EFFECT OF ANTI-PPCHIT1 ON SAND FLY FITNESS AND TRANSMISSION OF LEISHMANIA INFANTUM IN AMERICAN FOXHOUNDS BY SAND FLY BITE

by

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Abstract

Sand flies (Diptera:Psychodidae) are vectors of parasites if the genus *Leishmania*, the causative agent of leishmaniasis, a neglected tropical disease in several countries around the world. Sand flies transmit *Leishmania* to suitable vertebrates during the blood meal and following a complex development parasites undergo within the fly. Many aspects of the *Leishmania* development within the sand fly vector are well known, however details about how sand fly molecules affect the parasite are still not yet known. Our group previously identified that RNAi knockdown of PpChit1, a midgut specific chitinase from the sand fly *Phlebotomus papatasi*, led to a significant decrease in the load of *Le. major*. In this study, we assessed potential fitness effects of antisera anti-PpChit1 on three laboratory-reared sand fly species (*P. papatasi*, *Phlebotomus duboscqi*, and *Lutzomyia longipalpis*). Our results suggest that feeding sand flies with anti-PpChit1 sera led to a one day delay in the onset of oviposition, and also suggested that anti-PpChit1-fed flies survived on average up to three days longer that control flies. Analyses of the peritrophic matrix (PM) indicated a significant increase in thickness 72 hours post anti-PpChit1 feeding compared to control sera. Altogether the results suggest that feeding sand flies with anti-PpChit1 likely affects the kinetics of sand PM, which in turn affects the flow of nutrients and certain aspects of sand fly fitness.

In the course of this study, we also evaluated the ability of American Foxhounds naturally infected with *Leishmania infantum* to transmit these parasites via bites of phlebotomine sand flies to suitable vertebrates. Since 1999, an outbreak of canine visceral leishmaniasis (CVL) has been reported in the U.S especially among Foxhounds. The ability of sand flies to pick up and transmit this pathogen represents an important health risk for companion dogs and humans. Our results indicate that Foxhounds naturally infected with *Le. infantum* are highly infectious to sand flies and that the parasites are able to fully develop within these vectors and de successfully transmitted during blood feeding. Thus, the risk exists for these parasites to become endemic in North America where sand flies are also known to occur.

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Dedication

I dedicate this thesis to my family who is always with me even with the distance, for supporting my endeavors and encouraging me through life.

Chapter 1 - **Literature Review**

The Peritrophic Matrix (PM)

PM roles

The peritrophic matrix (PM) in insects is a non-cellular semipermeable layer separating the contents of the gut lumen from the digestive epithelial cells. By lining the midgut, the major roles ascribed to the PM include protection the midgut epithelium from abrasion, toxic compounds, and pathogens, as well as serving as a scaffold for proteases, peptidases, and glycosidases (Lehane, 1996; Pimenta et al., 1997; Terra, 2001; Toprak et al., 2010). In addition, the PM separates the midgut into the endoperitrophic space containing the food bolus surrounded by PM, and the ectoperitrophic space between PM and the midgut epithelium. The PM is associated with increasing the efficiency of the digestion process due to its semipermeable property allowing the selective migration of small molecules. Further, the porosity of the PM is an important determinant in regulating the movement of digestive enzymes from the surface of the midgut epithelial cells into the gut lumen (Lehane, 1996). In some hematophagous insects such as *Aedes aegypti* it was shown that the PM or PM-associated molecules are able to bind heme, a highly toxic prostetic group which is the byproduct of blood digestion (Pascoa et al., 2002; Walters et al., 1995). Later, Devenport et al. (2006) demonstrated that the *Ae. aegypti* PM-associated AeIMUC1 is able to bind heme. Recent studies by our group identified peritrophin PpPer1 and likely PpPer2 (see below) as playing a heme-detoxification role in the sand fly *P. papatasi* (Coutinho-Abreu et al., 2013)

Regarding its role as a physical barrier against parasites, Lewis (1953) described how most microfilariae of *Onchocerca volvulus* become trapped within the PM of *Simulium damnosum* and are eliminated. Many years later, studies described the role of the PM in preventing the escape and subsequent development of *Plasmodium* parasites in *Anopheles* mosquitoes (Billingsley and Rudin, 1992; Shahabuddin et al., 1993; Sieber et al., 1991). Similarly, it has been well documented that in sand flies the PM also serves as a barrier to *Leishmania* development; by trapping parasites within the endoperitrophic space and overpassed with the remnants of the blood meal (Coutinho-Abreu et al., 2010; Walters et al., 1992).

Synthesis and types of PM

The insect PM can be classified according to how they are synthesized as types 1 or 2. Type 1 PM (PM1) is formed along the entire midgut epithelium, in response to distention of the midgut during feeding (Peters, 1992; Toprak et al., 2010). This is a thick extracellular matrix secreted around the blood meal by midgut epithelial cells in the posterior midgut. Its thickness is approximately 1-20 μm and it is the most common type in bloodsucking insects, such as mosquitoes, sand flies, and black flies (Jacobs-Lorena and Maung Oo, 2004). Type 2 (PM2) is secreted by the cardia, a specialized organ near the foregut-midgut junction (Toprak et al., 2010). PM2 is a thin open-ended sleeve-like structure of approximately 1-2 μm think that lines the entire midgut and hindgut and it is secreted independently of the feeding status of the insect. Although most hematophagous insects secrete PM1 as adults, the larvae secrete PM2; likewise, many non-blood feeding dipterans adults secrete PM2 (Jacobs-Lorena and Maung Oo, 2004; Terra, 2001).

PM structure and composition

The PM2 is the most studied and its structure is characterized by distinct layers originated from different regions in the cardia (Jacobs-Lorena and Maung Oo, 2004). The PM1 generally does not contain discrete layered structures; instead, it is believed to be constructed by a 'template' generated by chitin fibrils secreted by the microvilli of the epithelial cells and other components. The regular arrangement of the microvilli is imprinted on the PM structure given its characteristic hexagonal (or honeycomb) and orthogonal texture. The assembly process of PM1 is thought to occur in several steps, starting with secretion of the chitin fibrils by the microvilli of the midgut epithelial cells; continuing with the maturation and formation of cross-linked network; and finally, the attachment of the matrix of probably proteins and proteoglycans that interlock the chitin fibrils (Lehane, 1996; Merzendorfer and Zimoch, 2003; Walters et al., 1993).

The PM consist mainly of chitin and proteins including glycoproteins and proteoglycans (Peters, 1992). The amount of these components varies depending of the insect species, life stage, as well as the maturation stage of the PM in one life stage (Lehane, 1996).

Proteins account for 21-55% of the total PM mass. PM-associated proteins are classified in four groups according to the ease with which they can be removed from the PM (Tellam et al., 1999; Wang and Granados, 2001). Class I proteins, elucidated from the PM of *Lucilia cuprina*

larva (PM2), can be removed with mild detergents as low or high ionic strength and physiological buffers, these proteins represent less than 1% of the total PM protein. Digestive enzymes can be found in this group. Class II proteins can be released with mild detergents such as Triton X-100, the detergent might be able to disrupt the protein-protein, protein-oligosaccharide, or protein-chitin interactions. Class III proteins are strongly attached to the PM but non-covalently, to remove them it requires strong denaturing agents such as urea, SDS or guanidine hydrochloride. These proteins are abundant, representing 11% of the total mass of the PM and are collectively referred to as peritrophins (Elvin et al., 1996; Tellam, 1996) (see below). Class IV proteins are those which are not solubilized with strong denaturants of high concentrations; in the case of PM2, this class of proteins is considered to be the most abundant after chemical analysis. These proteins are covalently cross-linked to themselves or to other constituents of the PM such as proteoglycans and chitin (Tellam et al., 1999).

Chitin

In the PM1, chitin, a polymer of β-(1,4)-N-acetyl-D-Glucosamine (GlcNAc) is considered a major component for structure maintenance (Hegedus et al., 2009; Shao et al., 2001). Chitin is always present in association as a chitin-protein complex. The chitin content is believed to be important for the tensile strength of the PM, while the proteoglycans might protect the matrix against enzymes and influence the permeability properties (Peters, 1992). The content of chitin in the PM varies between 3.5% to 13% of the total mass of the PM depending on insect species (Lehane, 1996). In the case of PM2, Tellam and Eisemann (2000) determined that the content of chitin in the larvae of *L. cuprina* was no more than 5.3% and the low content of chitin was responsible for the inability of chitinase and chitin synthesis inhibitors to disrupt the PM. In contrast, in other insects, chitin may be a greater component of the PM as chitin synthesis inhibitors and certain chitin binding compounds disrupt the integrity of the PM (Pimenta et al., 1997; Villalon et al., 2003; Wang and Granados, 2000).

Chitin is synthesized by chitin synthase (CS) through the addition of units of GlcNAc to the elongating end of the growing chain. Chitin can form three conformational structures: α-chitin composed of antiparallel chains; β-chitin, composed of parallel chains; and γ-chitin composed of three chitin chains in different orientations. The α- and γ-chitin chains seem to predominate in insect PM (Hegedus et al., 2009; Peters, 1992).

Proteoglycans

Proteoglycans consist of a core protein to which linear polysaccharides components are attached, in particular glycosaminoglycans (GAGs). GAGs are a family of macromolecules constituted by polymers of repetitive disaccharide units consisting of hexosamine and L-iduronic acid. Proteoglycans are present in most PMs and are evenly distributed. However, in dipterans, proteoglycans are concentrated into electron-dense layers which give the lamellar appearance to these PMs (Tellam et al., 1999). Proteoglycans are the filling molecules in the PM. The hydration of proteoglycans is believed to help in the formation of a gel-like meshwork in the PM which contribute to the strength of the PM and might be at the same time a determinant of PM permeability (Lehane, 1997).

Peritrophins

Peritrophins are integral and structural PM proteins that interact with the chitin matrix and are likely involved in determining the porosity, strength and elasticity of the PM. Peritrophins must have at least one chitin-binding domain (CBD) (see below), but also one or more mucin domains (MD) or commonly referred to as insect intestinal mucins (IIMs) (Toprak et al., 2010).

The molecular structure of three intrinsic PM proteins (perithropin-44, perithropin-48 and perithropin-95) was determined from *L. cuprina* (Elvin et al., 1996; Tellam et al., 1999). The amino acid sequences of perithropin-44, perithropin-48 shows some limited homology; each protein contains five domains of approximately 65-70 amino acids in length. Each domain is characterized by six cysteine residues likely forming three intra-domain disulphide bonds. Besides the conserve cysteine, there is a strong conservation of three hydrophobic amino acids or aromatic residues located at specific positions between cysteine residues. The common feature of these domains and other PM proteins is the strong conservation of the structure although not the overall amino acid sequences. All the amino acid sequences contain sites for potential for N-linked glycosylation but at different positions (Tellam, 1996; Tellam et al., 1999).

Three different types of CBD are known as Peritrophin-A, -B or –C domains and have motifs with six, eight, and 10 cysteine residues forming between three to five intra-domain disulphide bonds (Tellam et al., 1999) plus several conserved aromatic/hydrophobic amino acids.

The disulphide bonds in the PM contribute to protein stability and resistance to proteolysis in the gut (Devenport and Jacobs-Lorena, 2004). Peritrophin-A domains (PAD) are present among all insects, while Peritrophin-B (PBD) and Peritrophin-C (PCD) domains have been found only in dipteran larvae (Toprak et al., 2010). The potential for multiple CBDs in the PM may give them the capability to cross-linking with chitin fibrils in the PM in order to create a protein network throughout the chitin fibrils. The multiple ways of cross-linking of the peritrophins within the PM give strength and elasticity with limited thickness (Tellam, 1996; Wang and Granados, 2001).

