MOLECULAR ASPECTS OF SAND FLY-BASED VACCINE DEVELOPMENT

by

ILIANO VIEIRA COUTINHO ABREU GOMES

M.S., Federal University of Pernambuco, Brazil, 2006

AN ABSTRACT OF A DISSERTATION

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Abstract

The emergence and reemergence of vector-borne diseases pose significant threats to humans and other animals worldwide. Although vector control relies mostly on insecticides, the emergence of insecticide resistance urges for the development of new strategies to control the spread of such diseases. For sand fly-transmitted leishmaniasis, Transmission Blocking Vaccines (TBV) may constitute a feasible strategy to impair *Leishmania* transmission from infected to uninfected vertebrate hosts. Moreover, sand fly saliva-based vaccines represent an alternative or complementary approach as these vaccines protect different mammalian hosts against *Leishmania*. Based on the potential use of sand fly molecules as vaccines against leishmaniasis, we assessed the potential of *Phlebotomus papatasi* midgut secreted proteins as TBV candidates and the expression variability of sand fly salivary gland genes. Regarding the TBV approach, we took advantage of the RNA interference (RNAi) technique to evaluate the effects of knocking down *P. papatasi* midgut-specific genes on *Leishmania major* development within the sand fly midgut. Whereas peritrophin 1 (PpPer1) knock down led to increased *Le. major* load by 39%, knocking down chitinase 1 (PpChit1) reduced *Le. major* load in *P. papatasi* midguts by 63%. Thus, our data strongly suggest that PpChit1 constitutes a potential target for TBV approaches against *Leishmania* transmission in endemic areas. Concerning protective vaccines based on salivary gland secreted proteins, we searched for expression polymorphism in selected salivary gland genes in natural and colonized populations of *P. papatasi*. Significant differences in salivary gland gene expression were not only exhibited in *P. papatasi* specimens collected in different geographic habitats but also seasonal difference in gene expression was displayed by specimens belonging to the same population. As antigen dose is an important component of immune responses, different doses of salivary protein inoculated into host skin may interfere with vaccine protection. Thus, the efficacy of sand fly saliva-based vaccine upon exposure to different salivary protein doses must be evaluated before deployment in endemic areas. Our data also ruled out some biotic factors as responsible for fine-tuning the expression of such genes. Overall, this dissertation makes significant contribution to the development of sand fly-based vaccines against leishmaniasis.

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Dedication

I dedicate this dissertation to my wife, Patricia Cristina Monteiro de Barros, for her love, support, and encouragement throughout these years.

Preface

I started my Ph.D. at the Department of Biological Sciences at the University of Notre Dame in the Fall of 2007. Upon the transfer of my co-major professor, Dr. Marcelo Ramalho-Ortigao, to K-State in the Fall, 2008, I join his laboratory in the Department of Entomology at Kansas State University as a Ph.D. student. Thereby, this dissertation encompasses studies performed in both universities. In my view, such studies are complementary to the extent that they aimed to contributing to the potential development of sand fly-based vaccines against leishmaniasis.

At K-State, my research proposal focused on the identification of sand fly midgut antigens as candidates for the development of Transmission Blocking Vaccines (TBVs). Hence, I carried out the functional characterization of two midgut-secreted proteins of the sand fly *Phlebotomus papatasi* (Diptera: Psychodidae) involved in peritrophic matrix (PM) synthesis and degradation, and assessed their roles in sand fly vector competence to *Leishmania*. Despite the known role of the sand fly PM in vector competence, molecules involved in this process are yet to be identified. Using the RNA interference (RNAi), we analyzed the role of the *P. papatasi* midgut-specific chitinase 1 (PpChit1) and peritrophin 1 (PpPer1) on *Leishmania major* development in the sand fly midgut. In Chapter 1, an introduction to insect PM is presented. In addition, I also describe the role of the sand fly PM in vector competence. Manuscripts describing PpChit1 and PpPer1 functional characterization are found in Chapters 2 and 3, respectively. As an important outcome of studies related to vector-parasite interaction is the identification of potential targets to be used in strategies to control vector-borne diseases, such as TBVs, a manuscript is presented in the Appendix A, dealing with the effectiveness of vector and parasite molecules as TBV candidates.

At the laboratory of Dr. Mary Ann McDowell at Notre Dame, and under the supervision of Dr. Marcelo Ramalho-Ortigao, I was involved in a research project evaluating the polymorphism levels in sand fly salivary gland genes and the impact of these polymorphisms for sand fly saliva-based vaccine development. As the sand fly salivary proteins are immunomodulatory, vaccines based on these proteins may be used to switch the immune-response in the host skin from a non-protective Th2 to a protective Th1 immune-response against *Leishmania*. However, it is important to evaluate if these vaccines would be effective against

potential variations in antigen amount or epitope differences exhibited by sand flies. Hence, my research proposal comprised the assessment of expression polymorphisms of salivary gland-expressed genes in colonized and field populations of the sand fly *P. papatasi*. Besides the significance of this study for sand fly saliva-based vaccine development, the assessment of expression levels of salivary gland expressed genes in sand fly natural populations turned out to be a good model for ecological genetics studies regarding genes linked to vectorial capacity. An introduction to sand fly saliva, role of salivary components on immunomodulation, and ecological genetics is presented in Chapter 4. In Chapter 5, a manuscript describing gene expression polymorphisms of *P. papatasi* salivary gland-expressed genes caught in natural populations is shown. In Chapter 6, another manuscript emphasizing the role of aging and diet on the expression of such genes in colonized specimens of *P. papatasi* is presented. The assessment of the effects of aging and diet in *P. papatasi* salivary gland gene expression were important for understanding the ecological factors that might be involved in the expression modulation of such genes in natural habitats. Last, a summary of the findings is displayed in Chapter 7.

Overall, this dissertation covers important aspects related to sand fly-based vaccine development, from basic studies identifying antigens to be used in TBV strategies to more advanced ones dealing with assessment of antigenic polymorphisms in natural sand fly populations and their potential influences on vaccine efficacy.

Chapter 1 - Introduction I: The Potential of Sand fly Peritrophic Matrix-Associated Proteins as Transmission Blocking Vaccine Candidates

Introduction

The insect midgut is the only part of the alimentary tract not protected by a chitinous cuticle, and it is the place where food digestion and absorption take place (Tellam et al., 1999, Hegedus et al., 2009). This portion of the digestive tract of most insects, as well as other invertebrates, secretes a peritrophic matrix (PM), a noncellular semipermeable layer separating the contents of the gut lumen from the digestive epithelial cells. The presence of the PM was first noted in a caterpillar in 1762 by Lyonet (Tellam et al., 1999). By lining the midgut, the PM protects the midgut epithelium from abrasion, toxic compounds, and pathogens, as well as serving as a scaffold for proteases, peptidases, and glycosidases (Terra, 2001). Besides, the PM acts as a semi-permeable layer, separating the gut lumen into different compartments, thereby regulating the passage of molecules between such compartments and allowing the recycling of digestive enzymes (Terra, 2001). In light of its importance in insect physiology, the PM is a potential target in strategies to control pathogen transmission by insect vectors, such as Transmission Blocking Vaccines (TBVs). In this section, many aspects related to insect PM are portrayed, giving special emphasis to the role of the PM in sand fly vector competence. TBVs are thoroughly discussed in details in Appendix A.

Peritrophic Matrix Functions

An important PM function is the compartmentalization of the gut lumen in ectoperitrophic and endoperitrophic spaces (Terra, 2001), preventing enzymes that cleave polymers in the endoperitrophic space to be inhibited by the produced oligomers (Bolognesi et al., 2008). These smaller molecules cross the PM towards the ectoperitrophic space, reducing potential contact to polymer hydrolases, yet increasing contact with oligomer hydrolases, which are mostly localized in the ectoperitrophic space (Bolognesi et al., 2008). Hydrolysis of oligomers to monomer in the ectoperitrophic space also is advantageous as it increase the concentration of these molecules closer to the specific transporters localized on the midgut

epithelium (Bolognesi et al., 2008). Moreover, compartmentalization also allows the recycle of digestive enzymes (Terra, 2001). When the enzymes in the endoperitrophic space reach the PM posterior portion, they move to the ectoperitrophic space and are guided to the anterior portion of the midgut by the flow of fluid, where these molecules cross the PM back again to digest more food particles (Terra, 2001).

