose bisphosphate carboxylase large chain (*rbcL*) was assessed. Interestingly, the

predilection of several sand fly species such as *Lu. longipalpis* for feeding on *Cannabis sativa*, a presumably illegal plant in some countries, was found (Abbasi et al. 2018). However, there is still a lack of knowledge on how specific sugars from plants may affect *Leishmania* development in sand flies. It is already known that besides functioning as a source of energy, sugars may also be used by *Leishmania* during its establishment in the midgut.

MIDGUT PHYSIOLOGY

The sand fly gut is divided into three main regions: the foregut, the midgut, and the hindgut. The cardia separates the foregut from the midgut and the pyloric valve separates the midgut from the hindgut (Bates 2008). Most studies have focused on host-parasite interaction of suprapylarian *Leishmania* species (Assis et al. 2012). This development is restricted to the portion of the gut anterior to the pylorus, mainly in the thoracic and abdominal midgut (Lainson & Shaw 1987). This is different from *Viannia* species whose development occurs in the hindgut prior to migration to anterior parts. On the other hand, the mode of the gut development is poorly recognized by the subgenera *Mundinia* and *Sauroleishmania* (Espinosa et al. 2018). For this reason, more studies on how species from these subgenera behave in their respective vectors are needed. In this context, an early study (Luz et al. 1967) reported a suprapylarian development for *Le. enriettii* in *Pintomyia monticola*, the suspected vector. However, this species, together with *Le. orientalis* did not developed very well in *Lu. longipalpis* (Seblova et al. 2015b, Chanmol et al. 2019). Thus, studies with their suspected vectors (phlebotomine sand flies and/or ceratopogonids) can help to clarify this subject and are fertile fields for entomologists.

Molecular studies have contributed to understanding the events that occur during the establishment of *Leishmania* infection in sand flies (Ramalho-Ortigão et al. 2010). *Leishmania* molecules such as LPG (Pimenta et al. 1994, Svárovská et al. 2010), which binds to the sand fly midgut galectin receptor PpGalec (Kamhawi et al. 2004), sand fly digestive enzymes (Borovsky & Schlein 1987, Schlein & Jacobson 1998, Sant'Anna et al. 2009, Telleria et al. 2010) and the peritrophic matrix (PM) (Pimenta et al. 1997) contribute to the success of the infection. The PM is a chitinous structure that envelopes the bloodmeal along the entire midgut, separating the ingested food from the midgut epithelium. In most sand flies, this structure is formed between 12-24 h after blood ingestion and degraded after 72h, when digestion is completed (Secundino et al. 2005, Sádlová & Volf 2009). For more information about the *Lu. longipalpis* PM structure, composition, degradation and synthesis kinetics see Secundino et al. (2005). The authors also have described a midgut muscle network of *Lu. longipalpis*.

The PM degradation after blood digestion requires the activity of chitinases, which cleave the chitin microfibril components of the matrix. Although *Leishmania* chitinase is believed to take part in the escape of the parasite from the PM, it is likely that a sand fly-derived chitinase may also be involved. Ramalho-Ortigão & Traub-Csekö (2003) have isolated and characterized a cDNA encoding a chitinase (*Llchit1*) from midgut of *Lu. longipalpis*. Messenger RNA expression indicates that this gene is induced upon blood feeding and reaches a peak at approximately 72h post blood meal, presuming that this sand fly chitinase has a function in PM degradation (Ramalho-Ortigão et al. 2005). Besides that, Ortigão-Farias et al. (2018) have shown that

alternative splicing generates chitinases with different domain structures. *LlChit1A* is present in adult females post blood meal, L4 larvae and pre-pupae, whereas *LlChit1B* and *LlChit1C* are found in L4 larvae and disappear just before pupation.