Besides chitin-binding domains in PM, some proteins also display mucin-like domains. These domains display serine and threonine residues that frequently are the sites of O-linked glycosylation (Devenport and Jacobs-Lorena, 2004). An example is Peritrophin-95 which contains an additional carboxy-terminal domain of 100 amino acids characterized for proline and threonine- rich domains but absence of cysteine residues. The proline and threonine domains are extensively glycosylated with O-linked oligosaccharides (Tellam, 1996). Mammalian intestinal mucins are widely studied and known to have numerous functions such as protection from abrasion, hydrolytic enzymes, heavy metals, and pathogens, while allowing the passage of digestion products for absorption by intestinal epithelium. It is thought that insect intestinal mucins might play some of the same functions (Devenport and Jacobs-Lorena, 2004). In fact, the first insect intestinal mucin (IIM) identified in the lepidopteran *Trichoplusia ni* resembles mammalian intestinal mucins in aspects including high O-glycosylation, high concentrations of threonine, alanine, and proline, and resistance to proteases and probably shield other PM components from digestive enzymes (Wang and Granados, 1997). Insect intestinal mucins interact with chitin fibrils to create the PM, which facilitates the digestion process and protects the digestive tract form toxins (Rayms-Keller et al., 2000). In adult *Ae. aegypti* females, an intestinal mucin, AeIMUC1, protects against toxic reactive oxygen species produced during the process of hemoglobin degradation. To prevent the accumulation of these toxic metabolites, the cysteine-rich peritrophin-A domains (heme-regulatory motifs) in AelMUC1 promote binding of heme to the PM and thus its excretion (Devenport et al., 2006). Recent studies from our group on sand fly peritrophins led to the characterization of *P. papatasi* PpPer1, PpPer2, and PpPer3 (Coutinho-Abreu et al., 2013) and the identification of several putative peritrophins from *Lutzomyia longipalpis* (not shown). PpPer1 and PpPer2 display, respectively, four and one

chitin-binding domains (CBDs). PpPer3 on the other hand has two CBDs, one mucin-like domain, and a putative domain with hallmarks of a CBD, but with changes in key amino acids. Temporal and spatial expression analyses show that PpPer1 is expressed specifically in the female midgut after blood feeding. PpPer2 and PpPer3 mRNAs were constitutively expressed in midgut and hindgut, with PpPer3 also being expressed in Malpighian tubules. PpPer2 was the only gene expressed in developmental stages. Our results also demonstrated that recombinant rPpPer1 and rPpPer2 bind chitin, and that HRM identified as cysteine-proline dipeptides also were present in the predicted sequences for these proteins two proteins. Two HRM were identified (i.e., predicted) in PpPer1, one in CBD3 and one HRM in CBD4. A single HRM was identified in PpPer2. HRM also are predicted in the peritrophins identified form *Lu. longipalpis*. Collectively, these data suggest that sand fly peritrophins not only are part of the PM scaffold but also that they play a role in heme detoxification mechanism in sand flies.

Degradation of the PM

The degradation of the PM occurs as consequence of chitin degradation. Chitinolytic enzymes that fulfill this role include chitinases and β-N-acetyl-D-glucosamidases. Insect chitinases belong to the family 18 of the glycosylhydrolases superfamily and share a high degree of amino acid similarity. In contrast, β-N-acetyl-D-glucosamidases belong to family 20. Together, the synergistic action of family 18 and family 20 lead to rapid depolymerization of chitin in insects (Arakane and Muthukrishnan, 2010; Merzendorfer and Zimoch, 2003). Chitinases catalyze the random hydrolysis of internal bonds in chitin to produce smaller oligosaccharides. These oligosaccharides are further cleaved by β-N-acetyl-D-glucosamidases to liberate GlcNAc- from the non-reducing end of oligosaccharide (Suginta et al., 2000; Terra et al., 1996). Chitinases display molecular mass ranging from 20 to 90 kDa (Bhattacharya et al., 2007) and are present in broad group of organisms including bacteria, fungi, yeast, plants, humans, and arthropods. Chitinases are also involved in a variety of roles from digestion, to arthropod molting, to defense and innate immunity, and pathogenicity (Arakane and Muthukrishnan, 2010).

The structural organization of chitinases consists of multi-domains that include a catalytic region of 1-5 domains, a serine/threonine-rich linker region that can be highly glycosylated, and a 0-7 chitin-binding domain rich in cysteine. It is also predicted that chitinases have a hydrophobic signal peptide preceding the N-terminal region of the mature protein. The signal

peptide mediates secretion of the enzyme into the endoplasmic reticulum and it is cleaved off by signal peptides after the protein has been transported across the membrane (Arakane and Muthukrishnan, 2010; Merzendorfer and Zimoch, 2003).

In the case of the *P. papatasi* chitinase PpChit1, it belongs to family 18 of glycosylhydrolases and a highly conserve catalytic site FDGLDMDWEYPA besides several domains common of members of this family. Moreover, a signal peptide of 18 amino acids at the N-terminal comprises PpChit1; cleavage of this signal peptide results in the mature protein consisting of 452 amino acid residues and a molecular mass of 50.5 kDa. The C-terminal region has a Ser/Thr/Pro (S/T/P) rich region with multiple O-linked glycosylation sites. The S/T/P region if followed by a chitin-binding domain, with six highly conserved Cys residues (Ramalho-Ortigao et al., 2005).

In *Lu. longipalplis*, an ortholog of *PpChit1* also has been identified (Ramalho-Ortigao and Traub-Cseko, 2003). The *Lu. longipalpis Llchit1* complete cDNA sequence spans 1600 bp with an open reading frame (ORF) of 1425 bp encoding a mature protein of 474 amino acids and a mass of 51.6 kDa. The predicted LlChit1 displays the highly conserved catalytic domain FDGL(I/F)DV(L/I)DWEYP at the N-terminal indicative of family 18 glycosylhydrolases. The signal peptide is predicted to be cleaved at position Thr19 indicating the protein is secreted. Two Lys residues (Lys21 and Lys22) are potential trypsin activation sites. Furthermore, at the C-terminal, a small domain composed of serine/threonine/proline (STP) is a target region for O-linked glycosylation. After the STP region, there is a putative chitin binding domain (CBD) with three conserved cysteines and two aromatic residues similar to the CBD of *A. gambiae* AgChi-1 and those present in several other chitinases and PM binding proteins. Three N-glycosylation sites are predicted in the mature protein. Importantly, LlChit1 is secreted upon blood feeding with maximum expression level at approximately 72 h PBM (Ramalho-Ortigao and Traub-Cseko, 2003). Thus, LlChit1 is presumed to have similar function in PM type 1 formation and degradation as PpChit1 in *P. papatasi* and AgChi-1 in *A. gambiae*.

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Chapter 2 - **Effect of Anti-PpChit1 on sand fly fitness**

Abstract

Following a blood meal, sand flies (Diptera:Psychodidae) secrete a peritrophic matrix (PM) type 1 that compartmentalizes the blood meal, protects the epithelium, and serves as a barrier against parasites. It has been shown that sand flies also secrete a midgut-specific chitinase that modulates and degrades the PM. In *Phlebotomus papatasi*, knockdown of the midgut chitinase PpChit1 led to a reduction in the load of the parasite *Leishmania major* in the midgut of sand flies, but effects on the fitness of the flies were not investigated. The current study was focused on identifying potential fitness effects of feeding anti-PpChit1 to sand flies. *Phlebotomus papatasi, P. duboscqi* and *Lutzomyia longipalpis* were fed on red blood cells reconstituted with naïve or anti-PpChit1 sera and assessed for various parameters including, blood digestion, egg laying (oviposition) onset, number of eggs laid, egg bouts, average number of eggs per bout, and survival. No statistically significant differences were observed between treatments for blood digestion in all three species investigated. Similarly, no statistically significant differences were observed for the fitness parameters in *P. duboscqi* and *Lu. longipalpis*. However, in *P. papatasi*, the onset of egg laying was delayed by one day when comparing anti-PpChit1-fed to naïve serum-fed flies; likewise, the survival of treated flies was approximately three days longer compared to that of the control group. In *P. papatasi*, these differences approached significance in the analyses performed. Interestingly, feeding on anti-PpChit1 had a negative effect on overall ability of flies to lay eggs, as several gravid females from all three species were unable to lay any eggs despite having lived longer than control flies. Overall, this study suggests little, if any, effects on sand fly fitness by feeding anti-PpChit1. Notwithstanding, the inability of females that fed on anti-PpChit1 to lay eggs might have been due to changes in PM permeability affecting nutrient absorption.

Introduction

Sand flies (Diptera:Psychodidae) are vectors of leishmaniasis, a major neglected tropical disease caused by parasites of the genus *Leishmania* (Kinetoplastida:Trypanosomatida). *Leishmania* spp. are transmitted to a suitable vertebrate during the bite of an infected female sand fly. Leishmaniasis encompasses a spectrum of diseases that include cutaneous, mucocutaneous, and visceral forms. Currently, leishmaniasis is endemic in 98 countries putting 350 million people at risk, and with 2 million human cases worldwide and 40, 000 deaths estimated to occur annually (WHO, 2010).

There are over 40 species of *Leishmania* known (Ramalho-Ortigao et al., 2010), with more than 20 species involved in human infections (Esch and Petersen, 2013). Multiple animals, sylvatic and domestic, serve as hosts or reservoirs for the parasite. Dogs, in particular, are the most important risk factor predisposing human to infection with *Leishmania infantum*, a visceralizing parasite (Petersen and Barr, 2009)

Leishmania display a digenetic life-cycle, cycling between a suitable vertebrate host and sand fly vectors (Dostalova and Volf, 2012). In the vertebrate, *Leishmania* are obligatory intracellular parasites (amastigote form) and commonly found in macrophages and neutrophils, although other cells such as dendritic cells and mononuclear cells can also be infected. During blood feeding on the infected vertebrate, parasites are acquired by the female sand fly. Within the sand fly, *Leishmania* undergo a complex development. First, parasites burst out from the infected macrophages and change from the typically non-motile amastigotes to motile, and highly replicative, procyclic promastigotes. These two events take place within the endoperitrophic space that is formed by the secretion of the peritrophic matrix (PM) type 1 by the epithelial cells that line the female sand fly gut.

To complete their development, *Leishmania* encounter several barriers that must be overcome for successful establishment and subsequent transmission. First, parasites have to survive the proteolytic attack of digestive proteases secreted in the sand fly gut after blood feeding. Then, they must escape from the endoperithrophic space and attach to the midgut epithelia, in both cases to prevent excretion with remnants of the blood meal. Finally, parasites must detach from the midgut epithelia lining and migrate towards the thoracic midgut or to the foregut of the vector. During this final development stage, parasites transform into the infective

metacyclic promastigotes which will be deposited in the skin of the vertebrate host when the now infectious fly attempts the next blood meal (Bates, 2007).

The inability to evade one or more barriers within the sand fly gut has been associated with the vectorial capacity in sand flies. One classical example comes from the characterization of PpGalec, a receptor for *Leishmania major* lipophosphoglycan (LPG) identified in the midgut of the natural vector *Phlebotomus papatasi* (Kamhawi et al., 2004). PpGalec was shown to be responsible for *L. major* ability to attach to *P. papatasi* midgut preventing excretion. These data highlighted the close relationship that exists between *Leishmania* and sand flies, to the extent that some species of sand flies are able to transmit some species of *Leishmania*. Hence, sand fly species that are capable to sustain experimental infection of different *Leishmania* species are referred to as non-specific, or permissive; sand flies that are only infected with a single species of *Leishmania*, even under laboratory conditions, are referred to as specific or restrictive (Ramalho-Ortigao et al., 2010). A LPG-independent midgut attachment, driven by the level of glycosylation of midgut proteins expressed on the surface of the midgut epithelial cells, has been demonstrated for non-specific sand flies (Volf et al., 2007).

No vaccine is available to prevent human leishmaniasis despite much research. Protection against visceral and cutaneous leishmaniasis has been achieved in mice, hamster, dogs, and non-human primate using crude or defined antigens with appropriate adjuvants (Raman et al., 2012). Moreover, antigens derived as vectored DNA, plasmid DNA or recombinant proteins have been proven effective in animal models (Bethony et al., 2011). Nonetheless, an effective human vaccine that induces a strong and persistent T_h1 immune response is yet to be developed.