The insect PM also protects the midgut epithelium against damage caused by osmotic pressure (Lehane, 1997). The ingestion of high molecular weight food particles might exert a high osmotic pressure inside the midgut in the absence of PM, causing the swelling and rupture of the midgut epithelium cells (Lehane, 1997). In contrast, in the presence of a PM, hydrophilic proteoglycans attract osmotically active cations and in turn large amount of water, creating turgor pressure within the PM, which is counteracted by the chitin fibrils, reducing the osmotic pressure in the ectoperitrophic space (Lehane, 1997). Additionally, as digestion proceeds the solute concentrates on the PM luminal surface due to the PM permeability, followed by the solvent passage through the PM. In fact, the hydrostatic pressure within the PM may enhance the dehydration of watery foods, such as blood meal, by an increase in water and ions export to the ectoperitrophic space (Lehane, 1997).

Considering that the PM separates the ingested food from the midgut epithelium, it must be sufficiently permeable to allow digestive enzymes to cross and reach the food bolus and the products of the digestion to diffuse in the opposite direction to be absorbed by the epithelial cells (Lehane, 1997, Terra, 2001). The PM permeability cut-off for globular proteins are in the range of 6-200 kDa (or up to 7.5-9 nm) (Lehane, 1997, Terra, 2001). In the moth *Erinnyis ello*, smaller enzymes, such as trypsin and amylase, appear to be equally distributed inside (endoperitrophic space) and outside (ectoperitrophic space) of the PM, while bigger ones, like carboxypeptidase A and the secreted portion of N-acetylglucosaminidase, are mostly localized in the ectoperitrophic space (Lehane, 1997). Likewise, the PM of mosquito larvae has been estimated to be very permeable to particles smaller than 148 kDa (Edwards & Jacobs-Lorena, 2000).

The PM is also an important line of protection against toxic substances obtained through ingestion (Pascoa et al., 2002, Barbehenn & Stannard, 2004). In herbivorous insects, the PM acts as a sacrificial antioxidant by protecting the midgut epithelium from oxidative damage, becoming itself oxidized, and scavenging metal ions (Barbehenn & Stannard, 2004). Likewise, in

hematophagous insects PM sequesters heme, a toxic product of hemoglobin digestions, eliminating it along with the feces (Pascoa et al., 2002).

Peritrophic Matrix Structure

The insect PM is a highly regular orthogonal network of fibrils (Shi et al., 2004). The microfibrils contain between 20 and 400 individual chitin chains, organized in anti-parallel (α form) or parallel (β form) orientation (Merzendorfer & Zimoch, 2003). Each group of microfibrils assembles forming chitin bundles, which in turn form the backbone for PM assembly (Shi et al., 2004).

The midgut microvilli act as a template to produce chitin polymers by determining the space between chitin synthase enzymes (Lehane, 1997). Whether chitin chains are initially produced by chitin synthase localized on the midgut microvilli or in vesicles, where chitin fibers are accumulated and extruded into the midgut lumen, is yet to be determined (Merzendorfer & Zimoch, 2003).

The process of PM assembly appears to begin when chitin microfibrils are firstly secreted into the gut lumen (Lehane, 1997). As proteoglycan molecules are secreted, the chitin microfibrils are embedded in the proteoglycan matrix. Binding of various components, such as peritrophins, and layering of the PM probably occur during the gelling process (Lehane, 1997).

Peritrophic Matrix Types

Depending on their site of synthesis, PM is defined as Type 1 (PM1) or Type 2 (PM2). The two types of PM have a number of distinct properties, including morphology, composition, assembly, and function (Peters, 1991). The presence of each type of PM differs among insect species or developmental stages (larva versus adult); even when the same PM type is present in different life stages, there are differences in PM biochemistry between the different stages (Elvin et al., 1996).

PM type 1 (PM1)

The PM1 is synthesized by epithelial cells in the entire midgut and forms a bag-like structure containing the ingested meal (Peters, 1991). The thickness of the PM1 is typically in the range of 1-20 μm. The PM1 is the most common PM type in adult blood-sucking insects (Devenport & Jacobs-Lorena, 2005), with increasing thickness following the blood ingestion. No

PM1 is observed in sugar-fed insects (Devenport & Jacobs-Lorena, 2005). In nonhematophagous insects, the PM1 can be produced constitutively, as in *Locusta migratoria* (Tellam et al., 1999). The PM1 also is secreted by insects in the orders Dictyoptera (cockroaches), Orthoptera (grasshoppers), Coleoptera (beetles), and Hymenoptera (bees) (Terra, 2001).

PM Type 2 (PM2)

PM2 is produced by a specialized organ located at the junction of the cuticle-lined foregut and midgut, the cardia (Lehane, 1997, Tellam et al., 1999). This organ presents different cell types, and each cell type region appears to originate each layer of the PM2. The PM2 thickness is usually 0.1-2 μm, approximately 10 times thinner than the PM1 (Devenport & Jacobs-Lorena, 2005). Moreover, the PM2 constitutes an open-ended sleeve-like structure that lines the entire midgut and hindgut. It is constitutively secreted independently of the feeding status and is often more structured than the PM1. So, the PM2 may form as a single sleeve-like structure, as two concentric sleeves, or as three concentric sleeves, and each layer is often multilayered (Lehane, 1997). The larvae of higher dipterans and a few adult lepidopterans often produce this type of PM. Although the adult mosquito produces a PM1, the larva synthesizes a type 2 PM. Exceptions are the tsetse fly, *Glossina sp.*, and the stable fly *Stomoxys calcitrans*, in which adult stages secrete a PM2 (Lehane, 1997, Tellam et al., 1999, Terra, 2001, Devenport & Jacobs-Lorena, 2005).

PM Molecular Components

Proteins, including glycoproteins and proteoglycans, and chitin are the major components of the PM (Peters, 1991). Because the PM2 is easily obtained, its components have been the most studied (Tellam et al., 1999, Devenport & Jacobs-Lorena, 2005).

According to their solubility in a series of buffers of increasing ionic strength and denaturing ability, the PM2 proteins are separated in four classes (Tellam et al., 1999). The first group of proteins represents less than 1 % of the total PM proteins. These are easily removed using physiological or high-ionic-strength buffers and may represent digestive enzymes and proteins weakly attached to the PM. Proteins of the second group represent approximately 2 % of the total mass of the PM proteins. They are removed by relative gentle detergents that act disrupting the relatively weak protein-protein, protein-oligosaccharide, or protein-chitin interaction. The third group is extracted with strong denaturing agents (Urea, SDS or guanidine-

HCl) under non-reducing conditions and is constituted of integral/intrinsic membrane proteins or peritrophins. These proteins encompass approximately 11 % of the total PM mass. The fourth group is not extractable by treatment with strong detergents or denaturing agents and can make up a substantial portion of the total PM proteins (approximately 87 %). Probably, these proteins are covalently cross-linked either to themselves or to other constituents within the PM, such as chitin and proteoglycans (Tellam et al., 1999).

Chitin

Chitin, a linear polymer of β-(1,4)-*N*-D-acetylglucosamine (GlcNAc), is thought to be an important structural component of the PM. Chitin provides a scaffold onto which proteins and other components attach, thus providing strength and framework for assembly. Estimates of the PM chitin content range from 3 % to 40 %, though the tests used to measure it may not be accurate (Devenport & Jacobs-Lorena, 2005). Considering that chitinases can alter the structural integrity of the PM1, it serves as evidence for the presence of chitin in the PM1 (Villalon et al., 2003). In contrast, incubation of PM2 with chitinase did not present discernible changes in the structure of the PM (Edwards & Jacobs-Lorena, 2000). Thus, many of the structural characteristics of the type 2 PM may be derived from the interactions of the peritrophins with the GlcNAc-containing oligosaccharides of the other peritrophins (Edwards & Jacobs-Lorena, 2000).

Proteoglycans

Another PM component is proteoglycans (Lehane, 1997). These molecules have long and unbranched carbohydrate chains covalently attached to the protein core and encompass about 30 % of the PM. These chains are usually glycosaminoglycans (GAG) formed of repeating disaccharide units. One of the units is always a hexosamine and the other, an uronic acid residue of either D-glucuronic or iduronic acid. Some proteoglycans are sulfated, in which hyaluronan is probably the major component present. In the PM, proteoglycans seem to be the major space filling molecules, by forming hydrated gels. Such hydrated molecules, along with chitin microfibrils, account for the mechanical strength of the PM and might also be determinant of PM permeability characteristics (Lehane, 1997).

Peritrophins

Peritrophins are the most abundant component of the PM, in which they help create its scaffold by binding to chitin fibrils (Tellam et al., 1999). A common feature of these chitin binding proteins is the occurrence of cysteine-containing domains forming internal disulfide bonds (Elvin et al., 1996, Wang & Granados, 1997b, Shen & Jacobs-Lorena, 1998). These domains are similar to the chitin-binding domains (CBDs) of chitinases and are found alone or in combination. They also present some conserved aromatic amino acids between the cysteines, as well as some potential *N*-linked and *O*-linked glycosylation sites. The strong conservation of some amino acids residues is not observed in the overall amino acid sequence of peritrophins, and most peritrophins are characterized by acid isoelectric points (Elvin et al., 1996, Wang & Granados, 1997b, Shen & Jacobs-Lorena, 1998).