Serine proteases (trypsins and chymotrypsins) are the most abundant digestive enzymes in the midgut of sand flies. In addition to blood digestion, those proteases have been implicated in *Le. infantum* establishment in their respective insect vector, appearing to be detrimental to parasite survival during the first 48 hours prior to their increase after this period (Freitas et al. 2012). However, the same detrimental effect has not occurred in sand flies infected with *L. major* and *L. donovani*, suggesting that *Leishmania* mortality is not caused directly by sand fly proteases, but from toxic products of blood meal digestion. (Pruzinova et al. 2018). More studies are needed to better understand the effect of proteases on *Leishmania* establishment within sand flies. The opposite data may indicate distinct effects of proteases in *Leishmania* species. Sant'Anna et al. (2009) have reported that *Leishmania mexicana* was able to downregulate the trypsin secretion in *Lu. longipalpis* to its own advantage, promoting their establishment in the midgut. Likewise, a decrease of trypsin enzymatic activity in *Lu. longipalpis* infected by *Le. infantum* has been reported (Telleria et al. 2010). *Lutzomyia longipalpis* trypsin 1 gene knockdown through dsRNA microinjections into the thorax of females, seems to enhance the survival of *Le. mexicana* in comparison with mock-injected controls. Altogether, those data reinforce the inverse relationship between the expression and production of trypsin and the establishment of *Leishmania* in the sand fly midgut (Sant'Anna et al. 2009). Telleria et al. (2007) have identified and characterized two cDNAs, *Lltryp1* and *Lltryp2*, coding for trypsin-like proteins in *Lu. longipalpis*. *Lltryp1* expression remains undetected until blood feeding and reaches a peak at 12h post-blood meal, returning to pre-blood meal levels after 72h. *Lltryp2*, on the other hand, is constitutively expressed at high levels in the non-blood fed female but is reduced upon blood feeding. At the end of the digestive cycle, *Lltryp2* regains its pre-blood meal levels (Telleria et al. 2007). The pattern of trypsin expression in *Lu. longipalpis* differs from the results obtained for the Old-World species *Phlebotomus papatasi* (Ramalho-Ortigão & Traub-Csekö 2003). However, there is still lack of information on how proteases from *Ph. perniciosus* and from other natural vectors affect the development of *Le. infantum*. The transcriptome analysis has demonstrated that *L. infantum* infection can reduce the transcript abundance of trypsin PperTryp3 in the midgut of *Ph. perniciosus* (Dostálová et al. 2011). Although *Lu. longipalpis* and *Ph. perniciosus* may sustain infection with *Le. infantum*, the kinetics of proteases in those vectors in parallel are yet to be determined.

Studies on midgut pH as well as the mechanisms involved in pH control are extremely relevant, since *Leishmania* develops exclusively in the sand fly gut and the digestive processes are essentially enzymatic (Bates & Rogers 2004). There are three known mechanisms involved in the process of controlling gut pH. The first involves the loss of CO_2 from ingested blood and the transport of different ions through the plasmatic membrane of the enterocytes (Santos et al. 2008). Other physiological processes related to the alkalinization of the abdominal midgut involves the presence of blood in the abdominal midgut composed by proteins and amino acids. Those components cause midgut endocrine cells to release alkalinizing hormones, increasing gut pH favoring blood digestion (Santos et al.

2011). The third mechanism act involves Proton-Assisted Amino Acid Transporter (LuloPATs), removing H+ ions from the gut lumen into the cytoplasm of the enterocytes (Nepomuceno et al. 2020). However, alkalinization of the lumen may occur by the entry of some amino acids into the cytoplasm of enterocytes triggering a luminal alkalinization mechanism independent of LuloPATs (Nepomuceno et al. 2020). Some reports along the decades have shown the influence of *Leishmania* on sand fly physiology and such behavior most likely evolved to favor the development and transmission of the parasite. *Leishmania infantum* is able to reduce the alkalinization in the vector midgut, decreasing the activity of proteases like trypsin, resulting in a decreased supply of amino acids to the enterocytes favoring the development of the parasites during digestion (Santos et al. 2014).

There are few studies regarding midgut physiology of *Lu. longipalpis* larvae. The anatomy of the digestive tube of *Lu. longipalpis* larvae as well as the pH along the midgut have been described in Vale et al. (2007). The carbohydrases α-amylase, present in the anterior midgut and probably involved in the digestion of glycogen; α-glucosidase, that completes the digestion of glycogen in the posterior midgut, and a membrane bound trehalase, that probably acts in the digestion of trehalose, seems to be the most abundant within the midgut of the larvae (Moraes et al. 2012, Vale et al. 2012). The expression pattern of glycoside hydrolase genes in *Lu. longipalpis* larvae have been described by Moraes et al. (2014), where the catabolism of microbial carbohydrates in insects generally involves β-1,3-glucanases, chitinases and digestive lysozymes. This is interesting because *Le. infantum* LPG possess terminal β-1,3-glucoses that could be cleaved by those enzymes and perhaps contribute to the sand fly midgut sugar milieu (Soares et al. 2002, Coelho-Finamore et al. 2011).