One approach that has attracted attention in the last few years concerns the development of transmission blocking vaccines (TBVs). TBVs prevent pathogen transmission by targeting molecules expressed on the surface of pathogens essential to its development, or by targeting molecules expressed by the vector. By inducing the expression of specific antibodies in a suitable vertebrate host, vaccination with parasite or vector molecules (TBVs) lead to blocking of parasite development within the vector when acquired during the blood meal in the vaccinated individuals (Coutinho-Abreu and Ramalho-Ortigao, 2010). The sand fly PM, like its counterpart in mosquitoes, is a semipermeable layer formed by chitin fibrils, proteins and glycoproteins (Elvin et al., 1996; Pascoa et al., 2002; Peters, 1992). Generally, the PM's primary function involves compartmentalization of the blood meal and protection of the midgut epithelia against

abrasion during digestion of the food bolus (Elvin et al., 1996; Schlein et al., 1991; Secundino et al., 2005a). However, the sand fly PM was also shown to serve a dual role with regards to *Leishmania* infection: as a barrier and as protection to transitional amastigotes (when they are most vulnerable to the proteolytic attack). *Leishmania* development depends in part by the action of sand fly midgut chitinases (Ramalho-Ortigao et al., 2005) which can hydrolyze chitin in the PM, allowing the escape of the parasites.

PpChit1 is a midgut-specific chitinase presumably involved in modulation and degradation of the PM in *P. papatasi* (Ramalho-Ortigao et al., 2005), and escape of parasites from the endoperitrophic space (Dostalova et al., 2011). It has been demonstrated that anti-PpChit1 sera inhibited chitinolytic activity in the midgut of *P. papatasi*. Interestingly, these same antibodies displayed a cross-species effect, inhibiting chitinolytic activity in the midgut of *P. duboscqi and P. argentipes* (Ramalho-Ortigao et al., 2005). Recently, our group demonstrated that RNAi-induced knockdown of *PpChit1* leads to a significant reduction of *L. major* within *P. papatasi* midgut (Coutinho-Abreu et al., 2010). The results supported the potential use of PpChit1 as a TBV against transmission of *Leishmania* by sand flies.

The integrity of the PM is critical to protect the arthropod midgut from toxins, microbial infections, digestive enzymes and physical trauma (Ramos et al., 1994; Wang and Granados, 1997). Thus, we reasoned that feeding sand flies with anti-PpChit1 sera would led to changes in the structure of the PM, (i.e., increasing its thickness). As a consequence, the PM would remain intact for longer period of time influencing the rate of blood digestion and excretion, with possible effects on sand fly fitness. Thus, in order to address whether anti-PpChit1 antibodies do indeed affect sand fly fitness, the following study aims are proposed:

(1) Purify anti-chitinase1 antisera (anti-PpChit1)

(2) Assess the digestion of a blood meal in sand flies due to the effect of anti-PpChit1

(3) Study the fitness of three species of sand flies *P. papatasi* (PPIS), *P. duboscqi* (PDMA), and *L. longipalpis* (LLJB) upon anti-PpChit1 treatment in terms of egg laying onset (start of oviposition), number of eggs, survival, number of egg bouts, and average number of eggs per bout.

This research will serve to better understand the role of sand fly midgut chitinase in controlling the physiology and fitness of the sand fly; hence, it can be accounted towards parasite-vector interactions and the development of successful transmission blocking vaccines.

Materials and Methods

Ethics statement

The use of animals during this study was reviewed and approved by the Kansas State University Institutional Animal Care and Use Committee (KSU-IACUC).

PpChit1 antisera production

DNA plasmid (VR-2001-TOPO)

Plasmid VR2001-TOPO was derived from VR1020 (Vical, Inc.) a plasmid that has been approved for use as a DNA vaccine. VR-2001-TOPO was modified by the addition of topoisomerase to obtain a plasmid ("TOPO/TA") that contains a cloning site at the 3' end of the tissue plasminogen activator signal peptide of the VR1020 plasmid. The signal peptide cleavage site is preserved in this plasmid as well as the kanamycin-resistance gene and the cytomegalovirus promoter (Oliveira et al., 2006).

Plasmid preparation and purification

A glycerol stock aliquot of *E.coli* carrying the VR2001-TOPO plasmid with the mature PpChit1 cDNA was seeded into 1.5 L of LB kanamycin (50 mg/ml) and incubated overnight on a shaker at 37 ^{o}C. Plasmid purification was performed using the Endofree plasmid Mega Kit (Qiagen) following manufacture's specifications with the exception or the last step. Final plasmid purification was done using an Amicon Ultra-15 centrifugal filter unit with a 100 kDa cutoff (Milllipore) washed three times with ultrapure cell culture water and concentrated to a volume of approximately 500 µl. The cDNA sample concentration was measured on a Biotek Epoch Gen5 spectrophotometer (Biotek, Winooski, VT), and stored at -20 ^{o} C before immunization procedure. Prior to injection, sample was filter sterilized through a 0.2 µM filter unit (Millipore).

Mice immunization with DNA plasmid

Female BALB/c mice, 8-12 weeks old were bred in the animal facility of the Division of Veterinary Medicine, Kansas State University (Manhattan, KS) and maintained under pathogen-free conditions.

Mice were anesthetized with isoflurane and immunized subcutaneously in the ears using a 29.5-gauge needle with DNA plasmids VR2001-TOPO encoding the mature chitinase protein (Ramalho-Ortigao et al., 2005). Each mouse ear was injected three times in two-week intervals with 10 µg/µl of plasmid in a 10 µl volume. Two weeks after the last DNA immunization, approximately 300 µl of blood was collected from the submandibular vein ("cheek bleed") of immunized animals. Sera were separated following centrifugation at 750 rpm for 10 minutes.

Antibody titer

Antibody titer was measured with the Easy-Titer IgG Assay Kit (Pierce, Rockford, IL) following manufacturer's instructions and kept at -20 °C until use.

Sand fly rearing and blood feeding

Phlebotomus papatasi Israeli strain (PPIS), *P. duboscqi* Mali strain (PDMA), and *Lutzomyia longipalpis* Jacobina strain (LLJB) were reared in the Biology of Disease Vectors laboratory in the Department of Entomology, Kansas State University, at 26 ^{o}C and 70% humidity in a 12:12 light-dark cycle. Two groups, one experimental and one control, each containing four-to-five day old 150 females and 30 males sand flies were placed in a 500 ml plastic container (ø = 6.3 cm, height = 6.5 cm) (Thermo-Nalgene, Waltham, MA) covered with a piece of nylon mesh (0.5mm). Sand flies were blood fed in the laboratory using a glass feeding apparatus through a pig intestine membrane attached to the glass feeder. Two hours prior to blood feeding, a sample of fresh BALB/c mouse blood was collected by submandibular bleeding and mixed with heparin (Calbiochem, Billerica, MA) to prevent coagulation. Blood was centrifuged at 7500 rpm for 10 minutes (Eppendorf, Hauppauge, NY). The sera were removed and red blood cells were reconstituted with 195 µl of anti-PpChit1 (113 µg/ml) in the case of the experimental group and, naive sera previously collected for the control group.

Immediately after feeding for 1.5 h, fully engorged females were separated from the partial and non-fed. The presence or absence of blood in the sand fly digestive tract was verified by anesthetizing flies with CO_2 and observing the midgut distension under a stereomicroscope (Carl Zeiss, Thornwood, NY). Only fully fed sand flies of similar size were used for further examination. Flies were kept in paper cups (250 ml) (Huhtamaki, Fulton, NY), two cotton strips

(2 cm) with 20% sugar solution was added on top, and the containers were kept inside a Drosophila incubator (Percival, Perry, IA) at 26 ^{o}C and 70% humidity.

Assessment of hemoglobin concentration

The rate of hemoglobin excretion was assessed with a colorimetric assay using Drabkin's solution. Drabkin's solution was prepared by adding 5 µl of 30% Brij-35 solution (Sigma, Saint Louis, MO) to 10 ml of Drabkin's Reagent (Sigma, Saint Louis, MO) and mixing by inverting the tube several times, gently. Flies were blood fed as described above; at 24, 30, 36, 48, and 72 h post-blood meal a group of blood-fed flies from experimental and control groups were separated, midguts of sand flies were dissected and transferred into 250 µl of Drabkin's solution; homogenized with a hand-held homogenizer (Kimble Chase, Vineland, NJ) for approximately 30 sec; transferred to a 96-well microtiter plate and incubated for 15 min at room temperature (24 ºC). Absorbance at 540 nm was recorded on a Biotek Epoch Gen5 (Biotek, Winooski, VT). Known quantities of mouse blood were measured as a standard and each sample was measured in triplicate. The mean of individual midguts at each time point were analyzed with a two-tailed unpaired t test. Normality was tested with the Kolmogorov-Smirnov test. Pictures of dissected midguts were taken using an AM423X Dino-Eye camera (Dinolite, Songshan District.Taipei, Taiwan).

Sand fly oviposition

Flies were blood fed as described above. Three days post blood feed, individual blood-fed females from the anti-PpChit1 and the control groups were placed in plastic ovipots (ø = 6.3cm, height =6.5 cm) with 1.5-2 mm of laboratory plaster on the bottom, humidity of plaster was approximated to 65-70% for all ovipots, and one cotton strip (2 cm) with 20% sugar solution was added on top. The start day of oviposition (onset), number of eggs laid and survival of flies was recorded daily for each ovipot and until the fly died.

Statistical Analyses

Statistical analyses were conducted by individual species and then compared across all species as well. The general experimental design for an individual species was a randomized complete block design (RCBD) with sub-sampling. The anti-PpChit1 was the treatment factor,

the run was the blocking factor, and individual flies were the subsamples. The general experimental design across species was a randomized complete block design (RCBD) pooled over species and with sub-sampling. The anti-PpChit1 is the treatment factor, run nested within species was the blocking factor, and individual flies were the subsamples. Careful consideration was taken in selecting the type of probability distribution to be used with each response variable analyzed as indicated below. Due to an unequal number of replications and subsamples, the Between-within denominator degree of freedom method was used. Moreover, to help evaluate the antibody treatment by species interaction, simple effects of antibody treatment versus control were done for all species. Statistical analyses were conducted using SAS software version 9.3 (SAS Institute Inc., 2011) and Minitab software version 16.

Analysis for blood digestion response

To investigate the change in hemoglobin concentration over specified time intervals, an additional split plot of time had to be added onto the general experimental design described above. In detecting differences in the response blood digestion between the control and the treatment groups, a Mixed procedure was used as a preliminary analysis. Since the overall residuals appeared rightwardly skewed and tests of normality from the Univariate procedure (Shapiro-Wilk, Kolmogorov-Smirnov, Cramer-von Mises, and Anderson-Darling) showed residuals to be not normal, a generalized linear mixed model with a gamma distribution and a log link function in the Glimmix procedure were used.

Analysis for fitness responses

Due to the discrete nature of the measurements for onset of oviposition, survival, and egg bouts, the Glimmix procedure in SAS with a Poisson distribution and a Log link function. In the case of the fitness response variables for total number of eggs laid and average number of eggs per bout, a generalized linear mixed model with a gamma distribution and a log link function were used through the Glimmix procedure in SAS to account for upper skew of the data.

Flies that never laid eggs

To investigate differences in the proportion of flies that never laid eggs between the control and the experimental group for an individual species, a Fisher's Exact Test was conducted on Minitab software version 16.

Results

Antibody titer

Antibody was generated by injecting 10 µl of the plasmid for PpChit1 into nine BALB/c mice. After several sera collections, the titer of each mouse was measured and the sera corresponding to mouse number 4 was used for the analysis (Table 2.2).

Table 2.1 IgG Standards. IgG concentration in anti-PpChit1 sera collected from mice were calculated based on values of IgG concentration and absorbance determinate for six different concentrations.