The formation of disulfide bonds in each chitin binding domain in association with the glycosylation of the protein backbone contribute to the protein stability and resistance to proteolysis in the gut (Elvin et al., 1996, Wang & Granados, 1997b, Wang & Granados, 1997a). This feature has been observed that most of the peritrophin proteolytic cleavage sites are located in the CBD amino acid sequences (Wang et al., 2004). Thereby, the cleavage of these proteins in such sites is constrained by the presence of the disulfide bonds (Elvin et al., 1996, Wang et al., 2004). On the other hand, a few cleavage sites are found in the spacers between CBDs and are subjected to proteolytic digestion. Even so, the resultant CBD modules derived from proteolytic cleavage still participate in the PM assembly (Shi et al., 2004, Wang et al., 2004).

Peritrophins displaying a single CBD function as caps on the tips of individual chitin chains, protecting the chitin fibrils from degradation by exochitinases (Elvin et al., 1996, Shi et al., 2004). On the other hand, peritrophins exhibiting multiple CBDs have a role in PM scaffolding by cross-linking chitin fibrils and creating a three-dimensional molecular meshwork (Shen & Jacobs-Lorena, 1998, Shi et al., 2004).

Peritrophins may have a role in holding the intersecting chitin bundles through the help of some large spacers present between CBDs, such as in McPM1 (Shi et al., 2004). It was also suggested that CBDs might bind to the GlcNAc-containing oligosaccharide moieties of the other PM proteins, thus providing an alternative means for establishing a three-dimensional network (Shi et al., 2004). The protein-chitin network is likely to be important to maintain the PM's inherent physical properties, such as strength, elasticity, and porosity. Moreover, extra cysteine

residues present in some peritrophins are thought to be involved in intermolecular disulfide bridges (Wang & Granados, 1997a, Sarauer et al., 2003, Shi et al., 2004).

It was proposed that the three disulfide bonds of the chitin-binding domains may constrain the polypeptide to present the aromatic amino acids on the protein surface for interactions with the sugar residues within the chitin fibril and/or possibility the GlcNAc-containing oligosaccharides attached to other peritrophins (Wang et al., 2004). Site-directed mutagenesis demonstrated that the second and the sixth conserved cysteine residues of the chitin binding domains are the least important for the maintenance of the protein function, while the disruption of the third, fourth and fifth cysteines restrict the binding capacity of these proteins (Shao et al., 2005). The importance of the first cysteine remains to be elucidated (Shao et al., 2005).

Peritrophins also bear heme-regulatory motifs (HRM) composed of cysteine-proline dipeptides (Devenport et al., 2006). In the mosquito *Aedes aegypti* PM, the mucin-like peritrophin, AeIMUC, displays six HRMs that sequester heme. AeIMUC1bound to heme molecules also is capable of binding to chitin, demonstrating the dual role of such molecules in PM scaffolding and heme detoxification (Rayms-Keller et al., 2000, Devenport et al., 2006). Moreover, the over-expression of the AeIMUC1 gene in larval stages upon exposure to other heavy metals (Copper and Cadmium) suggests the participation of this protein in the detoxification of such compounds obtained in the aquatic habitats (Rayms-Keller et al., 2000).

Three different types of CBDs have been described and termed peritrophin-A, peritrophin-B, and peritrophin-C domains (Tellam et al., 1999).

Typically, the peritrophin-A domain structure displays 60-70 amino acids in length and contains six cysteines that form three internal disulfide bonds (Elvin et al., 1996, Shen & Jacobs-Lorena, 1998). Additionally, they present several conserved aromatic amino acids at specific positions between cysteines 1 and 2, 2 and 3, and 4 and 5 (Elvin et al., 1996, Shen & Jacobs-Lorena, 1998). The general consensus sequence for this domain is C-$X_{13-20}CX_{5-6}CX_{9-19}CX_{10-14}CX_{4-14}C$, where X is not a cysteine, and it is found in both PM1 and PM2 peritrophins, such as the mosquito *A. aegypti* AEMUC1 (Keller et al, 2000) and the *Mamestra configurata* McPM1(Shi et al., 2004) peritrophins.

Peritrophin-B domains are characterized by the presence of 8 cysteines (Tellam et al., 2003). The consensus sequence of such domains is $CX_{12-13}CX_{20-21}CX_{10}CX_{12}CX_{2}CX_{8}CX_{7-12}C$.

The peritrophins exhibiting peritrophin-B domain present an alkaline predicted isoelectric point (Tellam et al., 2003). The *Lucilia cuprina* peritrophins LCPM30 and LCPM55 display type B CBD domains (Tellam et al., 1999, Tellam et al., 2003).

Peritrophin-C domain is composed of approximately 72 amino acids and 6 conserved cysteines residues (Wijffels et al., 2001). The domain-C consensus sequence is $CX_{8-9}CX_{17-21}CX_{10-11}CX_{12-13}CX_{11}C$ with some conserved aromatic amino acids between cysteines 2 and 3 (F42W43 and Y48W49), cysteines 4 and 5 (F69), and cysteines 5 and 6 (F/W79XXW82XW84). The peritrophin-C domain is found in the peritrophin-15 of the *Chrysomya bezziana, L. cuprina*, and *Drosophila melanogaster* (Wijffels et al., 2001).

In addition to chitin-binding domains, some PM proteins have mucin-like domains, which are heavily *O*-linked glycosylated (Wang & Granados, 1997b, Wijffels et al., 2001). In some cases, glycosylation represents more than 50 % of the total protein mass (Wang & Granados, 1997a). The high degree of glycosylation contributes to a gel-like consistency to the PM, which is fundamental to their role in protecting the epithelium from abrasion, hydrolytic enzymes, heavy metals, and pathogens, as well as allowing the passage of digestion products for absorption (Wang & Granados, 1997a, Sarauer et al., 2003). It is likely that the mucin-binding domains share some of the protective functions performed by the mammalian intestinal mucins, which are usually associated with mucus layer of the respiratory, digestive and urogenital tracts (Wang & Granados, 1997a, Sarauer et al., 2003). In fact, the *A. aegypti* mucin-like peritrophin AeIMUC1 is potentially involved in susceptibility to *Plasmodium gallinaceum* infection (Morlais & Severson, 2001).

Although peritrophins are the main proteins forming the PM scaffold, these proteins also are expressed in other tissues, such as hindgut, Malpighian tubules, trachea, ovaries, and cuticular exoesqueleton, pointing to potential additional roles for peritrophins beyond PM scaffolding (Barry et al., 1999, Gaines et al., 2003, Jasrapuria et al., 2010).

PM Synthesis

Chitin is synthesized by a large 3-domain transmembrane protein, the chitin synthase (Merzendorfer & Zimoch, 2003). Domain A is composed of a variable number of transmembrane helices, Domain B contains the catalytic center, and Domain C encompasses seven transmembrane helices. The latter domain might also be involved in catalysis. In insects,

chitin is synthesized by chitin synthase A (CHSA) in epidermal cells and other ectodermic cells whereas chitin synthase B (CHSB) in involved in chitin synthesis in the midgut (Merzendorfer & Zimoch, 2003, Arakane et al., 2005).

PM Degradation

Chitinases and β-N-acetylglucosaminidases are the enzymes playing a major role in PM degradation (Merzendorfer & Zimoch, 2003). These enzymes act in concert, with chitinases digesting chitin chains forming chitoligomes that are further converted to monomers by β-N-acetylglucosaminidases (Merzendorfer & Zimoch, 2003).

Chitinases belong to the family 18 of the glycosyl-hydrolase superfamily (Merzendorfer & Zimoch, 2003). Insect chitinases involved in PM degradation are part of the group IV that is made of chitinases expressed in the midgut and fat body (Zhu et al., 2008). Chitinases are mainly characterized by a multidomain structure, consisting of catalytic domain(s) in the N-termini, linker(s) (PEST-like region), and chitin-binding domain(s) (CBDs) in the C-termini (Merzendorfer & Zimoch, 2003). The catalytic domain is formed by a TIM-barrel structure, encompassing eight β-strands, forming the barrel's core, along with eight α-helices surrounding the β-strands. A signature sequence (FDxxDxDxE) is exhibited in the groove of the catalytic domain, with a glutamate residue being essential for chitin catalysis (Merzendorfer & Zimoch, 2003). The linker region is enriched in proline, glutamate, serine, and threonine amino acids (Arakane et al., 2003). The latter amino acids are predicted sites for *O*-linked glycosylation, which confers solubility and resistance to proteolysis (Arakane et al., 2003). Chitinase CBDs exhibit the same domain structure as the peritrophin ones (Merzendorfer & Zimoch, 2003). The presence of CBDs in the chitinase structure results in increased catalytic activity towards insoluble chitin, but not towards the soluble chitin oligosaccharides (Arakane et al., 2003).