Early studies of Elnaiem have already focused on the effect of a second blood meal in the development of *Lu. longipalpis* (Elnaiem et al. 1992, 1994). Nowadays, most of the studies are interested in how a second bloodmeal affects *Leishmania* development. In this context, the effects of sequential blood meals on longevity, protein digestion, trypsin activity and *Leishmania* development within *Lu. longipalpis* midgut have been recently evaluated. The mortality of blood-fed females increases after a second blood meal as compared to sugar-fed females and the trypsin activity was lower during the second gonotrophic cycle (Moraes et al. 2018). The authors have not observed difference in the population size of *Leishmania* in the gut with sequential blood meals. However, Serafim et al. (2018) have reported that sequential blood meals promoted *Leishmania* replication and reversed metacyclogenesis to a leptomonad-like stage, the retroleptomonad promastigote, enhancing the *Lu. longipalpis* infectivity. Needless to say, this paper was a landmark study, bringing new information on parasite development after a second blood meal.

Salivary proteins

In general, female sand flies, except autogenic species, need to ingest blood for egg development and sugar for energy metabolism. Saliva is essential in both types of feeding, playing different roles since it contains sets of enzymes for blood and sugar feeding, as α-amylase (Cavalcante et al. 2006). Early studies by Volf have shown the effect of salivary gland proteins in Old World sand flies, in which the composition of sand fly saliva depend not only on sex, but also on the physiological state of the female (Volf et al. 2000). The salivary protein composition of *Lu. longipalpis* also depends on

age and diet (Prates et al. 2008). The protein content from unfed sand flies increased 94% from the first to the fifth day after emergence and such variation can be related to the synthesis of important enzymes for meal ingestion and initial digestion (Prates et al. 2008). A kinetic of protein content in salivary glands seems to occur after the blood meal, in which a depletion of total protein content has been observed with gradual increase in subsequent days, returning to similar basal values (Prates et al. 2008). The findings of Volf for Old World sand flies and the further records of Prates for New World ones could be generalized for sand flies worldwide, in view of the salivary content appears to follow the same pattern in several sand fly species.

Blood-feeding causes tissue damage creating a hemorrhagic pool resulting from probing and destruction of small capillaries. In this environment *Leishmania* and saliva interact with different host cells including peripheral blood and resident cells in the skin (Vasconcelos et al. 2014). It has been well documented that sand fly saliva possesses an array of potent pharmacological components, such as anticoagulants, anti-platelet, vasodilators, immunomodulators and anti-inflammatory molecules. For more details about the inflammatory role of *Lu. longipalpis* saliva in leishmaniasis see Prates et al. (2012).

To know the effect of these molecules, most studies on sand fly saliva have used experimental animals (mice), and to a lesser extent human cell. Salivary gland homogenates (SGH) of *Lu. longipalpis* induce an increase of IL-6, IL-8 and IL-12p40 and inhibits TNF-α and IL-10 production by human monocytes. SGH have also influenced the expression of cell surface molecules such as MHC class II, CD80 and CD86 on antigen-presenting cells, except on dendritic cells, representing a critical point for the development of a protective Tcell response (Costa et al. 2004). Moreover, SGH seem to increase the IL-17 expression in human peripheral blood mononuclear cells (Teixeira et al. 2018). Human volunteers exposed to laboratory-reared *Lu. longipalpis* bites developed both humoral and cell-mediated immune response against sand fly saliva, presenting increased frequency of CD4+CD25+ and CD8+CD25+ T cells as well as IFN-γ and IL-10 synthesis (Vinhas et al. 2007) and moreover, inducing heme oxygenase-1 expression at bite site (Luz et al. 2018). These studies confirm powerful immunomodulatory properties of saliva and help clarify how *Leishmania* takes advantage of them during the bite.

BALB/c mice exposed to repeated *Lu. longipalpis* bites have developed a diffuse inflammatory infiltrate characterized by neutrophils, eosinophils, and macrophages when challenged with SGH (Silva et al. 2005). Antibodies anti-saliva have also been detected in exposed mice, that presented significant increase of IgG and IgG1, but not IgG2a or IgG2b, suggesting a predominant Th2 response with a putative role for immune complexes in cell recruitment (Silva et al. 2005). *Lu. longipalpis* saliva is also capable of inducing neutrophil and macrophage recruitment and of modulating their function (Silva et al. 2005, Teixeira et al. 2005, Araújo-Santos et al. 2010, Prates et al. 2011, Carregaro et al. 2013). Neutrophil and macrophage activity seem to be impaired in the presence of saliva resulting in cell apoptosis, production of PGE2 and LTB4 promoting increased parasite survival (Monteiro et al. 2005, Araújo-Santos et al. 2010, Prates et al. 2011). *Lutzomyia longipalpis* saliva enhances *Le. amazonensis* infection affecting the macrophage function by upregulation of IL-10 and downregulation of NO production (Norsworthy et al. 2004). The same regulation pattern of immune response has been described in BALB/c mice experimentally infected with *Le.*