ID	IgG Concentration (ng/ml)	Absorbance 340nm	Mean	Std Dev	CV (%)
STD1	500	0.547	0.562	0.021	3.77
	500	0.577			
STD2	250	0.676	0.665	0.016	2.45
	250	0.653			
STD3	125	0.797	0.802	0.007	0.882
	125	0.807			
STD4	62.5	0.967	0.965	0.003	0.293
	62.5	0.963			
STD5	31.2	1.13	1.148	0.025	2.22
	31.2	1.166			
STD6	15.6	1.317	1.311	0.008	0.647
	15.6	1.305			

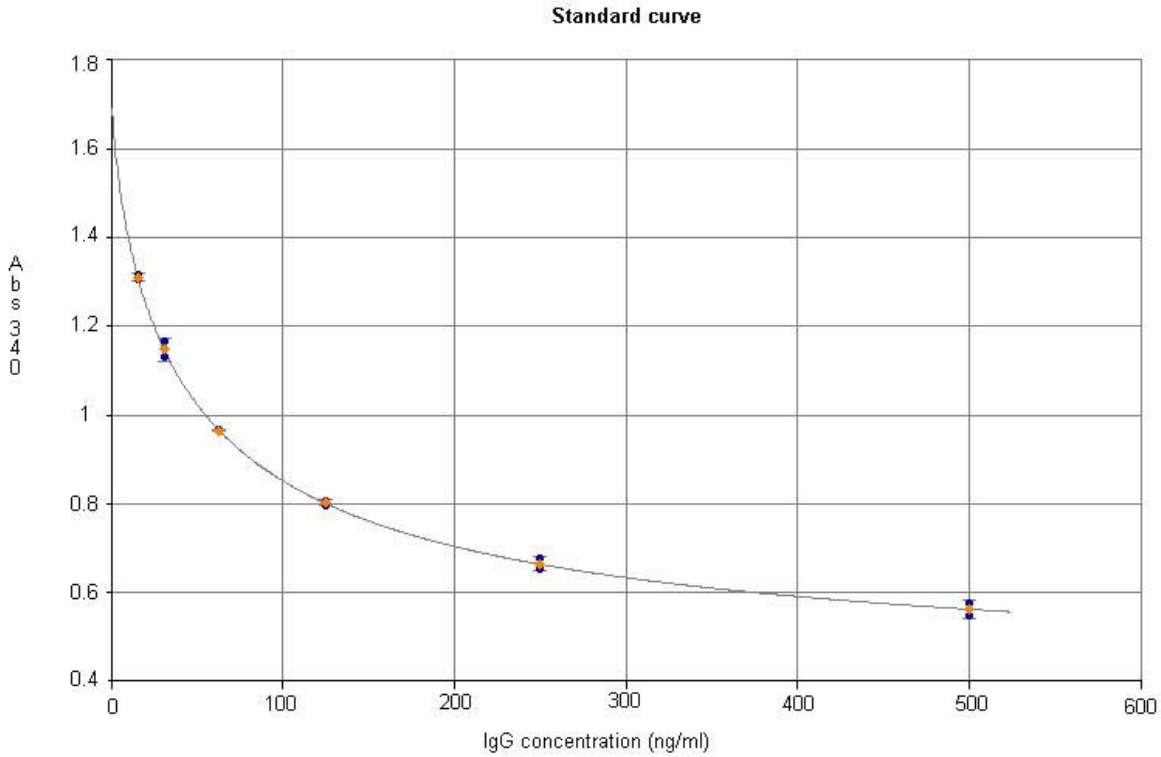

Figure 2.1 Standard curve for antibody titer. Corresponding IgG concentration for anti-PpChit1 was determined by extrapolating the absorbance readings shown on the graph.

Table 2.2 Antibody titers of nine mice injected with plasmid. The total concentration of IgG for each mouse was calculated based on absorbance at 340 nm of a 1:10,000 dilution. ODs were plotted on graph of figure 2.1 to determine each individual concentration. Each sample was done in duplicate. Standard deviation and coefficient of variance are shown for each pair of sample readings.

Name	OD340	IgG concentration (mg/ml)	Total IgG concentration (mg/ml)	Std Dev	CV (%)
mouse1	0.817	1.25	0.59	0.11	9.21
	0.788				
mouse2	0.875	0.91	0.25	0.01	0.589
	0.873				
mouse3	0.907	0.78	0.12	0.02	2.01
	0.914				
mouse4	0.854	1.24	0.58	0.36	28.7
	0.762				
mouse5	0.815	1.11	0.45	0.1	8.61
	0.843				
mouse6	0.85	0.98	0.32	0.04	3.89
	0.863				
mouse7	0.928	0.72	0.06	0.02	2.26
	0.936				
mouse8	0.867	0.94	0.28	0	0
	0.867				
mouse9	0.835	1.03	0.37	0.06	6.05
	0.855				
Naïve mouse sera	0.943	0.66	0	0.04	6.43
	0.966				

Effect of anti–PpChit1 on blood digestion

To test the effect(s) of anti-PpChit1 on blood digestion, two groups of sand flies were fed, one with fresh red blood cells (RBCs) reconstituted in naïve sera (control) and the other with RBCs reconstituted in anti-PpChit1. As a measure of blood meal loss over time, the hemoglobin concentration (of dissected midguts) was determined for each of the three species of sand flies included in the study. For each species, five replicates for PPIS and PDMA, and three replicates for LLJB were performed. The values were analyzed by pooling together the replicates per each species. Overall, there was no statistical significant difference in blood retention in midguts from naïve blood-fed flies compared with anti-PpChit1 blood-fed flies when examined over time (Fig. 2.2 and Table 2.3 - 2.5).

Interestingly, we observed a faster excretion of the blood meal in treated PDMA flies at the beginning with a decrease later during digestion. Twenty-four hours PBM, the blood retained in the midgut of treated flies was equivalent to the control group, at 30 h and 36 h PBM these treated flies begun to excrete the blood meal faster than the control group. However, at 48 h they seemed to slow down the digestion process compared to the control group; yet, these results are not statistically significant.

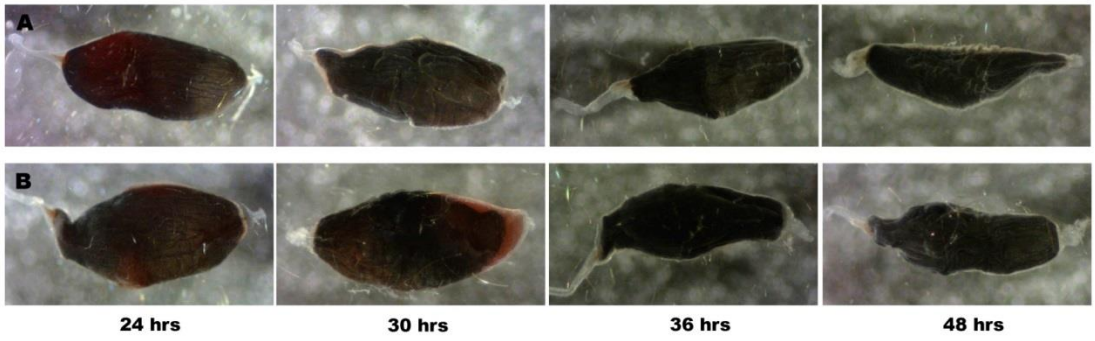

Figure 2.2 Sand fly midgut digestion process over time. Midguts of sand fly fed on RBCs reconstituted in naïve (A) or anti-PpChit1 (B) sera and dissected at 24 h, 30 h, 36 h and 48 h post blood ingestion (PBM).

Table 2.3 Amount of blood in control and anti-PpChit1 groups for *P. papatasi* (PPIS). Five replicates were measured and pooled to generate the average of hemoglobin concentration in whole midgut at different time points PBM.

Digestion time (h)	Control			anti-PpChit1			P-value
	Sand fly PPIS (n)	[Hb] mg/ml	95% CI	Sand fly PPIS n)	[Hb] mg/ml	95% CI	
30	30	0.268	0.200 - 0.359	32	0.262	0.196 - 0.351	0.863
36	30	0.258	0.193 - 0.346	32	0.282	0.211 - 0.378	0.497
48	36	0.221	0.165 - 0.295	36	0.221	0.165 - 0.296	0.982
72	32	0.132	0.098 - 0.177	52	0.15	0.112 - 0.200	0.326

Table 2.4 Amount of blood in control and anti-PpChit1 groups for *P. duboscqi* (PDMA). The amount of hemoglobin in whole midgut was calculated by testing five replicates and pooling them to calculate the average hemoglobin concentration at different time points PBM.

Digestion time(h)	Control			anti-PpChit1			P-value
	Sand fly PDMA (n)	[Hb] mg/ml	95% CI	Sand fly PDMA (n)	[Hb] mg/ml	95% CI	
24	25	0.261	0.216 - 0.315	25	0.261	0.216 - 0.316	0.987
30	25	0.266	0.220 - 0.322	25	0.244	0.202 - 0.295	0.364
36	26	0.247	0.204 - 0.298	26	0.215	0.178 - 0.260	0.142
48	26	0.198	0.164 - 0.239	26	0.222	0.183 - 0.268	0.225

T**able 2.5 Amount of blood in control and anti-PpChit1 groups for *Lu. longipalpis* (LLJB)**. Three replicates were performed and pooled to generate the average of hemoglobin concentration in whole midguts at different time points PBM.

Digestion time (h)	Control			anti-PpChit1			P-value
	Sand fly LJB (n)	[Hb] mg/ml	95% CI	Sand fly LLJB (n)	[Hb] mg/ml	95% CI	
24	20	0.265	0.189 - 0.371	22	0.283	0.202 - 0.396	0.620
30	20	0.272	0.194 - 0.381	20	0.307	0.219 - 0.43	0.368
36	20	0.252	0.180 - 0.354	22	0.304	0.217 -0.425	0.179
48	22	0.261	0.186 - 0.366	21	0.281	0.201 - 0.394	0.577

Sand fly fitness

To test the effect of anti-PpChit1 on the PM and its relationship to the fitness of sand flies, the onset of oviposition, survival, total number of egg laid per female, egg bouts, and the average number of eggs per bout were recorded and compared between females of the control and the anti-PpChit1 group for each sand fly species. In this case, four replicates were completed for PPIS, three replicates for PDMA, and three replicates for LLJB. The values were analyzed by pooling the replicates corresponding to each species. Based on statistical analyses, a difference was observed for onset of oviposition ($p = 0.074$) for PPIS (Table 2.6). This difference indicates that the group of flies treated with anti-PpChit1 take one extra day to start oviposition (7.5 days) compared to the control group (6.4 days). Similar difference was observed for the survival of PPIS ($p = 0.077$), indicating that treated flies with anti-PpChit1 live almost three days longer (10.6) compared the control group (7.73), no significant difference was detected for the other fitness parameters. The analysis for PDMA and LLJB species did not show a statistically significant difference in any of the fitness parameters between the control and the anti-PpChit1 groups (Tables 2.6).

Gravid sand flies that never laid eggs

One of the effects observed following feeding with anti-PpChit1 was that several gravid sand flies from all three species (PPIS, PDMA, and LLJB) never laid any eggs even if these sand flies were able to survive for several days in the laboratory (Table 2.7). Statistical analyses using the Fisher's exact test, suggested an approaching level of significance for the results obtained for at least two sand fly species: PDMA ($p = 0.058$) and LLJB ($p = 0.06$). Because of the experimental design used in our studies, and due to the data obtained it was not possible to statistically compare the survival of the flies that never laid eggs with the flies that did lay eggs. Nevertheless, the average number of days that the flies lived under the experimental conditions assessed are indicated in Table 2.7.

Table 2.6 Effect of anti-PpChit1 on fitness parameters for *P. papatasi* (PPIS), *P. duboscqi* (PDMA), and *Lu. longipalpis* (LLJB). Fitness parameters were calculated by combining four, five, and three experimental replicates for each species respectively.