Upon chitin digestion by chitinases, the resulted oligomers are converted to monomers by exo-splitting β-N-acetylglucosaminidases; the latter enzyme belongs to the glycosyl-hydrolase family 20 (Merzendorfer & Zimoch, 2003). β-N-acetylglucosaminidase activity has been detected in the midgut of the mosquito *A. aegypti* (Filho et al., 2002) and the tobacco hornworm *Manduca sexta* (Zen et al., 1996). Likewise, two genes encoding β-N-acetylglucosaminidases are predominantly expressed in the midgut of the red flour beetle *Tribolium castaneum* (Hogenkamp et al., 2008).

PM Modification

Chitin deacetylase converts chitin into chitosan via *N*-deacetylation reactions that might contribute to the binding of some proteins to chitosan in chitinous structures (Dixit et al., 2008). The chitin deacetylase domain organization is characterized by a chitin-binding domain (CBD), a low density lipoprotein receptor domain (LDLa), and a deacetylase domain, though some variants lack CBD and/or LDLa domains (Dixit et al., 2008). This protein belongs to the carbohydrate esterase family CE4, with the midgut-specific chitin deacetylases being members of group V (Dixit et al., 2008). Chitin deacetylases in Group V display only a catalytic domain (Dixit et al., 2008). In the insect gut lumen, this protein has a role defining PM porosity (Jakubowska et al., 2010), possibly being involved in defense against fungi (Toprak et al., 2008, Arakane et al., 2009). Other evidences pointing to a role of chitin deacetylases in insect gut physiology were shown by the abilities of a recombinant *Mamestra configurata* (bertha armyworm) chitin deacetylase (McCDA1) to deacetylate chitin (Toprak et al., 2008) as well as recombinant chitin deacetylases of *Tricoplusia ni* (cabbage looper; TnPM-P42) and *Bombyx mori* (silkworm; BmPM-41 and BmPM-43) to bind to chitin (Guo et al., 2005). In *T. castaneum*, four chitin deacetylase transcripts are differentially expressed not only along the length but also in different cell types of the late larval gut, suggesting specific roles for each of these proteins (Arakane et al., 2009).

PM-like Structures

PM is not secreted in a few insect species, for instance, most adult bees and wasps, most adult moths and butterflies, some adult ants, lice, and adult fleas (Terra, 2001). Some of these species (lepidopteran and hymenopteran) feed on sugars, low molecular weight molecules, not requiring compartmentalization of digestion (Terra, 2001). Other insect species, for instance, *Locusta migratoria* and *Zabrotes subfrasciatus*, secrete a peritrophic gel that lacks chitin and exhibits larger pore sizes (Terra, 2001). The localization of this structure can be restricted to anterior midgut, midgut caeca, or surrounding the whole midgut (Terra, 2001). The secretion of a peritrophic gel appears to be a physiological adaptation of those insects that display influx of digestive enzymes to crop (requiring larger pores), secrete chitinases as an anti-fungus defense in the anterior midgut (lined by a peritrophic gel lacking chitin), or feed on plants that produce defensive substances, as vicilins, that are detrimental to chitin in PMs (Terra, 2001). Hemipteran

and thysanopteran secrete a lipoprotein perimicrovillar membrane that buds off from the Golgi complex and coat the midgut microvilli (Silva et al., 2004). The perimicrovillar membrane also is capable of compartmentalizing food digestion as well as immobilizing digestive enzymes (Silva et al., 2004).

Evolution of the PM

It was speculated that the PM evolved from a mucosal lining of the insect intestine (Terra, 2001). The midgut epithelial cells of the ancestral insects must have been lined with a mucous layer similar to that found in vertebrates (Terra, 2001). Then, the peritrophins evolved from mucins by acquiring their chitin-binding domains. Based on this theory, secretion of the PM by the whole midgut epithelium would be the ancestral condition whereas secretion by specialized regions (cardia) would have evolved more recently (Terra, 2001).

Regarding the peritrophins, it has been postulated that the chitin binding domains (CBDs) are basic evolutionary modules that expand themselves by domain duplications, and also combine with other protein sequences to generate or modify existing functions (Shen & Jacobs-Lorena, 1998). Such a hypothesis was suggested based on the similarities between the chitin binding domain of *A. gambiae* AgChit-1 chitinase and Ag-Aper1 peritrophin (Shen & Jacobs-Lorena, 1998), between CBDs in *Trichoplusia ni* CBP1 and CBP2 peritrophins (Wang et al., 2004), and the six tandem repeated modules, encompassing two CBDs and two spacers, in the moth *Mamestra configurata* McPM1 peritrophin (Shi et al., 2004).

PM and Pathogens

The insect PM is an important line of defense against pathogen infection (Hegedus et al., 2009). However, some pathogens can bypass this barrier by taking advantage of distinct mechanisms (Devenport & Jacobs-Lorena, 2005). Whereas some pathogens escape from entrapment within the PM by reaching the midgut epithelium before PM synthesis or after PM breakdown, other pathogens secrete enzymes capable of degrading the PM, allowing pathogen escape from the endoperitrophic space (Wang & Granados, 1997a, Shi et al., 2004, Devenport & Jacobs-Lorena, 2005).

Most viruses and microfilaria are capable of attach and/or cross the midgut epithelium before secretion of the PM (Devenport & Jacobs-Lorena, 2005). *Leishmania*, on the other hand, survives in the endoperitrophic space until the PM breaks down (Walters et al., 1993, Walters et

al., 1995), allowing parasites to escape to the ectoperitrophic space and attach to the midgut epithelium, preventing excretion with the fecal pellets (Pimenta et al., 1992).

Regarding pathogens that escape from PM entrapment by secreting enzymes, they rely either on chitin or peritrophin digestion (Wang & Granados, 1997a, Shi et al., 2004, Devenport & Jacobs-Lorena, 2005). The *T. ni* granulosis TnGV virus and *M. configurata* MacoMNPV baculovirus express a metalloprotease, named enhancin. That disrupts the structural integrity of the PM by digesting peritrophins, enhancing viral infection (Wang & Granados, 1997a, Shi et al., 2004). Likewise, *Plasmodium*, the causative agent of malaria, secretes a chitinase necessary for penetrating and crossing the PM matrix (Shahabuddin et al., 1993, Dessens et al., 2001, Tsai et al., 2001, Devenport & Jacobs-Lorena, 2005).

PM and Insect Control

Targeting insect PM (or specific PM proteins) seems to be a feasible strategy to control insect pest and disease vector via the development of specific inhibitors, transgenic plants, and vaccines (Baum et al., 2007, Mao et al., 2007, Khajuria et al., 2010, Zhang et al., 2010).

Several studies have applied RNA interference and shown its feasibility of targeting the PM in insect control strategies (Baum et al., 2007, Mao et al., 2007, Khajuria et al., 2010, Zhang et al., 2010). Transgenic plants expressing double-strand RNA molecules (dsRNA) targeting physiological processes essential for insect larva survival suffered reduced root damage due to reduced larval feeding (Baum et al., 2007, Mao et al., 2007). By the same token, PM-related genes can be targeted by RNAi through feeding dsRNA particles, reducing survival of the target species (Khajuria et al., 2010, Zhang et al., 2010). For instance, feeding larvae of the mosquito *Anopheles gambiae* on dsRNA targeting the chitin synthase B (*CHSB*) gene increased the harmful effects of diflubenzuron against these insects (Zhang et al., 2010). Similarly, inhibition of a gut chitinase in the European corn borer (*Ostrinia nubilalis*) larvae via dsRNA feeding increased chitin content in the PM and led to reduced larval growth (Khajuria et al., 2010).

The PM in pest insects also is targeted by the defense mechanism produced by plants (Mohan et al., 2006). A maize germplasm that is resistant to a number of stem-boring lepidopterans expresses a 33 kDa cysteine protease (Mir1-CP) that acts on *Spodoptera frugiperda* PM, causing holes and fissures and reducing nutrient utilization (Mohan et al., 2006). Moreover, transgenic maize overexpressing Mir1-CP reduces larval growth by 70 % (Pechan et

al., 2000). A feasible alternative to the control of lepidopteran pests is the co-expression of Mir-CP and Bt-toxins as feeding four different species of lepidopteran larvae on a mixture of MIR-CP and Bt-CryIIA increases mortality by 50-60 % (Mohan et al., 2008).