major, in which a considerable increase of IL-10 and IFN-γ was detected, inducing preferentially type-2 cytokines and the sequential migration of neutrophils, eosinophils, and CD4+ $CD45RB^{low}$ cells (Monteiro et al. 2007). However, Laurenti et al. (2009) have reported that SGH from wild-caught *Lu. longipalpis* have determined lower production of IL-4 and IL-10 but higher IL-12 levels in C57BL/6 compared with laboratory-reared SGH. These findings may indicate a probable bias by using SGH from laboratory-colonized sand flies instead of wild-caught vector SGH. In addition, it indicates differences on immune response of the most used experimental models for studies concerning saliva effects (Laurenti et al 2009). However, it is important to note that sand flies also inject their microbiota together with the salivary content, and the presence of distinct bacteria within laboratory-colonized sand flies compared to wild-caught ones, can also influence the immune response. The presence of SGH from *Lu. longipalpis* was able to differentially modulate the course of the lesion and macrophage differentiation in *Cavia porcellus* caused by avirulent and virulent *Le. enriettii* strains (Pinheiro et al. 2018). Several basic studies, especially those that used needle models, were very important for understanding the *Leishmania* infection. However, there is an urgent need that from now on, transmission needle studies use saliva at least from a colonized sand fly vector. Although, for obvious reasons, it is not possible to use the natural pairs depending on the *Leishmania* species, most of the properties of the saliva of different sand flies share similar effects.

Most of the studies above have used SGH, but it seems that the search for specific molecules has been the target for by several groups. Consistent with this observation, the structure and function of LJM11 has been described by Xu et al. (2011). A protective immunity driving a strong Th1 type immune response was observed in immunized C57BL/6 mice infected with *Le. major* (Xu et al. 2011) and in BALB/c mice infected with *Le. braziliensis* (Cunha et al. 2018). Immunization with salivary protein LJM19 induced protection in hamsters challenged with *Le. braziliensis* (Tavares et al. 2011). The presence of smaller lesion sizes as well as reduced parasite burdens both at lesion sites and in the draining lymph nodes, was associated with a significant decrease in the expression levels of IL-10 and TGF-β and increased IFN-γ expression have been reported (Tavares et al. 2011). Both LJM17 and LJL143-immunized dogs have presented a mixed (Th1/Th2) immune response and moreover, increased IFN-γ production (Abbehusen et al. 2018), providing immune responses qualitatively similar to those previously obtained by Collin et al. (2009). Although knowing specifically the activity of a given molecule, the use of several antigens that do not exhibit antagonistic properties could help the development of more potent saliva-based vaccines.

Valenzuela et al. (2004) have isolated and identified the most abundant secreted proteins from the salivary glands of *Lu. longipalpis* using massive cDNA sequencing, proteomics and customized computational biology approaches. However, several proteins coded by their corresponding salivary gland transcripts remain without a defined function until today (Valenzuela et al. 2004, Anderson et al. 2006). Likewise, some biological functions described in the salivary gland have not been associated with a specific protein. For example, the anticoagulant of *Lu. longipalpis* remained elusive for decades until Collin et al. (2012) describe Lufaxin (*Lutzomyia longipalpis* Factor Xa inhibitor). This recombinant protein has potent and specific anticoagulant activity toward FXa, impairing protease-activated receptor 2 activation and, consequently inhibiting the

inflammation and thrombosis in C57BL/6 mice. New insights of recombinant hyaluronidase (LuloHya) and *Lutzomyia* NET destroying protein (Lundep), the proteins responsible for the hyaluronidase and endonuclease activities have been described (Chagas et al. 2014, Martin-Martin et al. 2018). Lundep seems to increase *Le. major* survival, destroy neutrophil traps and inhibits XIIa contact activation in human plasma. The relationship between *Leishmania* parasites and sand flies hyaluronidase was first described by Volfova et al. (2008). The authors have shown that co-inoculation of parasites with hyaluronidase enhances *Leishmania* infection. Altogether, those data indicate that saliva is an endless subject and several factors are still to be defined and how to block those molecules is an open field for alternative tools against transmission.