Species	Group	Sand flies (n)	Egg laying onset (days)	P	Survival (days)	P	No. eggs	P	Egg bouts	P	Average No. eggs per bout	P
PPIS	Control	53	6.40	0.074	7.73	0.077	45	0.064	1	0.304	37	0.230
	Anti-PpChi1	53	7.49		9.60		55		1		43	
PDMA	Control	44	9.27	0.560	9.44	0.561	28	0.227	1	0.948	25	0.249
	Anti-PpChi1	47	8.14		8.42		36		1		31	
LLJB	Control	32	5.54	0.469	7.01	0.8052	32	0.999	2	0.513	19	0.513
	Anti-PpChi1	36	5.27		6.9		32		2		16	

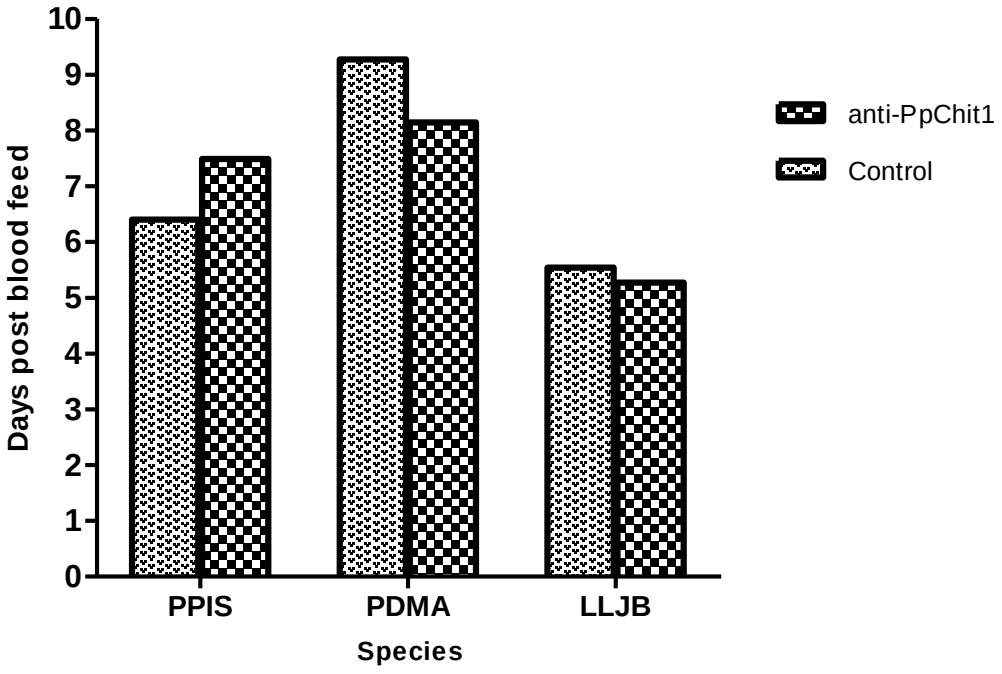

Figure 2.3 Graphical representation of the effect of anti-PpChit1 on egg laying onset for PPIS, PDMA, and LLJB. The effect of the antibody on PPIS is approaching significance level (p=0.074), on average the treated flies take one day longer (7.5 days) to start laying eggs compared to the control group (6.4 days). PDMA and LLJB flies are not being affected.

Table 2.7 Gravid sand flies that never laid eggs. Comparing the number and percentage of flies that never laid eggs, and the survival (days) for flies that never laid eggs vs. flies that laid eggs per species.

Sand fly species	Group	Sand flies (n)	# flies never laid eggs	Proportion (%)	Lifetime not laying: Mean (SD)	Lifetime laying: Mean (SD)
PPIS	Control	53	7	13.2	15.14 (4.14)	7.49 (1.23)
	Treatment	53	3	5.66	15.66 (4.72)	9.6 (1.7)
PDMA	Control	44	1	2.27	10 (-)	9.27 (2.34)
	Treatment	47	7	14.89	12.71 (5.53)	8.14 (1.9)
LLJB	Control	32	1	3.13	10 (-)	7.0117 (0.86)
	Treatment	36	7	19.44	11.1 (3.39)	6.9 (0.82)

Table 2.8 Approximate number of eggs that sand fly did not lay. After each sand fly died, it was dissected and the number of eggs still in ovaries were counted.

Species	Group	Sand flies (n)	Average # eggs in fly
PPIS	control	7	62
	anti-PpChit1	3	72
PDMA	control	1	33
	anti-PpChit1	2	81

Effect of anti-PpChit1 across sand fly species

The effect of anti-PpChit1 was also evaluated across the three species of sand flies for the blood digestion and all the fitness parameters mentioned above. After statistical analysis it was only obtained an approaching significance level (p=0.0647) on the survival of PPIS (Fig.2.4). The blood digestion and other fitness parameters were not statistically significant.

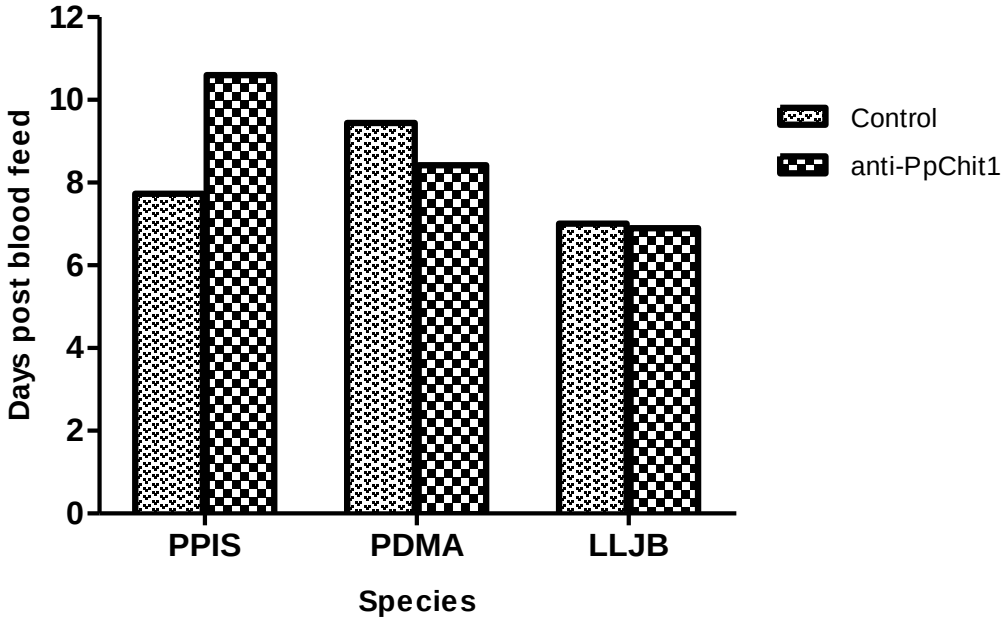

Fig 2.4 Graphical representation of the effect of anti-PpChit1 on survival of LLJB, PDMA and PPIS. It is noticeable that survival of PPIS treated flies is longer (10.6 days) than the control group (7.75 days). LLJB and PDMA are minor affected.

Discussion

In adult sand flies, secretion of the PM type I occurs following distention of the midgut during blood feeding. However, the kinetics of PM secretion and formation can change according to species (Pimenta et al., 1997; Sadlova and Volf, 2009). Indication that the PM is modulated by the action of chitinases was obtained by feeding chitinase inhibitors, such as allosamidin to insects. This led to thicker and more persistent PMs, similarly observed in sand flies (Pimenta et al., 1997) and mosquitoes (Shahabuddin et al., 1993; Shen and Jacobs-Lorena,

1997). Following the characterization of sand fly midgut chitinases (Ramalho-Ortigao et al., 2005; Ramalho-Ortigao and Traub-Cseko, 2003), and more recently, our RNAi knockdown studies of PpChit1 (Coutinho-Abreu et al., 2010) and PpPer1 (Coutinho-Abreu et al., 2013), we reasoned that changes in the PM permeability caused either by silencing (RNAi) or blocking (antibodies) of chitinase or other PM-related molecules affect digestion/excretion of the blood meal process in sand flies and consequently impact the fitness of the sand fly. The studies described here were focused on the effects of feeding antibodies specific to PpChit1, a midgut-specific chitinase secreted in the midgut of the sand fly vector *P. papatasi*. Anti-PpChit1 sera was fed to three different sand fly species, *P. papatasi* (PPIS), *P. duboscqi* (PDMA), and to *Lu. longipalpis* (LLJB) using an artificial blood feeding apparatus. Parameters assessed included physiological responses, such as blood digestion, or excretion; fitness response was evaluated using parameters such as onset of oviposition, total number of egg laid, eggs bouts, number of eggs per bout, and sand fly overall survival. Suggestive differences were observed between the three species with regards to several parameters assessed. However, it may be important to keep in mind that the flies used in this study are colony flies, with free and continuous access to sugar and maintained at constant temperatures. Moreover, fluctuation in colony numbers of sand fly species happens and that some experimental replicates were done at different periods. Thus, certain results obtained through the analyses should be taken in that context. Results obtained from the various parameters investigated are further discussed below.

Effect of PpChit1 on blood digestion

No statistical significant differences were observed regarding blood digestion between anti-PpChit1 fed or naïve sera fed flies for all three sand fly species tested (PPIS, PDMA and LLJB). These results are similar to observations by Pimenta et al. (1997) in which no difference in blood meal loss/excretion was observed when comparing between blood fed flies, infected blood fed flies, and blood fed treated with chitinase. However, Pimenta et al (1997) did observe differences in blood meal loss in chitinase-treated infected flies compared to the other groups. Moreover, greatest decline of hemoglobin occurred between 48 h and 72 h with complete loss of the blood meal by 96 h.

An apparent difference was detected in the excretion of the blood in PDMA flies at 30h and 36h PBM (Table 2.4), as indicate by the concentration of hemoglobin in comparison to PPIS

and LLJB (Tables 2.3 and 2.5). In this case, in PDMA fed on anti-PpChit1 sera displayed a rate of digestion that was faster that naïve sera fed flies whereas the opposite was observed in PPIS and LLJB for the same time points. Though no statistical difference was observed in any of the treatments in all three flies, it is plausible that in *P. duboscqi* the PM kinetics affected the results observed. Another possibility is that upon feeding, chitinase is secreted into the gut lumen as an inactive pro-enzyme that is later activated by trypsin (Shen and Jacobs-Lorena, 1997). Thus, if inhibitors of trypsin are present in the gut between 24 h and 48 h PBM, the activation of chitinase might not be achieved until the levels of this inhibitors decrease.

In *Anopheles stephensi* and *Ae. aegypti*, lack of PM following feeding with anti-PM antibodies led to a faster digestion of the blood meal in these mosquitoes (Villalon et al., 2003) In sand flies, digestion of the blood meal appears to be affected by the absence of the PM (Oliveira de Araujo et al., 2012). Possibly, digestion may also be affected by either a thicker or thinner PM.

Effect of anti-PpChit1 on sand fly fitness

Sand flies feed on plant nectar and vertebrate blood. Hematophagy provides females with essential nutrients used for egg development (Secundino et al., 2005b; Villalon et al., 2003). A slower blood digestion, to some extent, expected after feeding with anti-PpChit1, would then lead to slower egg production (oviposition) due to longer lasting PM affecting absorption of nutrients. In our results, however, no difference in blood digestion between control and experimental flies of the three species (PPIS, PDMA, and LLJB) was observed.

Regarding the number of eggs laid by females fed either with anti-PpChit1 or naïve sera, no difference was observed for any of the three sand fly species, PPIS, PDMA, or LLJB. In a similar fashion, no difference in reproduction and fecundity (egg production) were observed in *A. stephensi* and *Ae. aegypti* fed with antisera targeting the PM (Kato et al.(2008); Villalon et al. (2003).