The sheep blowfly *L. cuprina* larva is the causative agent of cutaneous myiasis in sheep (Tellam et al., 2000). The excretion or regurgitation of *L. cuprina* PM in the site of myiasis induces antibody-mediated immune response by sheep naturally infected with *L. cuprina* larvae (Tellam et al., 2000). As this immune response is harmful to larval development, sheep immunization using peritrophins as antigens can protect these animals against sheep blowfly myiasis (Tellam et al., 2000).

Ingestion of anti-peritrophin-44 antibodies by *L. cuprina* larvae blocks the PM pores, preventing the flow of food from the endo- and to the ectoperitrophic space (Willadsen et al., 1993). This finding suggests that peritrophin-44 is involved in PM permeability (Elvin et al., 1996). Likewise, ingestion of anti-peritrophin-55 or anti-peritrophin-95 antibodies by *L. cuprina* larvae inhibits larval growth and slightly increases mortality, acting on PM in the same way as the anti-peritrophin-44 antibodies (Casu et al., 1997, Tellam et al., 2003). Regarding protection mediated by anti-peritrophin-95 antibodies, it also is mediated by antibodies targeting the carbohydrates linked onto peritrophin-95 molecular surface (Tellam et al., 2001).

PM and Sand fly Vector Competence

Leishmaniasis is a vector-borne diseases caused by 21 *Leishmania* species and transmitted to mammalian hosts by 30 species of sand flies of the genera *Lutzomyia* and *Phlebotomus* (Herwaldt, 1999). *Leishmania* development in its insect vector is completely accomplished within the sand fly gut (Ramalho-Ortigao et al., 2010). Upon feeding on an infectious blood, sand flies take up *Leishmania* amastigotes that become procyclic promastigotes in the posterior midgut after surviving the harmful actions of digestive enzymes (Schlein & Romano, 1986, Borovsky & Schlein, 1987, Pimenta et al., 1997, Secundino et al., 2010, Svarovska et al., 2010). Procyclic promastigotes become nectomonads, which multiply in the endoperitrophic space and escape towards the ectoperitrophic space between 48h and 96h post blood meal (PBM) (Pimenta et al., 1997). Once in contact with the midgut microvilli, *Leishmania* binds to the midgut epithelium by a LPG-dependent (Pimenta et al., 1992, Wilson et al., 2010) or LPG-independent (Myskova et al., 2007, Volf & Myskova, 2007) mechanism, so as

not to be excreted along with the fecal pellets (Pimenta et al., 1992, Kamhawi et al., 2000, Soares et al., 2002). After the digested blood is passed, *Leishmania* detaches from the midgut epithelium, migrates towards the anterior midgut, where it develops in haptomonad and metacyclic (infective) forms. The latter forms secrete a plug consisted of filamentous proteophosphoglycans (fPPG) (Stierhof et al., 1999, Rogers et al., 2002), and degrades the stomodeal valve's intima (Schlein et al., 1991, Volf et al., 2004). The damaged stomodeal valve along with the *Leishmania*-secreted plug disrupt the mechanism of fluid sucking in the sand fly, leading to regurgitation of the parasites into the biting site upon blood feeding (Schlein et al., 1992).

Adult female sand flies are hematophagous and secrete a type I peritrophic matrix (PM) after ingesting a blood meal (Blackburn et al., 1988, Secundino et al., 2005). The secretion of the PM in *P. papatasi* begins at four hours PBM, becoming multi-layered at 48 h PBM. At 72 h to 96 h PBM, the PM starts to break down at the posterior end when digested blood along with fragments of PM are passed (Blackburn et al., 1988). In *L. longipalpis*, the secretion of the PM components starts as soon as 1h after the blood ingestion; however, a PM is first observed 12 h PBM, and a thick well-formed PM is observed at 24 h PBM. The latter is composed of two distinct layers: a thin fibrillar layer, in contact to the epithelium, and a thick granular layer facing the bloodmeal. After 36 h PBM, the PM undergoes a progressive shrinkage (Secundino et al., 2005).

In order to survive in the sand fly midgut, *Leishmania* must escape from the PM. This was first suggested by results showing that *Le. donovani* and *Le. panamensis* were unable to escape from the endoperitrophic space in *Phlebotomus mongolensis* and *P. papatasi*, respectively, and the infection was lost as the blood remnants were passed (Feng, 1950, Walters et al., 1992). Although Schlein *et al* (Schlein et al., 1991) pointed out that the *P. papatasi* PM degradation in infected sand flies is anticipated by the action of a *Leishmania*-secreted chitinase, sand fly midgut transcriptome studies demonstrated the presence of endogenous chitinase transcripts in the sand flies *P. papatasi* and *L. longipalpis* (Ramalho-Ortigao et al., 2001, Ramalho-Ortigao et al., 2007). Additionally, the comparison between the pattern of mRNA expression and protein activity suggests that *P. papatasi* chitinase protein is stored in midgut epithelial cells, possibly as a zymogen, and secreted in the midgut cavity at 48-72 h PBM (Ramalho-Ortigao et al., 2005), at the time when the sand fly PM breaks down (Blackburn et al.,

1988). Similar mRNA profile is displayed by the *L. longipalpis* midgut chitinase (Ramalho-Ortigao & Traub-Cseko, 2003). In fact, *P. duboscqi* PM breaks down at the same rate and mode (by the posterior end, despite accumulation of parasites at the anterior end) in both *Le. major* infected and noninfected flies, suggesting that *Leishmania* chitinase plays a minor role, if any, in parasite escape from the endoperitrophic space (Sadlova & Volf, 2009). Despite initial results showing that transgenic *Leishmania* overexpressing a chitinase displayed greater ability to escape from the PM (Rogers et al., 2008), a chitinase-knockout *Leishmania* line would be a better tool in order to demonstrate if *Leishmania* relies on its own chitinase (Schlein et al., 1991) or takes advantage of sand fly chitinases to escape from the endoperitrophic space (Ramalho-Ortigao & Traub-Cseko, 2003, Ramalho-Ortigao et al., 2005).

Although the sand fly PM consists of a barrier for *Leishmania* development, it protects *Leishmania* against the action of digestive enzymes in the initial hours after a blood meal (Pimenta et al., 1997). Unlike amastigotes and procyclic promastigotes, the transitional stage between these two *Leishmania* forms is highly susceptible to digestive enzymes (Pimenta et al., 1997). This coincides with the expression patterns of *P. papatasi* and *L. longipalpis* midgut trypsins and chymotrypsins, which peak at the first hours after blood feeding (Ramalho-Ortigao et al., 2003, Telleria et al., 2007). Accordingly, when *P. papatasi* females were fed with an infected blood meal along with exogenous chitinases, most of the parasites were killed in the initial stages of blood digestion (Pimenta et al., 1997). On the other hand, when a trypsin inhibitor was added to infected blood with exogenous chitinase, parasite survived, demonstrating trypsins are responsible for parasite killing in the absence of a PM (Pimenta et al., 1997).

Specific Aims and Significance

Leishmaniasis is a multi-spectrum disease (Herwaldt, 1999). Every year, 500,000 new human cases are diagnosed in 88 countries, and 350 million people are at risk of becoming infected (Herwaldt, 1999, TDR/WHO, 2002). The DALY burden for leishmaniasis is 2 million (TDR/WHO, 2002). Despite the high morbidity and mortality caused by *Leishmania* infection, no vaccine is available and vector control relies almost exclusively on insecticide spraying (Herwaldt, 1999). The emergence of insecticide resistance in many disease vectors compels for the development of new strategies to control vector-borne diseases (Hill et al., 2005), including sand fly-transmitted leishmaniasis. Transmission Blocking Vaccines (TBVs) seem to be a viable

strategy to control pathogen transmission by insect vectors (Appendix A). TBVs rely on the immunization of a host population with pathogen or insect vector-derived proteins (antigens); hence, specific antibodies are produced by the host aiming to block pathogen development in the insect vector's digestive tract (Appendix A). The identification of antigens is an initial and crucial step in the development of TBVs (Appendix A). For the sand fly *P. papatasi*, the *Le. major* midgut receptor, PpGalec, bears the features of a TBV candidate as ingestion of PpGalec antiserum blocked the development of *Le. major* in the *P. papatasi* midgut (Kamhawi et al., 2004). Although Kamhawi and colleagues (2004) have demonstrated the proof-of-concept that TBVs are a feasible strategy to fight *Leishmania* transmission, the specificity of PpGalec as a receptor for *Le. major* precludes its use as a TBV against transmission of other species of *Leishmania*. In contrast to PpGalec, antiserum against PpChit1, a *P. papatasi* midgut-specific chitinase, inhibits chitinolytic activity in midgut extracts of *P. papatasi*, its sister species *P. duboscqi,* as well as *P. argentipes*, a sand fly belonging to another sand fly subgenus (Ramalho-Ortigao et al., 2005). These data point to the potential use of PpChit1 as across-species TBV (Ramalho-Ortigao et al., 2005).