Lutzomyia longipalpis is able to feed on several mammal and bird species (Afonso et al. 2012). For this reason, an arsenal of complement inhibitors is needed to protect this species. In this context, *Lu. longipalpis* saliva was able to inhibit the serum complement activation from a wide range of vertebrates, including dogs, guinea pigs and rats (Mendes-Sousa et al. 2013). Studies involving the human complement inhibition by *Lu. longipalpis* saliva have shown at least two inhibitors of the classical pathway in this species. The first is a Salivary Anti-complement from *Lu. longipalpis* (SALO) (Ferreira et al. 2016), considered a leishmaniasis vaccine candidate (Asojo et al. 2017) and the second, a soluble intestinal inhibitor (Saab et al. 2020).

One of the most studied salivary peptides is the potent vasodilator maxadilan (MAX). MAX also seems immuno-modulate the host immune response. MAX treatment reduced the surface expression of CD80 on $CD11c^{+}$ dendritic cells and resulted in a concomitant increase in CD86 expression on a subpopulation of these cells. Moreover, MAX seemed to upregulate the cytokines associated with a type-2 response (IL-10, IL-6, and TGF-β) and downregulated type-1 cytokines (IL-12p70 and TNF-α), NO and CCR7. This enhanced parasite survival in the vertebrate host in the early stages of infection (Brodie et al. 2007, Wheat et al. 2008). MAX was also able to drive plasma leakage via PAC1–CXCR1/2-pathway (Svensjö et al. 2009, 2012). A protective effect against *Le. major* infection in murine models has also been reported for MAX (Wheat et al. 2017).

Anti-saliva antibodies can be used to assess exposure of humans and other *Leishmania* hosts to sand fly bites (Rohousova et al. 2005, Bahia et al. 2007, Vinhas et al. 2007, Hostomska et al. 2008, Fraga et al. 2016). These anti-saliva antibodies seem to be species-specific as shown by Volf & Rohousova (2001) and Rohousova et al. (2005). The antibodies of hosts bitten by Old-World sand flies did not cross-react with *Lu. longipalpis* SGH. Therefore, this specificity of anti-saliva antibodies enables to measure/estimate the exposure to a particular species. Also, the protective effect of immunization by saliva have been species-specific as shown by Thiakaki et al. (2005): mice have been protected against co-inoculation of *Leishmania* with *Lu. longipalpis* saliva only if they were preimmunized by SGL of *Lu. longipalpis* but not if preimmunized by SGL of *Phlebotomus* species. Nine recombinant salivary proteins were developed and tested for immunogenicity and specificity in mammalian hosts (Teixeira et al. 2010). The recombinant proteins LJM17 and LJM11, both belonging to the insect "yellow" family of proteins, were potential markers of exposure to sand fly bite (Souza et al. 2010). LJM17 was recognized by human, dog, and fox sera and LJM11 by humans and dogs. Notably, LJM17 and LJM11 were specifically recognized by humans exposed to *Lu. longipalpis* but not by individuals exposed

to *Nyssomyia intermedia* (Teixeira et al. 2010). A recent paper has shown that one of the salivary proteins of *Ny. intermedia*, LinB-13, could be a useful marker for the development of a more severe cutaneous leishmaniasis (Carvalho et al. 2017a). This study opens the possibility that similar mechanisms could also happen in the viscerotropic *Leishmania* species transmitted by *Lu. longipalpis*, especially in canine infection, that is a very susceptible host compared to humans.

HOST-PATHOGEN INTERACTIONS

Laboratory studies on sand fly competence to *Leishmania* parasites suggest that the sand flies fall into two groups. Several species are termed specific/restricted vectors that support the development of one *Leishmania* species. On the other hand, permissive vectors are susceptible to various *Leishmania* parasites (Volf & Myskova 2007, Dostálová & Volf 2012). The presence of the permissive vector *Lu. longipalpis* in Latin America was crucial for the establishment of *L. infantum* from Mediterranean to this continent (Volf & Myskova 2007). Another factor that seems to affect the establishment of *Leishmania* in sand flies is the temperature. *Leishmania infantum* and *Le. braziliensis* have developed well in *Lu. longipalpis* at 20 and 26 degrees C, while *Le. peruviana*, a mountain species, developed well in sand fly females kept at 20 degrees C (Hlavacova et al. 2013). Previous studies have suggested that for 'specific' vectors, successful parasite development is mediated by parasite surface glycoconjugates and sand fly lectins. However, Myšková et al. (2007) have shown that interactions involving 'permissive' vectors, as *Lu. longipalpis* utilize other molecules of the midgut epithelium as a parasite ligand. The *Helix pomatia* agglutinin (HPA), a lectin specific for terminal N-acetyl-galactosamine (GalNAc) present on O-linked glycoconjugates, bound to midgut proteins from permissive but not from specific vectors (Myšková et al. 2007). The characterization of O-linked glycoconjugate of *Lu. longipalpis* has revealed the presence of mucin-like properties, GPI-anchored in the membrane of enterocytes and localized it on the luminal side of the midgut (Myšková et al. 2016).