Interestingly, we observed a difference which may be considered as “approaching significance” with regards to the onset of oviposition in PPIS flies fed with anti-PpChit1. PPIS fed on anti-PpChit1 sera took approximately one extra day to start laying eggs in comparison to the flies that were fed the naïve sera. Recently, it has been shown that in *Lu. longipalpis* fed on blood containing exogenous chitinase oviposition onset occurred sooner and produced fewer

eggs than control fed flies (Oliveira de Araujo et al., 2012). Taken together these data suggest that, following digestion of the blood meal, the presence of the PM in sand flies somehow slows down the absorption of nutrients that are used for egg development and egg laying.

Regarding survival, a slight difference which we considered as "approaching significance" (p=0.065) was observed in PPIS flies that fed on blood containing anti-PpChit1 in comparison to flies that fed on naïve sera: PPIS flies fed on anti-PpChit1 lived approximately three days longer. In contrast, complete removal of the PM by exogenous chitinase (Oliveira de Araujo et al., 2012) seems to have no effect. In our case, at least for PPIS, the feeding of anti-PpChit1 leading to a thicker PM possibly provided additional heme binding sites (HRM from peritrophins associated with the PM), to further reduce the toxic effects of this molecule.

Gravid flies that never laid eggs

Several gravid sand flies in each of the three groups (PPIS, PDMA and LLJB) treated with anti-PpChit1 did not lay eggs even when surviving longer than flies that laid eggs. To us, that raised the question of whether the effect was due to the anti-PpChit1 treatment. *p*-values "approaching significance" in LLJB and PDMA somewhat suggest that anti-PpChit1 is able to affect fertility in these flies. During the process of egg development, secretion of vitellogenin (Vg) and other yolk proteins is activated in the fat body, and these proteins are stored in the primary oocytes supporting egg maturation. The production of yolk protein is activated by amino acids obtained from the digested blood meal, and by the ecdysteroid hormone (EDH) secreted by the ovaries. These steps are also regulated by the release of neuropeptides from the brain in response to blood ingestion (Gulia-Nuss et al., 2011). Thus, in flies that did not lay eggs perhaps one or more of the components of this complex network of proteins, protein signaling molecules, hormones, and neuropeptides leading to egg development and egg laying might have been negatively affected by feeding with anti-PpChit1. Further studies to clarify the effect of anti-PpChit1 on treated flies are necessary to address this question.

Overall, the fitness parameters analyzed in this study complement the observations made when treating sand flies with exogenous chitinase that leads to disruption of the PM (Oliveira de Araujo et al., 2012). Therefore, it is reasoned that with a thicker and more persistent PM, the fitness of the fly is influenced, in this case, a slower acquisition and absorption of nutrients can occur; therefore, a delay or absence in egg production is observed.

Conclusion

PpChit1 is a sand fly midgut specific chitinase involved in the modulation and degradation of the PM. Specific anti-PpChit1 sera was produced to feed sand flies in order to evaluate its effect on the fitness. These results suggest that the structure of sand fly PM was modified by anti-PpChit1 causing the PM to be thicker and last longer and that likely interfered with certain fitness parameters in the sand fly. Statistical analyses suggest that the digestion process of the blood meal is not altered by the treatment with anti-PpChit1. However, for PPIS sand flies, the fitness responses such as onset of oviposition, and survival were affected. Despite the lack of statistical significance in the fitness parameters between control and anti-PpChit1 for PDMA and LLJB, several gravid flies in each of the three species that were treated with anti-PpChit1 never laid eggs even though these insects live between 3-7 days longer than the flies that were able to lay eggs.

Future research

Future research will be aimed at investigating the rate of absorption of lipids and/or proteins by enterocytes (in the midgut) and trophocytes (in the fat body) and how this rate affects egg development. We shall also investigate whether the thickening of the PM following feeding with anti-PpChit1 changes the profile of expression of certain genes regulated by blood feeding, including vitellogenin.

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Chapter 3 - **Literature Review**

Phlebotomine Sand Flies and *Leishmania*

Sand flies as vectors of leishmaniasis

Phlebotomine sand flies (Diptera:Psychodidae) are the principal vectors of leishmaniasis in tropical and semitropical zones around the World, including Central, South America, the Mediterranean, Middle East, and the Indian Subcontinent (Munstermann, 2005; Sacks et al., 2008). Leishmaniasis is a multi-spectrum disease ranging from self-healing cutaneous lesions to visceral disease and in terms of severity, from asymptomatic to fatal cases (Dostalova and Volf, 2012; Ramalho-Ortigao et al., 2010). The global burden has been estimated to be approximately 500,000 cases of visceral leishmaniasis (VL) and close to 1.1-1.5 million cases of cutaneous leishmaniasis (CL) per year (Reithinger, 2008; WHO, 2010). Sand flies are typically not anthropophilic and the preferred host is the easiest available. Thus, humans are an auxiliary host for sand flies that have developed the capacity to adapt in urban environments (Munstermann, 2005).

Sand flies are essential to support the development of *Leishmania* parasites (Sacks et al., 2008). Approximately 900 sand flies species have been identified but only 93 species are proven or probable vectors of human and animal diseases (Ramalho-Ortigao et al., 2010). Primarily, species and subspecies of *Phlebotomus* in the Old World and *Lutzomyia* in the New World (WHO, 2010) are involved in the transmission cycle of *Leishmania* spp.

Sand flies-*Leishmania* interaction

Sand flies can be categorized into permissive (or non-restrictive or non-specific), and non-permissive (or specific or restrictive). Permissive sand fly vectors are those that support development of multiple species of *Leishmania*. An example is *Phlebotomus arabicus* which has been shown to allow development of such *Leishmania* species as *Le. tropica* (Svobodova et al., 2006), as well as *Le. major* and *Le. infantum* (Myskova et al., 2007). The relationship between permissive vectors and parasites is due to the role of O-glycosylated proteins that have a terminal N-Acetyl-galactosamine and a lectin-like activity on the surface of *Leishmania* for attachment to microvilli surface of the sand fly midgut (Evangelista and Leite, 2002; Myskova et al., 2007).

Restrictive or specific sand fly vectors refer to sand flies that only allow the development of a single *Leishmania* species Examples of restrictive sand flies include *Phlebotomus papatasi*, the principal vector of *Le. major* (Killick-Kendrick et al., 1994; Pimenta et al., 1994), and *Phlebotomus sergenti*, which is a vector of *Le. tropica* (Kamhawi et al., 2000). Most sand fly species support the development of a broad range of *Leishmania* species making them permissive vectors (Myskova et al., 2007).

When a female sand fly takes an infected blood meal from the host, the blood containing amastigotes reach the midgut via the stomodeal valve that regulates the fluid flow into the gut (Sacks et al., 2008). Although *Leishmania* are obligatory intracellular parasites when within the vertebrate host, within the sand fly vector *Leishmania* development occurs in, and is generally restricted to the midgut lumen. Moreover, *Leishmania* development within the lumen can be divided according which portions of the sand fly midgut they are localized. Peripylarian parasites establish an initial infection in the hindgut (pylorus) with promatigotes, the flagellated and motile form, attaching to the cuticular lining. Further during the course of development these parasites migrate to the anterior parts including the midgut and foregut. *Le. braziliensis* (subgenus *Viannia)* is an example of such development. In contrast, suprapylarian parasites development takes place strictly in the midgut and foregut of the sand fly host. Most of the *Leishmania* causing human leishmaniasis display suprapylarian development within the sand fly vector (Dostalova and Volf, 2012).

During its development within the sand fly vector, *Leishmania* parasites are faced with some formidable barriers. These barriers include a proteolytic attack by proteases secreted upon blood feeding; the need to escape the endoperitrophic space likely through the peritrophic matrix (PM) in order to prevent excretion with the blood meal; the required attachment to the midgut epithelia also to prevent excretion; followed by detachment from the epithelia and the need to migrate to the anterior portion of the midgut (stomodeal valve) and mouth parts for transmission during the next blood feeding attempt (Rogers et al., 2008; Schlein et al., 1992; Volf et al., 2004); (Ramalho-Ortigao et al., 2010; Ready, 2013).

Sand fly molecules during *Leishmania* infection

Digestive enzymes secreted into the midgut of sand flies following the ingestion of the blood meal can contribute to the success of *Leishmania* development. Trypsins, chymotrypsins

(Ramalho-Ortigao et al., 2003), serine proteases, metallocarboxypeptidases, and astacin-like metalloproteases (Jochim et al., 2008; Ramalho-Ortigao et al., 2007) are up-regulated after a blood meal and known to influence survival of *Leishmania* in sand fly midgut (Pimenta et al., 1997). Recently, Sigle and Ramalho-Ortigao (2013) reported two Kazal-type serine proteinase inhibitors, PpKzl1 and PpKzl2, in *P. papatasi* that are up-regulated after a blood meal. Further, PpKzl2 was shown to inhibit α-chymotrypsin, α-thrombin and, trypsin, and is likely involved in regulating digestive enzymes that can affect *Leishmania* development within the sand fly.

Leishmania molecules promoting infection

It has been shown that *Leishmania* is able alter the behavior of its sand fly host. One example of such change is due to the secretion of a proteophosphoglycan (PPG), mucin-like gel, known as promastigote secretory gel (PSG) that accumulates in the sand fly gut and mouthparts (Rogers, 2012). PSG blocks the lumen of the anterior midgut of the fly and the stomodeal valve, which in addition to the damage caused by a chitinase secreted by the parasites (Schlein et al., 1991), causes the valve to remain open. As the stomodeal valve is no longer able to function during attempts to blood feed, regurgitation by the infected sand fly ensues, and infectious metacyclic promastigotes, as well as fragments of the PSG are transmitted (Rogers et al., 2002; Sacks et al., 2008; Stierhof et al., 1999). As this "blocked" sand fly cannot acquire sufficient blood in its attempt, it continues to probe, and transmit parasites during these attempts. Thus, the infected sand flies probe more frequently, take more time to feed, and likely ingest incomplete blood meals compared to non-infected sand flies (Killick-Kendrick et al., 1977; Maia et al., 2011; Rogers and Bates, 2007; Rogers et al., 2002).

It has also been shown that glycosylphosphatidylinositol (GPI)-anchored molecules are important for *Leishmania* attachment to the midgut of sand flies. Hajmova et al. (2004) described that a zinc metalloproteinase gp63 on the surface of *Leishmania* could play an important role in early development of parasites and sand fly infection. It was observed that over-expression of the gp63 protease promoted early development of parasites compared to slow development with down-regulation of gp63. Moreover, sand fly infections using parasites with down-regulated gp63 had a low parasite load compared to normal *Le. amazonensis* in *Lutzomyia longipalpis* (Hajmova et al., 2004). These results were in contrast to Joshi et al. (1998) suggesting that gp63 was not essential for development and survival of *Le. major* in *P. papatasi* and *P. argentipes*.

More recently, Jecna et al. (2013) demonstrated the role of gp63 on *Le. amazonensis* development in *Lu. longipalpis* by an *in vitro* competitive binding assay. Down-regulation of gp63 in *Le. amazonensis* transfectants led to non-efficient binding of the parasites to the midgut of *Lu. longipalpis*. Moreover, their results also suggested a critical role for gp63 in *Le. mexicana* attachment to *Lu. longipalpis* midgut as 99% of wild type (WT) parasites were bound to the midgut whereas only 25% of the *Le. amazonensis* that did not express GPI-anchored proteins including gp63, but with normal levels of lipophosphoglycan (LPG), bound to the *Lu. longipalpis* gut. These data together with previous reports on the role of gp63 in *Leishmania* attachment (Sadlova et al., 2006) are a clear indication of the role of these proteins during *Leishmania* development within the sand fly vector.