In light of the importance of PM-associated molecules in sand fly vector competence, this study was focused on assessing the role of proteins involved in *P. papatasi* PM scaffolding and degradation as molecular barriers against *Le. major* development. Thereby, we aimed at identifying potential candidates to be used in TBV strategies against leishmaniasis. The specific aims of this study were:

(1) Evaluate the effects of knocking down the expression of *PpChit1*, a *P. papatasi* midgut-specific chitinase, on the development of *Le. major* in the midgut of the sand fly *P. papatasi*

(2) Describe the molecular features of three *P. papatasi* putative peritrophins, PpPer1, PpPer2, and PpPer3

(3) Assess the effects of *PpPer1* knock down on *Le. major* development in *P. papatasi* midgut

Pinpointing molecules that participate in the *P. papatasi*-*Le. major* interaction can reveal new targets to be disrupted in strategies to control sand fly-transmitted leishmaniasis, such as TBVs.

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Chapter 2 - Targeting the Midgut Secreted PpChit1 Reduces *Leishmania major* Development in its Natural Vector, the Sand Fly *Phlebotomus papatasi*

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Abstract

Background: During its developmental cycle within the sand fly vector, *Leishmania* must survive an early proteolytic attack, escape the peritrophic matrix, and then adhere to the midgut epithelia in order to prevent excretion with remnants of the blood meal. These three steps are critical for the establishment of an infection within the vector and are linked to interactions controlling species-specific vector competence. PpChit1 is a midgut-specific chitinase from *Phlebotomus papatasi* presumably involved in maturation and degradation of the peritrophic matrix. Sand fly midgut chitinases, such as PpChit1, whether acting independently or in a synergistic manner with *Leishmania*-secreted chitinase, possibly play a role in the *Leishmania* escape from the endoperitrophic space. Thus, we predicted that silencing of sand fly chitinase will lead to reduction or elimination of *Leishmania* within the gut of the sand fly vector. *Methodology/Principal Findings:* We used injection of dsRNA to induce knock down of *PpChit1* transcripts (dsPpChit1) and assessed the effect on protein levels post blood meal (PBM) and on *Leishmania major* development within *P. papatasi*. Injection of dsPpChit1 led to a significant reduction of *PpChit1* transcripts from 24 hours to 96 hours PBM. More importantly, dsPpChit1 led to a significant reduction in protein levels and in the number of *Le. major* present in the midgut of infected *P. papatasi* following a infective blood meal. *Conclusion/Significance:* Our data supports targeting PpChit1 as a potential transmission blocking vaccine candidate against leishmaniasis.

Author summary

For a successful development within the midgut of the sand fly vector, *Leishmania* must overcome several barriers which are imposed by the vector. The ability to overcome these barriers has been associated with species specificity, and interference with the sand fly vector-parasite balance can change the outcome of the infection in the vector. Recently, our group has carried out a transcriptome assessment of the sand fly *Phlebotomus papatasi* midgut, uncovering many transcripts possibly associated with the barrier to *Leishmania* development. In order to validate the role of such genes, we have developed a dedicated RNA interference (RNAi) platform to assess whether RNAi targeting such genes can reduce *Leishmania major* development. PpChit1 is a midgut-specific chitinase presumably involved in the maturation/degradation of the peritrophic matrix in the gut of the sand fly after a blood meal. Our results show that knockdown of *PpChit1* via RNAi led to a significant reduction of *Le. major* within the gut, supporting the potential use of PpChit1 as a target for transmission blocking strategies against sand fly-transmitted leishmaniasis.

Introduction

Emerging and reemerging vector-borne diseases pose significant threats to human and animal health (Hill et al., 2005). The emergence of insecticide resistance as well as the lack of other efficient insecticidal tools to control disease vectors imply that new methodologies need to be developed in order to reduce vector-borne disease transmission (Hill et al., 2005). For this, the study of vector-pathogen interaction pinpointing factors underlying vector competence can reveal new molecular targets to be disrupted, preventing pathogen transmission (Coutinho-Abreu et al., 2009, Coutinho-Abreu & Ramalho-Ortigao, 2010).

In sand flies, midgut molecules are known or believed to be involved in defining a species ability to transmit *Leishmania* in nature. For a successful development within the midgut of the sand fly vector, *Leishmania* must overcome several barriers that include an early proteolytic attack (Dillon & Lane, 1993a, Dillon & Lane, 1993b, Pimenta et al., 1997, Schlein & Jacobson, 1998), the need to escape the peritrophic matrix (PM)(Feng, 1950-51, Lawyer et al., 1990, Walters et al., 1992, Pimenta et al., 1997, Sadlova & Volf, 2009), and attachment to the midgut epithelia to prevent excretion with the remnants of the blood meal (Pimenta et al., 1992, Pimenta et al., 1994, Kamhawi et al., 2004, Anderson et al., 2006).

Attachment to midgut epithelia has long been associated with the type of lipophosphoglycan (LPG) present on the surface of *Leishmania*, and is associated with defining sand fly-*Leishmania* pairs in nature (Pimenta et al., 1992, Pimenta et al., 1994). For *Leishmania major* V1 strain, with LPG displaying highly decorated side chains with prominent galactose residues, we demonstrated that PpGalec, a *P. papatasi* galactose-binding protein, is the docking site for *Le. major* on the midgut epithelium of *Phlebotomus papatasi* (Kamhawi et al., 2004). Recently, LPG-independent midgut binding has been associated with the degree of glycosylation detected on proteins expressed by midgut epithelial cells (Myskova et al., 2007).

For events leading up to the midgut binding, such as early parasite survival during the proteolytic attack and escape of the endoperitrophic space, some investigators suggested that midgut proteases, such as trypsins and chymotrypsins, also are responsible for defining vector-*Leishmania* specificity (Borovsky & Schlein, 1987, Dillon & Lane, 1993a, Dillon & Lane, 1993b, Schlein & Jacobson, 1998). Such proteases were shown to be specially harmful to transitional stages amastigotes (Pimenta et al., 1997).

A role of the PM on sand fly vector competence was suggested through comparisons of *Leishmania* development in different sand fly species displaying different PM degradation rates (Feng, 1950-51, Lawyer et al., 1990, Walters et al., 1992). Studies later revealed a dual role for the sand fly PM in parasite development; protecting *Leishmania* from digestive enzymes in the beginning of blood digestion, yet becoming a barrier to parasite escape when mature (Pimenta et al., 1997). Recent data also indicate that an anterior PM plug located at the junction between the anterior and posterior midgut acts as a barrier to *Leishmania* migration towards the stomodeal valve (Sadlova & Volf, 2009).

Regarding *Leishmania* escape from the PM, it was firstly proposed to be solely accomplished by a parasite chitinase (Schlein et al., 1991). Further work demonstrated that a *Le. mexicana* chitinase-overexpressing strain had an accelerated escape from the PM in *Lutzomyia longipalpis* (Rogers et al., 2008). However, since the characterization of a blood induced chitinolytic system in the sand fly midgut (Ramalho-Ortigao et al., 2005), it became apparent that the parasite must take advantage of the sand fly peak chitinolytic activity within midgut, approximately 40-48 hours after a blood meal, for their escape (Ramalho-Ortigao et al., 2005, Sadlova & Volf, 2009).

PpChit1 is presumably involved in PM maturation/degradation in *P. papatasi* (Pimenta et al., 1997). Based on the fact that *Leishmania* must escape the PM, and that this escape may be aided by the vector's own chitinase, we predicted that *PpChit1* knock down (via RNAi) would interfere with *Le. major* development. Our data indicates that dsRNA-mediated silencing of *PpChit1* transcripts leads to a reduction in the parasite load within the midgut of *P. papatasi*, pointing to the role of this molecule in *P. papatasi* vector competence and its potential for the development of a transmission-blocking vaccine.

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).

Sand fly rearing, dissection, and infection with Le. major

P. papatasi (Israeli strain -PPIS) was reared in the Department of Entomology, Kansas State University, according to conditions described (Ramalho-Ortigao et al., 2005). For all experiments, three-to-five day old female sand flies were used. Blood feeding was performed through a chicken skin membrane attached to a feeding device. Prior to sand fly feeding, fresh mouse blood was heat inactivated for 30 min at 56 °C and supplemented with 50 µl/ml of Pen/Strep solution (MP Biomedicals, Solon, OH, USA) as well as 1mM ATP (MP Biomedicals). Sixteen to twenty four hours after blood feeding, fully engorged females were separated from partially engorged and non-blood fed by anesthetizing flies with CO_2 and examining the midgut distension under a stereoscope microscope. Only fully fed individuals were maintained for further analyses.