As *Leishmania* undergo metacyclogenesis and acquire infectivity within the sand fly gut, they secrete a unique class of serine-rich proteophosphoglycans (PPGs); which condense to form a gel in which the parasites are embedded (Rogers & Bates 2007). PPGs are synthesized by all species of *Leishmania in vitro* and the promastigote secretory gel (PSG) has been observed in all *Leishmania*-sand fly combinations examined to date. The *Le. infantum* PPGs regurgitated by the bite of *Lu. longipalpis* promote parasite establishment in mouse skin and skin-distant tissues, reinforcing PSG as an important part of *Le. infantum* transmission and visceral infection (Rogers et al. 2010). The binding of *Leishmania* promastigotes to the midgut epithelium is regarded as an essential part of the lifecycle in the sand fly vector, enabling the parasites to persist beyond the initial blood meal phase and establish the infection. Wilson et al. (2010) have shown that *Leishmania* gut binding is strictly stage-dependent and is a property of those forms found in the middle phase of development (nectomonad and leptomonad forms) but is absent in the early blood meal and final stages (procyclic and metacyclic forms). Furthermore, the adhesion is affected by glycoconjugates on *Leishmania* surface, especially LPG and gp63 (Jecna et al. 2013).

Significant advances have been made in exploring *Leishmania*-vector interactions throughout the last two decades, especially

on permissiveness of *Lu. longipalpis*. The development of *Le. infantum* from establishment of infection to metacyclogenesis as well as the transmission dynamics by the bite to BALB/c mice and golden hamster have been described (Maia et al. 2011, Freitas et al. 2012, Secundino et al. 2012). For the first time *Ph. perniciosus* and *Lu. longipalpis* have been co-infected with transgenic promastigotes of *Le. donovani* strains carrying hygromycin or neomycin resistance genes (Sadlova et al. 2011). Seblova et al. (2015a) have tested the development of *Le. infantum*/*Leishmania donovani* natural hybrid (CUK strain) in *Lu. longipalpis* and the biological behavior appeared similar to what has been observed in the natural vector *Phlebotomus tobbi*. The phenotype impact of miltefosine-resistant *Le. infantum* has been evaluated on *Lu. longipalpis* showing a significant reduction in sand fly infection, stomodeal valve colonization and differentiation into metacyclic forms compared to the isogenic parent susceptible strain (Bockstal et al. 2019). Paromomycin-resistant *Le. infantum* (MHOM/FR/96/LEM3323-cl4) has behaved similar to those WT, in terms of infection and parasite location within *Lu. longipalpis*, and are able to colonize the stomodeal valve with metacyclic forms (Hendrickx et al. 2020). However, the mechanisms underlying drug-resistance phenotype during infection in the sand fly are yet to be determined.

In laboratory conditions *Lu. longipalpis* supports infection of other *Leishmania* species, besides *Le. infantum*. However, aflagellated *Le. amazonensis* promastigotes (Ld ARL-3A-Q70L-overexpressing) did not survive in experimentally infected *Lu. longipalpis*, in contrast to untransfected or native Ld ARL-3A overexpressing cells (Cuvillier et al. 2003). The role of *Leishmania* flagellar proteins in establishment of the parasite in the vector have been recently explored by Beneke et al. (2019). In mixed infections of the permissive sand fly *Lu. longipalpis*, paralyzed promastigotes and uncoordinated swimmers of *Le. mexicana* were severely diminished in the sand fly after the blood digestion. Furthermore, the parasites have not reached the anterior regions of the midgut, suggesting that *L. mexicana* needs directional motility for successful colonization of sand flies (Beneke et al. 2019). The relationship between the zinc protease gp63 and the parasite development in the sand fly vector has been evaluated (Hajmová et al. 2004). *Leishmania amazonensis* gp63-downregulated have presented a weak development especially in the early phase of infection, indicating that gp63 may protect promastigotes from degradation by the midgut digestive enzymes, favoring parasite survival. More recently, trying to understand the concomitant roles of gp63 and LPG, Soares et al. (2017) evaluated those two glycoconjugates using the midgut *in vitro* system (Pimenta et al. 1992) and LL5 cells. Both glycoconjugates were equally responsible for inhibiting parasite attachment in those models reinforcing their importance for interaction with the invertebrate host.