One of the best studied molecules responsible for *Leishmania* attachment to the midgut epithelium is LPG. The crucial role of LPG in *Le. major* binding to microvilli lining the *P. papatasi* midgut has been previously demonstrated (Kamhawi et al., 2000; Pimenta et al., 1994; Pimenta et al., 1992; Sacks et al., 1995). LPG is crucial for attachment to the midgut of the restrictive vector *P. papatasi*. It is also apparently important for the binding and development of *Le. infantum* in its natural vector, *P. perniciosus* (Jecna et al., 2013*)*. However, when this group tested the binding of LPG deficient *Le. infantum* and wild type (WT) in *P. sergenti*, they observed that no parasites in both groups bind to the midgut of *P. sergenti*. Thus, suggesting that in this pair *Le. infantum-P. sergenti* LPG is not a determinant (or independent of LPG) for attachment. Therefore, confirming the specificity of *P. sergenti* for *Le. tropica*. In brief, *Leishmania* LPG and protease GP63 are important GPI-anchored components for attachment to the sand fly midgut.

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Chapter 4 - **Transmission of *Leishmania infantum* in American Foxhounds by sand fly bite**

Abstract

Phlebotomine sand flies are the principal vectors of leishmaniasis, a neglected tropical disease caused by parasites of the genus *Leishmania*. Endemic transmission of leishmaniasis has been reported in 98 countries with an annual incidence of 2 million human cases. Since 1999, several cases of canine visceral leishmaniasis (CVL) have been reported in Foxhounds in North America. Strong evidence points to vertical and horizontal transmission of the parasite *Leishmania infantum* among these dogs, however a sand fly transmission route is also thought to occur. To this extent, a model with naturally infected Foxhounds was used to assess the development of *Le. infantum* in a sand fly vector and the subsequent transmission of the parasites to a naive vertebrate host following the bite of infected sand flies. The results in this study indicate that VL-symptomatic Foxhounds (naturally infected) are highly infectious to laboratory-reared *Lutzomyia longipalpis* sand flies. Moreover, parasites fully develop within these flies and are transmitted to naïve vertebrate hosts following the bite of infected sand flies. Thus, the potential exists for parasite transmission to humans and the risk of visceral leishmaniasis to become endemic in North America.

Introduction

Leishmaniasis is a zoonotic disease in which dogs are the most important reservoir of human visceral leishmaniasis (VL) caused by *Le. infantum* (Ashford et al., 1998). Parasites are thought to be generally transmitted by phlebotomine sand flies but another rare modes of transmission include congenital and parenteral via blood transfusion and, needle injection (Magill et al., 1993). An outbreak of canine visceral leishmaniasis (CVL) was reported in 1999 in North America (Gaskin et al., 2002), especially among Foxhounds. Strong evidence points to vertical (Boggiatto et al., 2011) and suspected horizontal transmission of *Le. infantum* among these dogs. However, vector-borne transmission by sand flies could be the mode that explains visceral leishmaniasis in foxhounds.

Fourteen species of *Lutzomyia* sand flies have been recorded in North America, and at least three species (*Lutzomyia anthophora, Lutzomyia diabolica,* and *Lutzomyia shannoni*) have been shown to be *Leishmania* vectors. *Leishmania mexicana* for example is considered endemic in south-central Texas (Petersen, 2009) and the cause of cutaneous leishmaniasis in Texas and Mexico. *Lu. diabolica* is believed to be the primary vector for *Le. mexicana* in the New World (Young and Perkins, 1984) but it is uncertain if this sand fly species represent a vector of leishmaniasis at this site. However, cats, dogs, and people have been diagnosed with cutaneous leishmaniasis in Texas (Trainor et al., 2010; Wright et al., 2008). More recently, Clarke et al. (2012) reported the emergence of autochthonous cases of cutaneous leishmaniasis in northeastern Texas and southeastern Oklahoma.

The principal sand fly vector of *Le. chagasi*, the causative agent of visceral leishmaniasis in the New World, is *Lu. longipalpis.* This sand fly has never been reported in North America. *Lutzomyia vexator,* another native species of sand fly, and *Lu. shannoni*, have been recently identified in two additional states of the U.S. (Weng et al., 2012). As far as we know, *Lu. vexator* is currently found in 23 states in the U.S. and *Lu. shannoni* have been reported in 16 states (Claborn et al., 2009; Haddow et al., 2008; Minter et al., 2009; Price et al., 2011; Young and Perkins, 1984).

The objective of this study was to assess whether the *Le. infantum* parasites circulating among Foxhounds in the U.S are able to fully develop within sand flies vectors and be able to be transmitted to a suitable vertebrate host during feeding.

Materials and Methods

Ethics statement

The use of animals during this study was reviewed and approved by Iowa State University Laboratory Animal Resources (LAR).

Experimental animals

Two *Le. infantum*-infected American Foxhounds donated to Dr. Christine Petersen's laboratory at Iowa State University were used in this study. The dogs were one 6 years old female and one 7 years old male. Both animals had tested positive for *Le. infantum* by PCR and serology via the indirect immunofluorescent antibody test (IFAT) with the recombinant antigen K39. Additionally, both dogs were considered polysymptomatic for visceral leishmaniasis, with signs including weight loss, rough hair coat, lymphanomegaly, splenomegaly, and cutaneous lesions. Healthy hamsters were purchased under the care of Iowa State University-LAR

Sand fly rearing and infection with *Le. infantum*

Lutzomyia longipalpis (Jacobina strain -LLJB) was reared in the Biology of Disease Vectors laboratory at the Department of Entomology, Kansas State University, at 26 ^{o}C and 70 % humidity in a 12:12 light-dark cycle. In order to evaluate sand fly transmission, two-to-three day old female sand flies were fed on the ventral part of the ears of two naturally infected with *Le. infantum* Foxhound housed at Iowa State University. Immediately after feeding, engorged females were separated from non-fed flies. The presence or absence of blood in the sand fly digestive tract was verified by anesthetizing flies with CO_2 and observing the midgut distension under a stereomicroscope (Carl Zeiss, Thornwood, NY). In order to assess parasite development, blood fed flies were dissected daily after 72 h post blood meal (PBM). The midgut of three flies was dissected, homogenized in 1X phosphate-buffer salt solution (PBS), and observed for parasite development in a hemocytometer chamber. The development of the parasite in the sand fly was monitored for approximately 12 days.

DNA extraction and PCR to detect *Leishmania* DNA in sand flies

Genomic DNA of individual *Lu. longipalpis* females was extracted using 10 % Chelex 100 resin beads (Bio Rad). Sand flies were homogenized individually in 20 µl of molecular grade

water, heated in 120 µl of 10 % Chelex solution at 95 °C for 30 min, centrifuged briefly (6 sec) at 14,000 × g, and the supernatant transferred to a new tube. DNA extraction was confirmed by amplification of *Leishmania* 145 bp fragment targeting kDNA minicircle (10,000 copies) to *Le. donovani* complex using GoTaq Colorless Master Mix (Promega, Madison, WI), 10µM each forward RV1 (5'-CTTTTCTGGTCCCGCGGGTAGG-3') and reverse RV2 (5'-CCACCTGGCCTATTTTACACCA-3') primers, in 25 µl reaction. PCR conditions were set at 94 °C for 2 min, followed by 40 cycles of denaturation (94 °C; 1 min), annealing (59 °C ; 1 min), and extension (72 °C; 1 min), with a final extension of 72 °C for 10 min (Lachaud et al., 2002). PCR products were visualized following electrophoresis on 1.5 % agarose gel with ethidium bromide.

Hamster infection through sand fly bite

On day thirteen, three infected and two non-infected flies were put in a small plastic vial (cole-palmer: 47.6 mm height x 19.1 mm diameter) covered with a thin-nylon fabric mesh at one end. One vial was placed against the ventral side of the left ear and one on the ventral side of the right ear of an anesthetized (isoflurane) hamster. Clamps were used to attach the plastic vial to the ear of the hamster and to create a flat surface for easy access of the fly to the site of feeding; the process was performed for approximately 1 h in dark. After direct feeding by sand flies, animals were monitored daily by LAR staff for five months and examined for clinical signs of leishmaniasis.

Hamster blood and tissue collection

Blood samples were collected prior to sand fly infection followed by collections at 2 weeks, and 1, 2, 3, 4, and 5 months post-sand fly exposure.

Hamsters were placed under anesthesia via inhalation of isoflurane. Blood samples (roughly 0.33 ml) were collected from the lateral saphenous vein in capillary tubes and cryo-preserved at -80 ^{o}C for further analysis of parasite load by real time quantitative PCR (RT-qPCR).

Five months post-sand fly exposure, blood samples were collected and the hamsters were humanely sacrificed via intraperitoneal injection of pentobarbital (1 ml/ 10 lbs). Tissues samples including those from spleen, liver, and lymph nodes were collected to ascertain parasite

infection. Tissue samples were used in impression smears, histopathological examination, RT-qPCR analysis, and parasite culture.

DNA extraction

DNA from blood samples was isolated using the Qiagen blood DNA isolation kit (Qiagen Sciences, Maryland) according to the manufacturer's instructions.

DNA from tissues was extracted using a modification of the Qiagen blood DNA isolation kit (Qiagen Sciences, Maryland). Small sections of tissue were placed into a sterile tissue grinder with 80 µl of PBS and mechanically disrupted. Samples were transferred to 1.5 ml centrifuge tubes and added 100 µl of buffer AL and 20 µl of protease. The sample was vortex and incubated at 56 ^{o}C overnight. The sample was briefly centrifuged and 100 µl of buffer AL was added. After this point, DNA isolation was performed following the manufacture's protocol at the incubation step. DNA concentration was measured by a NanoDrop ND1000 spectrophotometer (Wilmington, DE).

Detection of *Le. infantum* by RT-qPCR

Le. infantum (LIVT2, ATCC) was grown in complete Grace's medium (incomplete Grace's supplemented with 20% fetal bovine serum, 100 U/ml penicillin, 100 µg/ml streptomycin, and 2 mM L-glutamine). Parasites were harvested by centrifugation at 2500 x g for 15 min at 4 ^{o}C, washed twice with PBS, and resuspended in PBS to a concentration of 10^{7} parasites per milliliter. Fifty microliters of this solution was spiked into 150 µl of negative canine blood.

Le. infantum kinetiplast DNA (kDNA) was detected using specific primers and probes F 5'-CCGCCCGCCTCAAGAC, R 5'-TGCTGAATATTGGTGGTTTTGG, (Integrated DNA Technologies, Coralville, IA) and, Probe 5'-6FAM-AGCCGCGAGGACC-MGBNFQ (Applied Biosystems, Foster City, CA) (FAM: laser-activated reporter dye; MGBNFQ: 3'-minor-groove binder non-fluorescent quencher). BLAST analysis indicated that these primers and probe were specific for *Le. infantum.* DNA from *Le. amazonensis* or *Le. major* parasites did not amplify using this primer and probe set.

Leishmania SSU rRNA was identified using specific fluorogenic probe LEIS.P1 (5'-6-carboxyfluorescein [6-FAM]-CGGTTCGGTGTGTGGCGCC-3') and its flanking primers

LEIS.U1 (5'-AAGTGCTTTCCCATCGCAACT-3'); and LEIS.L1(5'-GACGCACTAAACCCCTCCAA-3' previously designed (Wortmann et al., 2001) and obtained from Applied Biosystems, Foster City, CA. Blood DNA samples were analyzed by qPCR in duplicate using a 96-well format of two concentrations, whole blood and a dilution 1:10. Amplification was performed using a Stratagene Mx3005P qPCR system, Perfecta qPCR super Mix (Quata Biosciences, Gaithersburg, MD), and Low ROX master mix (Quanta Biosciences, Gaithersburg, MD). Primers were used at 775 nM and probe at 150 nM with thermocycling at 95 ^{o}C for 3 min, followed by 50 cycles at 95 ^{o}C for 15 s, and 60 ^{o}C for 1 min. Results were analyzed by MxPRO QPCR software version 4.01.