Fully engorged sand fly midguts were individually dissected on RNase free (cleaned with ELIMINase, Fisher Scientific, Pittsburgh, PA, USA) glass slides, transferred to 50 µl of 1X PBS buffer (RNase free, pH 7.4; Fisher Scientific), and thoroughly homogenized using a hand held tissue homogenizer and RNAse-free pestle. Half the homogenate volume (25 µl) was transferred to 350 µl of RLT buffer (supplemented with 1 % β-mercaptoethanol) provided by the RNA extraction kit (RNAeasy mini kit, Qiagen, Valencia, CA, USA) and stored at -80°C for quantitative real-time PCR assays. The remaining 25 µl of midgut homogenate was used in Western blot assays, as described below.

Infections of sand flies with *Le. major* amastigotes V1 strain were done by addition of 5 $x10^6$ parasites/ml into the blood meal. *Le. major* amastigotes were harvested from BALB/c mouse footpads lesions formed roughly 30 days after inoculation with 5 x 10^5 parasites from late phase culture according to (Sacks, 2003).

dsRNA synthesis and injection

dsRNA for *PpChit1* were synthesized using the primers PpChit1/T7i_2–F (5'–TAATACGACTCACTATAGGGAGAATGAAGATATCATTGTGTGC-3') and PpChit1/T7i_2–R (5'– TAATACGACTCACTTAGGGAGATCAGCATTGGACCAGGAAGG-3'), which contain the complete T7 promoter and amplify the full length sequence encoding the mature PpChit1. PCRs were performed with 0.5 pmoles of each primer along with 1 µl of cDNA (synthesized from midgut dissected at 72 h post-blood meal, PBM), and 10 µl of GoTaq PCR

master mix (Promega, Madison, WI, USA). The 20 µl PCRs were carried according to the conditions: 10 cycles of 95°C for 1 min, 55°C for 1 min, and 72°C for 1 min and 15 sec, followed by 35 cycles 95°C for 1 min, 65°C for 1 min, and 72°C for 1 min and 15 sec. The reaction products were purified and concentrated using the YM-100 filters (Millipore, Billerica, MA, USA), and 1 µg DNA was used for dsRNA synthesis using the Megascript RNAi kit (Ambion, Austin, TX, USA). dsRNA synthesis reactions were performed for four hours at 37°C, and the products were further purified following manufacturer's recommendations. Thereafter, dsRNAs were suspended in ultra-pure water and further purified and concentrated to approximately 3.5 mg/ml or 4.5 mg/ml using the YM-100 filters (Millipore). The positive control provided by the Megascript RNAi kit (Ambion; used in Real-Time PCR and Western blot assays) or a dsRNA specific to a green fluorescence protein gene (dsGFP (Arakane et al., 2008); for parasite counting assays) was used as controls for dsRNA injection assays.

For dsRNA injections, individual females were anesthetized with CO_2, kept on a cold dish, and injected intra-thoraxically with either 23 nl (3.5 mg/ml, 80.5 ng) or 32 nl (4.5 mg/ml, 144 ng) of dsRNA using Nanoject II microinjector (Broomall, PA, USA). Immediately following injection, flies were transferred to a 500 ml plastic container, provided with 30 % sugar embedded cotton, and maintained inside a high humidity chamber (85-95 % humidity at 25°C). Flies were allowed to recover for 48 hours and blood fed on an uninfected blood meal through a chicken membrane, as described above.

RNA isolation and cDNA synthesis

Total RNA was isolated from individual midguts dissected as described above. RNA extraction was carried out using the RNAeasy mini kit (Qiagen) following manufacturer's instructions. Following extraction, the Turbo DNA-*free* kit (Ambion) was used to eliminate DNA contamination. After quantification, 25 ng total RNA was used for cDNA synthesis using 200 units of SuperScript III Reverse Transcriptase (200 u/µl), 2.5 µM Oligo $(dT)_{20}$ primer, and 0.5 µM dNTPs (10 mM). These reagents were incubated at 65°C for 5 minutes (min) and kept in ice for at least 1 min. This step was followed by addition of a mix containing 4 µl 5X SuperScript III Reverse Transcriptase First-Strand Buffer, 5 mM DTT (0.1M), 20 Units of RNaseOUT to the reaction. The mixture was incubated for one hour at 50°C and stored at -20°C. All the reagents for cDNA synthesis were purchase from Invitrogen (Carlsbad, CA, USA).

Quantitative real-time PCR analyses

Real-Time PCRs were performed using BioRad SYBR green and BioRad iCycler (BioRad, Hercules, CA, USA). The reactions were carried out in duplicate using 0.5 µl cDNA, 6 pmoles of each primer (10 µM), 10 µl of 2X SYBR green, and 8.3 µl of Ultra Pure DNase/RNase-Free Water (Invitrogen). The primers used for chitinase amplification were PpChit_137F (5' - ATGATCTGCATGGTTCTTGG - 3') and PpChit_137R (5' - GGAGCTCCATTTCGAATCC - 3') while the S3 primers (Pp40S_S3_136F: 5' - GGACAGAAATCATCATCATG – 3' and Pp40S_S3_136R: 5' – CCTTTTCAGCGTACAGCTC – 3') were used for amplifications of the housekeeping control gene (encoding the protein S3 of ribosomal subunit 40S). The reaction cycle of 94°C for 1 min, 57°C for 1 min, and 72°C for 30 sec was repeated 40 times, and the amplification profiles were assessed using the BioRad iCycler software (BioRad).

PpChit1 anti-sera and Western blot analyses

Polyclonal anti-PpChit1 sera were obtained by injecting three month old female BALB/c mice subcutaneously into the ears. Mice were injected three times in two weeks intervals with approximately 10µg of purified VR2001 plasmid (Oliveira et al., 2006) encoding the mature chitinase protein (Ramalho-Ortigao et al., 2005) per mouse ear. Blood was collected from the submandibular vein ("cheek bleed") of injected animals and antibody levels accessed via Easy-Titer IgG Assay Kit (Pierce, Rockford, IL, USA). Sera were maintained at -20°C until used. For Western blots, seven midgut extracts from flies injected with dsPpChit1 and dsControl were pooled together in RNasefree microcentrifuge tubes containing 1µl of complete protease inhibitor (Thermo Scientific, Rockford, IL, USA) and concentrated using the YM-10 filters (Millipore). Total protein concentration in midgut extracts was quantified using BCA Protein Assay Kit (Thermo Scientific). Similar proteins amounts (5 µg per lane) from midguts of dsPpChit1 and dsControl injected sand flies were fractionated on 10% Bis-Tris NuPAGE gels (Invitrogen). Proteins were transferred to a nitrocellulose filter (Whatman, Dassel, Germany), incubated with PpChit1 antisera (1:100 dilution) overnight at 4°C, washed three times in TBS-T (1X TBS buffer with 0.1% tween-20) for 15 minutes each time. Blot was incubated with anti-mouse conjugated to alkaline phosphatase (1:10,000 in TBS-T) antibodies (Promega) for one hour at room temperature and washed in TBS-T as indicated above. The protein bands (56 kDa,

(Ramalho-Ortigao et al., 2005) were visualized using the Western Blue substrate for Alkaline Phosphatase (Promega). Alternatively, Western blot was incubated with anti-mouse-Horseradish Peroxidase secondary antibody (1:10,000) and detected with SuperSignal West Pico Chemiluminescence Substrate (Thermo Scientific) in chemiluminescence assays. Densitometry analysis was performed using the TotalLab TL100 software (Nonlinear Dynamics, Durham, NC, USA).

P. papatasi *dissection and parasite counting*

In order to assess the *PpChit1* knockdown effects on *Le. major* development, 80.5 ng of dsRNA was injected intra-thoraxically into *P. papatasi*, and flies were fed on an infected blood meal as described above. Midguts from fully engorged-only flies were dissected at 48 h and 120 h after the infective blood meal and homogenized in 30 µl of PBS buffer (pH 7.4). Parasites were counted with a hemocytometer. Two independent experiments were carried out for each time point.

Statistical analysis

Mann-Whitney U test was performed to compare expression profiles as well as parasite numbers between sand flies injected with either dsRNA targeting *PpChit1* transcripts (dsPpChit1) or the dsRNA control (dsControl) injected flies. D'Agostino & Pearson omnibus normality test was performed to assess whether parasite numbers followed a normal distribution. The Chi-square test (or Fisher's exact test) was performed in order to assess whether dsPpChit1-injected flies exhibit altered *Le. major* load compared to the dsControl-injected flies. Parasite infection load in flies dissected at 48 h post infection was scored according to parasite numbers in the sand fly midgut as no parasite, light infection (1-1,000 parasites), moderate infection (1,001-10,000), or heavy infection (>10,000), in accordance to (Svarovska et al., 2010). For flies dissected at 120 h PBM parasite loads were categorized in two groups: zero or light infections (0-1,000 parasites) was arranged as one group, and moderate infection (>1,000 parasites) as another. Differences were considered statistically significant at $p< 0.05$, and tests were carried out using GraphPad Prism v. 5.01 software (GraphPad Software, Inc).