Parasites of the subgenus *Leishmania* (*Mundinia*) (Espinosa et al. 2018) are becoming increasingly important to human health, since some species have been reported to infect humans, such as *Le. martiniquensis*, *Le.* "Ghana strain", and *Le. orientalis* (previously called "*Le. siamensis*") (Pothirat et al. 2014, Chiewchanvit et al. 2015, Kwakye-Nuako et al. 2015, Jariyapan et al. 2018). The two other known species, *Le. enrietti*, have been found in guinea pigs (*Cavia porcellus*), and *Le. macropodum* (previously called "*Le.* sp. AM-2004"), have been found in red kangaroos and other macropods (Rose et al. 2004, Dougall et al. 2011, Barratt et al. 2017). Some authors have evaluated the biological behavior of *Leishmania (Mundinia)* parasites in permissive vectors, such as *Lu. longipalpis* in view of the uncertainty

about the probable natural vector. Seblova et al. (2015b) have described that both *Le. enrietti* and *Le. macropodum* were able to develop late-stage infections in *Culicoides sonorensis* and *Lu. longipalpis*. However, *Le. orientalis* was able to establish infection in *Cu. sonorensis* midges but not in *Lu. longipalpis* (Chanmol et al. 2019), suggesting that the biting midges might be natural vectors of some *Leishmania (Mundinia)* species. This is of importance, because those insects were once not considered as vectors of *Leishmaniasis*. However, *Cu. sonorensis* achieved 5 out 6 criteria of Killick-Kendrick (1990) in the work of Dougall et al. (2011). Still, transmission is yet to be demonstrated for those *Mundinia* species (Paranaiba et al. 2017).

Insects cell lines have been used as a value tool to understand host-parasite interactions *in vitro*. There is two established *Lu. longipalpis* cell lines derived from embryonic tissues, LL5 (Tesh & Modi 1983) and Lulo (Rey et al. 2000). When LL5 cells were transfected with double stranded RNA (dsRNAs), they developed a nonspecific antiviral response (Pitaluga et al. 2008). Secreted molecules implicated in immune response in LL5 cell line have been described, such as phospholipid scramblase, an interferon-inducible protein and forskolin-binding protein, a member of the immunophilin family (Martins-da-Silva et al. 2018). A complex immune response in LL5 line cell has also been detected when challenged by different pathogens, as bacteria, yeast and *Leishmania* (Tinoco-Nunes et al. 2016). The Lulo cell line can be infected by *Le. infantum* (Bello et al. 2005) and moreover, other *Leishmania* species were also able to adhere to Lulo cells at different rates (Côrtes et al. 2011). The mechanisms involved in the adhesion of parasites to Lulo cells remains unclear. Côrtes et al. (2012) have described the participation of heparin binding proteins from the surface of *Le. braziliensis* promastigotes to Lulo cells, by their glycosaminoglycans, through heparan sulfate participation. However, lectin-like activity specific for heparin has been previously described by (Svobodová et al. 1997). Although the development of those cells could help to understand some aspects of the interaction of the parasites, there are few published papers using those models in the past years or replacement for *in vivo* studies have decreased their use along the years.

Finally, the presence of naturally infected sand fly by non-*Leishmania* trypanosomatids and other microorganisms have been reported throughout the last decades, reinforcing the role of these insects as multi-pathogens host (Shaw et al. 2003). Despite this fact, there is a lack of information about the biological behavior and infectivity of these pathogens in sand flies. Flagellates of *Endotrypanum schaudinni* were able to infect the abdominal midgut, pylorus, ileum, and rectal ampulla but a scarcity of infection has been observed near the stomodeal valve in *Lu. longipalpis* (Barbosa et al. 2006). Moreover, the presence of *Le. guyanensis* in a mixed infection has inhibited the development of *Endotrypanum*, suggesting the effect of selective pressures that have already been reported previously, among co-cultivated trypanosomatids (Barbosa et al. 2006). Also, *Lu. longipalpis* seems to be the host for gregarines, fungi and nematodes (Secundino et al. 2002, Matos et al. 2006, Caligiuri et al. 2014), but also the vector of other pathogens, including viruses and bacteria. Carvalho et al. (2018) have detected and isolated a putative new *Phlebovirus* (Viola *Phlebovirus*) from *Lu. longipalpis* in Brazil. Phylogenetic analysis revealed proximity with viruses causing disease in humans, rodents and isolated from sand flies belonging to phlebotomus fever serogroup. Moreover, the isolation of Viola virus in mammalian cells indicates that this virus is not an insect-specific