Results

Sand fly infection

In order to assess if *Le. infantum* are able to develop in the sand fly, approximately 300 female sand flies were fed on a naturally infected Foxhound housed at Iowa State University. Seventy two hours post blood feeding (PBF), development of parasites and infection of flies was confirmed by observing *Le. infantum* promastigotes in the midgut of dissected flies. A total of 41 flies were dissected during 11 days that development of parasites was monitored. Apparently, 11 flies (27%) did not feed since no blood was observed in the midgut of these flies and no eggs were developed. Thirty flies (73%) had at least one parasite in the midgut. Replication of parasites and change of stages was observed as the infection was advancing. Ten days PBF, it was clearly evident the migration and attachment of metacyclic promastigotes to the stomodeal valve (Fig. 4.1) which was a hallmark of infection.

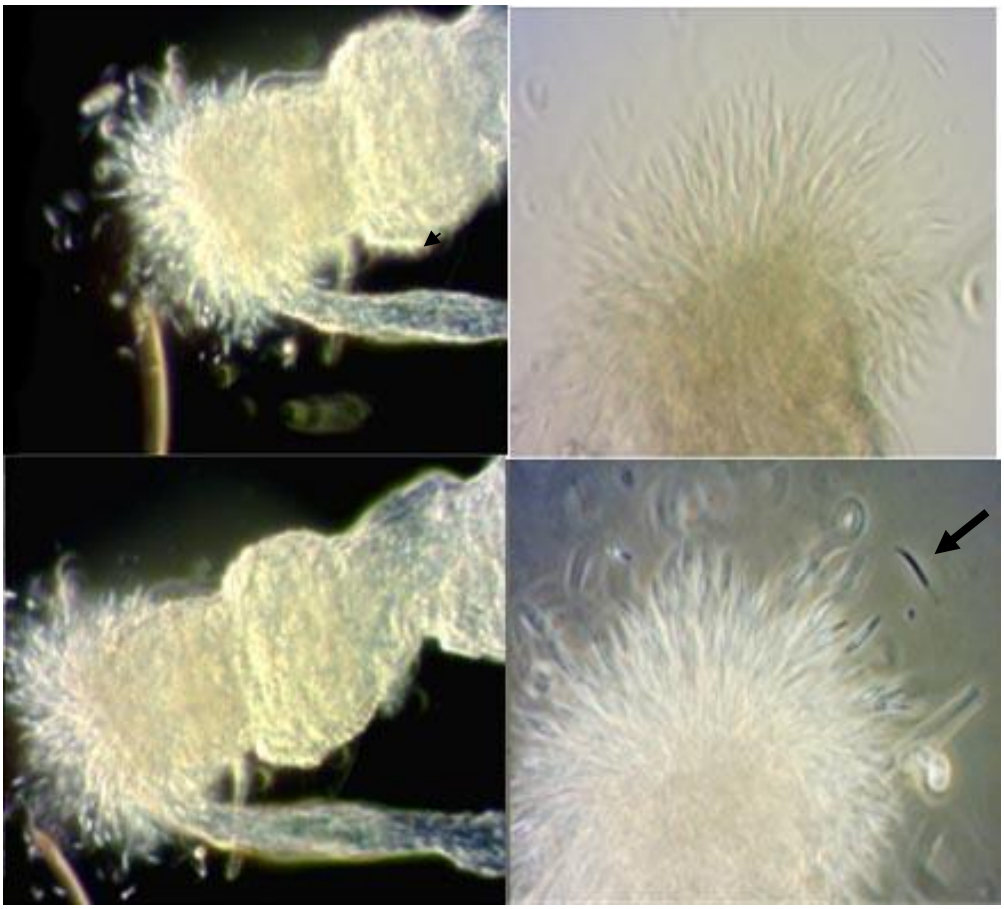

Figure 4.1 Hallmark of mature *Le. infantum* infection of laboratory-reared sand flies (*Lu. longipalpis*). Picture shows parasites colonizing the sand fly stomodeal valve 10 days after feeding on naturally infected Foxhound. The pictures illustrate the blockage of the stomodeal valve by *Le. infantum*. Arrow points at a free swimming metacyclic promastigote parasite.

Le. infantum transmission to a vertebrate host

With the attempt to transmit *Le. infantum* to a vertebrate host, 13 days PBF and infection of sand flies, a group of these flies was brought back to Iowa State University to feed on seven naïve hamsters. Four days PBF, the midgut of sand flies that fed on hamsters and were still alive were dissected to confirm *Leishmania* development microscopically or by PCR (Table 4.1). A total of 30 flies attempted to feed on hamsters, 11 flies fed and were recovered for *Leishmania* DNA detection. PCR results indicated that five flies were positive for *Leishmania.* Thus, these results confirmed that flies feeding on three hamsters had *Le. infantum* in the midgut. This was a quick analysis that anticipated the possible outcome of infection in the hamsters.

Table 4.1 Representation of sand flies that fed on corresponding hamster and were tested for *Leishmania* DNA. Five sand flies were set to feed on the ears of hamsters; three infected and two non-infected. Sand flies that fed on the hamster were tested to confirm that the fly had parasites and potentially transmitted them to the host.

hamster #	Total # of sand flies with blood assessed by PCR	Results of sand flies assessed by PCR
1	1	1 Negative
2	2	2 Positive
3	1	1 Negative
4	No flies fed on this animal	No flies fed on this animal
5	3	2 Positive
		1 Negative
6	2	1 Positive
		1 Negative
7	2	2 Negative

Detection of *Le. infantum* by RT-qPCR

After infection, blood samples of hamsters were collected at two weeks and each month until 5 months. RT-qPCR was performed to test for *Le. infantum* DNA. Two months after infection, *Le. infantum* was detected in hamster 1 and hamster 5 with a threshold cycle (Ct) of 43.88 and 34.38 respectively compared to positive control with Ct=25. Three months later, a sample of hamster 2 amplified for *Leishmania* DNA with a Ct=28.27. This indicated that hamster 2 had more parasites than hamster 1 and 5. Additionally, three months after infection, a single cutaneous lesion became apparent on the nose of hamsters 5, this was consistent with signs of *Leishmania* infection (Fig. 4.2). Four months later, hamster 6 was confirmed positive for *Le. infantum* since a sample amplified for *Leishmania* DNA given a Ct=45. Five months after infection, the facial lesion on hamster 5 appeared to have healed. Hamsters were euthanized at this point. No other clinical signs of disease were noted throughout this trial.

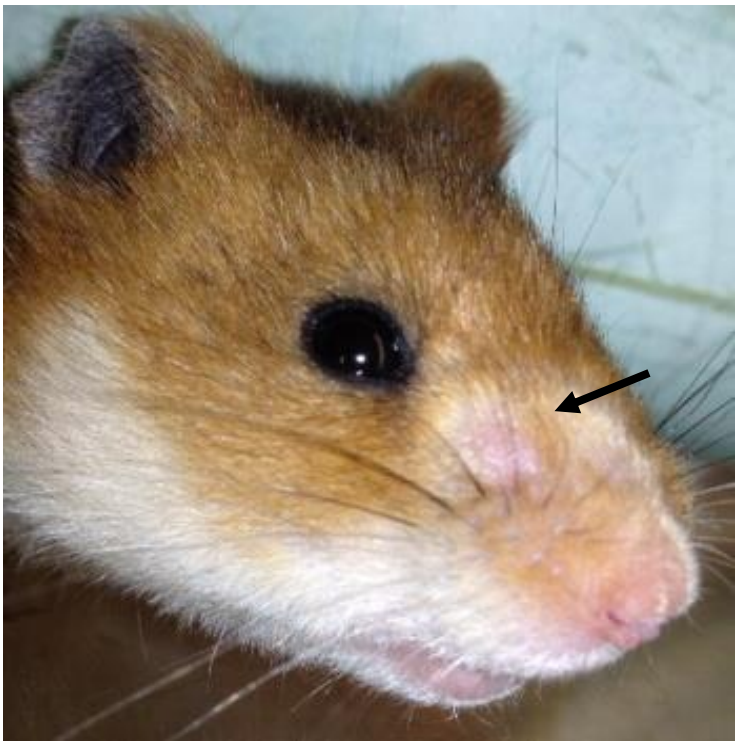

Figure 4.2 Dermal (skin) lesion on hamster infected with *Le. infantum* via sand fly bite. Pictures shows hamster #5, infected via the bite of sand flies with the *Le. infantum* strain circulating among Foxhounds and responsible for the current outbreak of canine VL in the dogs. Arrow points to (unusual) skin lesion caused by the infection with *Le. infantum*.

Furthermore, section of liver and spleen from each hamster were sent to the center for disease control and prevention (CDC) for the culture of parasites; however, there was no growth of parasites.

Discussion

The relevance of this study is to understand 1) the potential of *Le. infantum* strain originally from the Mediterranean basin and currently found in infected Foxhounds in the U.S. to development in a sand fly vector, 2) to prove the transmission of the parasite to a healthy vertebrate host and, 3) to follow the progression of the disease. Results of this study indicate that VL-symptomatic Foxhounds, naturally infected, are highly infectious to laboratory-reared sand flies. Moreover, parasites fully develop within these flies and are transmitted to naïve vertebrate hosts following the bite of infected sand flies. Previous studies have shown that *Le. infantum* is

able to develop within sand flies after blood feeding on naturally infected dogs (Killick-Kendrick et al.(1994). According to Killick-Kendrick et al (1994), development of *Le. infantum* promastigotes in *Lu. longipalpis* was observed between three and nine days after blood feeding. In addition, infection of dogs also was performed by needle injection of metacyclic promastigotes that developed and were isolated from sand flies. All dogs that were inoculated with parasites were confirmed to be infected. Some developed skin lesions between six and 14 weeks after inoculation, but spontaneously healed three to four months later. In our study, at least one of the hamsters used for transmission of *Le. infantum* by the infected sand flies also developed a skin lesion (Fig 4.2) which also spontaneously healed five months later; thus, consistent with previous observations.

The capacity to acquire *Le. infantum* from naturally infected dogs was compared between *Lu. shannoni* and *Lu. longipalpis* (Travi et al. (2002). It was shown that in spite of the low infection rates observed in *Lu. shannoni* (9%), the intensity of infection (200-500 promastigotes/fly) was higher in comparison to *Lu. longipalpis*. Although that study pointed out the ability of *Le. infantum* to develop in *Lu. shannoni*, it did not assess the transmission of the parasites to vertebrate hosts by sand fly bites.

Canids such as coyotes (*Canis latrans),* red fox (*Vulpes vulpes*), and grey fox (*Urocyon cinereoargenteus*), as well as opossum (*Didelphis virginiana)* are potential reservoirs of visceral leishmaniasis. These animals are widely distributed throughout North America and are commonly found near humans (Rosypal et al., 2010; Travi et al., 2002). *Lu. shannoni* is a native sand fly species in North America whose range expansion has been the subject of several studies, including recent state records in Kansas and Missouri (Weng et al., 2012). The occurrence of *Lu. shannoni*, potential vectors of *Leishmania*, in areas where these flies overlap with the distribution of the potential reservoirs indicated above, and of naturally infected Foxhounds could bring together the components necessary for the establishment of a sylvatic cycle of *Le. infantum* in North America, thus becoming an endemic area for this pathogen.

Conclusion

This research provides evidence that *Le. infantum* currently circulating among American Foxhounds are able to fully develop in sand flies (i.e. *Lu. longipalpis*) after blood meal, and be transmitted to a suitable vertebrate host. Moreover, it is clear from our results that symptomatic

dogs are highly infectious to the sand flies. We reason that the *Le. infantum* strain (previously identified as MON-1) circulating in North America has maintained all the necessary requirements or "molecular machinery" (such as LPG and other GPI-anchored proteins) to fully develop within sand fly vectors. However, it remains to be determined if these molecular signatures are indeed conserved between *Le. infantum* circulating among the Foxhounds and those circulating in Europe or South America. The overlap of potential sand fly vectors such as *Lu. shannoni* and *Le. infantum*-infected Foxhounds puts domestic or companion dogs and humans at a risk, and also poses a risk for the parasite to become endemic in North America.

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