virus and represents a novel species with unknown vertebrate host (Carvalho et al. 2018). In general, *Lu. longipalpis* was able to support the *Bartonella bacilliformis* infection and seems to be a user-friendly, live vector/host model system (Battisti et al. 2015). Rocha et al. (2018) have reported for the first time the occurrence of *Wolbachia pipientis* in a natural population of *Lu. longipalpis* from the State of Bahia, Brazil. Recently, the endosymbiont bacterium *Wolbachia* has been used as an alternative strategy to control vector-borne diseases, through the reduction or blocking of pathogen infections. However, Gonçalves et al. (2019) have shown that the *Wolbachia* introduction into *Lu. longipalpis* cell lines has not affected the infection with *Le. infantum*. Endosymbiotic bacteria present in sand flies, especially in the midgut, can affect their capacity to transmit *Leishmania* (Telleria et al. 2013). Moreover, the microbiota is able to differentially infect the larval digestive tract and regulate the immune response in *Lu. longipalpis* larvae (Heerman et al. 2015). Pires et al. (2017) have described the native microbiota of wild-caught *Lu. longipalpis* under distinct physiological conditions including a *Leishmania*-infected group. The amplicon oriented metagenomic profiling revealed five phyla (Actinobacteria, Bacteroidetes, Firmicutes, Proteobacteria and Spirochaetes), 64 bacterial genera and 46 families associated with wild-caught *Lu. longipalpis* (Pires et al. 2017). The gut microbiome of laboratory-reared *Lu. longipalpis* was recently shown to be essential for survival of the parasite (Kelly et al. 2017). The authors have shown that an antibiotic-mediated decrease in midgut microbiota impaired *Le. infantum* survival in the sand fly, inhibited parasite growth, and decreased differentiation to the infectious metacyclic form was observed (Kelly et al. 2017). Furthermore, when *Lu. longipalpis* was pre-fed with *Pseudozyma*, *Asaia* or *Ochrobactrum*, a reduced parasite survival rate has been observed by Sant'Anna et al. (2014). Still, more field-studies using such bacteria are important to establish their biological role as possible alternative control measures.

FINAL CONSIDERATIONS

The genome annotation of *Lu. longipalpis* is still underway and most of the omics approaches are very scarce. Dillon et al. (2006) analyzed expressed sequences tags (ESTs) of *Lu. longipalpis* to investigate the critical proteins underlying the host-parasite relationship and recently, an improved annotation of *Lu. longipalpis* genome has been published (Yang & Wu 2019). Besides that, a global approach for the identification of midgut ESTs via random, uni-directional sequencing of clones from cDNA libraries obtained using mRNAs extracted from midguts of *Lu. longipalpis* have been published (Jochim et al. 2008, Pitaluga et al. 2009). Moreover, transcriptome analysis of the salivary and pheromone glands as well as annotation of both female and male adults have brought important insights into the repertoire of molecules expressed in the vector (Oliveira et al. 2009, Azevedo et al. 2012, González-Caballero et al. 2013, McCarthy et al. 2013). It seems likely that in the next decade, these approaches, and perhaps more advanced ones will bring additional information of functional aspects on how molecular biology of *Lu. longipalpis* affects its interactions with vertebrate host and parasites. The establishment of VL in urban areas, where until recently, the disease did not occur, is closely related to the adaptation of the natural vector *Lu. longipalpis* to this environment. Several factors are involved in the difficulty to control VL such as the presence of sibling species in the *Lu. longipalpis* complex, as

well as differences on vectorial capacity among populations. Moreover, the presence of another vector species has been reported in Brazil especially in absence of the main vector (de Carvalho et al. 2010, Dias et al. 2013, Guimarães et al. 2016; Rêgo et al. 2020). Studies on biological behavior of the vector, salivary components, gut physiology as well as host-parasite interaction represent a wide and important field to better understand several aspects involved in the transmission and establishment of *Leishmania* parasites in permissive vectors. Omics approaches are also added in this context, even in its initial phase, but providing tremendous opportunities for the research on sand flies and *Leishmania* species in the Americas.

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