Results

dsPpChit1 effects on mRNA levels

Injection of 80.5 ng of dsRNA into the sand fly thorax targeting the midgut expressed *PpChit1* gene led to a significant decrease in *PpChit1* mRNA levels in comparison with the control dsRNA-injected flies (Figure 2.1). Reduction of *PpChit1* expression after a blood meal varied over time. Twenty four hours after blood meal (and 72 h after injection of dsPpChit1), a 27 % reduction of *PpChit1* transcripts was detected (Figure 2.1A). At 48 h PBM (previously shown to be the maximum activity for PpChit1 (Ramalho-Ortigao et al., 2005) and at 72 h PBM, reductions of 58 % and 53 % on average of the *PpChit1* expression were observed (Figure 2.1A). Finally, at 96 h PBM (120 h after dsRNA injection), when no chitinolytic activity was detected (Ramalho-Ortigao et al., 2005), the reduction in *PpChit1* expression was 72 %.

On the other hand, injection of 144 ng of dsPpChit1 into *P. papatasi* thorax displayed a weaker reduction in *PpChit1* expression levels than injection of 80.5 ng (Figure 2.1B). Although similar expression reduction at 24 h PBM was exhibited (26 %, Figure 2.1B), expression differences between dsPpChit1 and dsControl injected flies at 48 h and 72 h PBM were lower (13 % and 43 %, respectively) than detected at the same time points when 80.5 ng of dsRNA was injected (Figure 2.1B). These differences could be occurring due to a still obscure feedback loop for transcription activation upon knock down, as proposed elsewhere (Belles, 2010).

dsPpChit1 effects on protein levels

Silencing of the *PpChit1* message RNA produced a concomitant reduction in the amount of PpChit1 protein as determined by Western blots (Figure 2.2). Similar to the Real-Time PCR data, reduction in PpChit1 protein levels in dsPpChit1 injected flies was detected at 48 h and 72 h PBM (Figures 2.2A-C) when either 80.5 ng or 144 ng of dsRNA was injected. No PpChit1 expression was detected at 24 h PBM (Figure 2.2B). Likewise, densitometry analysis of blot developed using a chemiluminescence method displayed 95 % reduction in PpChit1 protein levels at 48 h PBM when 144 ng dsPpChit1 (Figure 2.2C and 2.D). Interestingly, the corresponding time point only led to 13 % reduction of PpChit1 mRNA levels, as shown in Figure 2.1B.

dsPpChit1 effects on Le. major *development within* P. papatasi *midgut*

As injection of either 80.5 ng or 144 ng of dsRNA targeting *PpChit1* transcripts are capable of significantly reducing *PpChit1* expression levels in the midgut of *P. papatasi* (Figure 2.1 and 2.2), we assessed the effects of injecting 80.5 ng of the dsRNA on *Le. major* development within the injected flies. Following the injection of the PpChit1 dsRNA, flies were provided an infective blood meal, and dissected at different time points after feeding. Our results demonstrate that dsPpChit1-targeted knock-down resulted in significant reductions in parasite load within the sand fly midgut as the numbers of *Le. major* were reduced by 46 % (or 1.85 fold) at 48 h post infection (Figure 2.3A) and by 63 % (or 2.70 fold) at 120 h PBM post infection (Figure 2.3B).

The injection of dsPpChit1 also affected the range of parasite loads. An analysis of the range of parasite load at 48 h and 120 h post infection points to a normal distribution of parasite numbers in the dsControl-injected flies (48 h PBM, $p=0.51$, and 120 h PBM, $p= 0.26$, D'Agostino & Pearson omnibus normality test), whereas for dsPpChit1-injected flies this distribution was significantly affected (48 h PBM, $p=0.004$, and 120 h PBM, $p< 0.0001$, D'Agostino & Pearson omnibus normality test).

Changes in *P. papatasi* infection levels following silencing of PpChit1 were further confirmed by comparing infection prevalence. For instance, injection of dsPpChit1 reduced the prevalence of heavy infection from 47 % (dsControl-injected) to 19 %, and of light infection from 19 % (dsControl-injected) to 6 % at 48 h post blood feeding (Figure 2.4A). Likewise, moderate infections levels were reduced from 57 % (dsControl-injected) to 14 % at 120 h post infection (Figure 2.4B).

Discussion

After a blood meal, sand flies synthesize a PM type 1 that is fully developed at approximately 36-40 h PBM (Secundino et al., 2005). In addition to compartmentalizing the blood meal and protecting the epithelia, the sand fly PM serves an additional dual role regarding *Leishmania* infection: as a barrier to these parasites but also as protection against proteolytic attack on transitional-stage amastigotes (Pimenta et al., 1997, Ramalho-Ortigao et al., 2003, Telleria et al., 2007, Rogers et al., 2008, Sant'anna et al., 2009). In order to successfully complete its cycle within the sand fly, *Leishmania* nectomonads must escape from endoperitrophic space,

through the PM, to prevent being passed together with remnants of the digested blood meal (Pimenta et al., 1997).

We have previously characterized a functional, blood-induced chitinolytic system, in the midgut of *P. papatasi* and *L. longipalpis* sand flies (Ramalho-Ortigao & Traub-Cseko, 2003, Ramalho-Ortigao et al., 2005). We also demonstrated that polyclonal antibodies to PpChit1 inhibit the midgut chitinolytic activity *in vitro*, and this effect also was shown across different sand fly species (Ramalho-Ortigao et al., 2005). PpChit1 is presumably involved in the maturation and degradation of *P. papatasi* PM (as is its ortholog in *L. longipalpis*, LlChit1) (Ramalho-Ortigao & Traub-Cseko, 2003, Ramalho-Ortigao et al., 2005), and addition of allosamidin, a chitinase inhibitor to the infective blood meal of this sand fly led to entrapment of *Le. major* within the peritrophic space (Pimenta et al., 1997). Although allosamidin may have also inhibited chitinase secreted by *Leishmania*, taken together, these data suggested that PpChit1 also can be involved with *Leishmania* escape from the endoperitrophic space.

To address whether silencing of *PpChit1* transcripts via RNAi-induced pathway would affect *Le. major* development within its natural vector, *P. papatasi*, we synthesized a dsRNA specifically targeting *PpChit1*.

Injection of dsRNA targeting specific transcripts has now been widely applied in disease vectors and proven an invaluable tool for the understanding of underlying events in pathogen-vector relationships (Molina-Cruz et al., 2008, Gonzalez-Lazaro et al., 2009, Pinto et al., 2009). In sand flies, gene silencing with dsRNA was first applied to *L. longipalpis* cell culture (Pitaluga et al., 2008), inducing a non-specific antiviral response. Recently, dsRNA injection of adult sand flies led to a specific reduction of Xanthine dehydrogenase expression (Sant'anna et al., 2008), and to an effect on *Le. mexicana* development when a midgut trypsin produced by *L. longipalpis* was silenced (Sant'anna et al., 2009).

The midgut chitinase PpChit1 is only expressed following a blood meal (Ramalho-Ortigao et al., 2005). Thus, following injection of dsPpChit1 double-stranded RNA, sand flies were blood fed and midguts dissected at different intervals after feeding. Specific silencing of *PpChit1* transcripts was detected by quantitative real-time PCR analyses (Figure 2.1), with concomitant knock down of PpChit1 protein levels assessed by Western blots (Figure 2.2).

Based on the presumptive role of PpChit1 in the maturation and degradation of the PM1, we expected that silencing of this gene would lead to entrapment of *Leishmania* within the

endoperitrophic space. Our results are consistent with this hypothesis, as *Le. major* load was reduced 120 h PBM in midguts of dsPpChit1 injected *P. papatasi* (Figures 2.3 and 2.4) suggesting that PpChit1 is indeed involved in PM1 degradation. Moreover, reduction of the *Le. major* load at 48 h PBM in dsChit1 compared to control-injected flies might have been caused by at least two scenarios: 1) a reduction in nutrient availability in the endoperitrophic space as the PM may be less permeable to proteolytic enzymes, or in the contrary, 2) to inability of parasites to escape leading to longer exposure to digestive enzymes inside the peritrophic space. Regardless of the mechanism, it still remains to be determined.

Future studies will assess whether this is a feasible approach in preventing transmission from an infected animal to a naïve host. Moreover, the results support the targeting of PpChit1 as a mean to interfere with *Leishmania* development within the sand fly – a candidate transmission-blocking vaccine.

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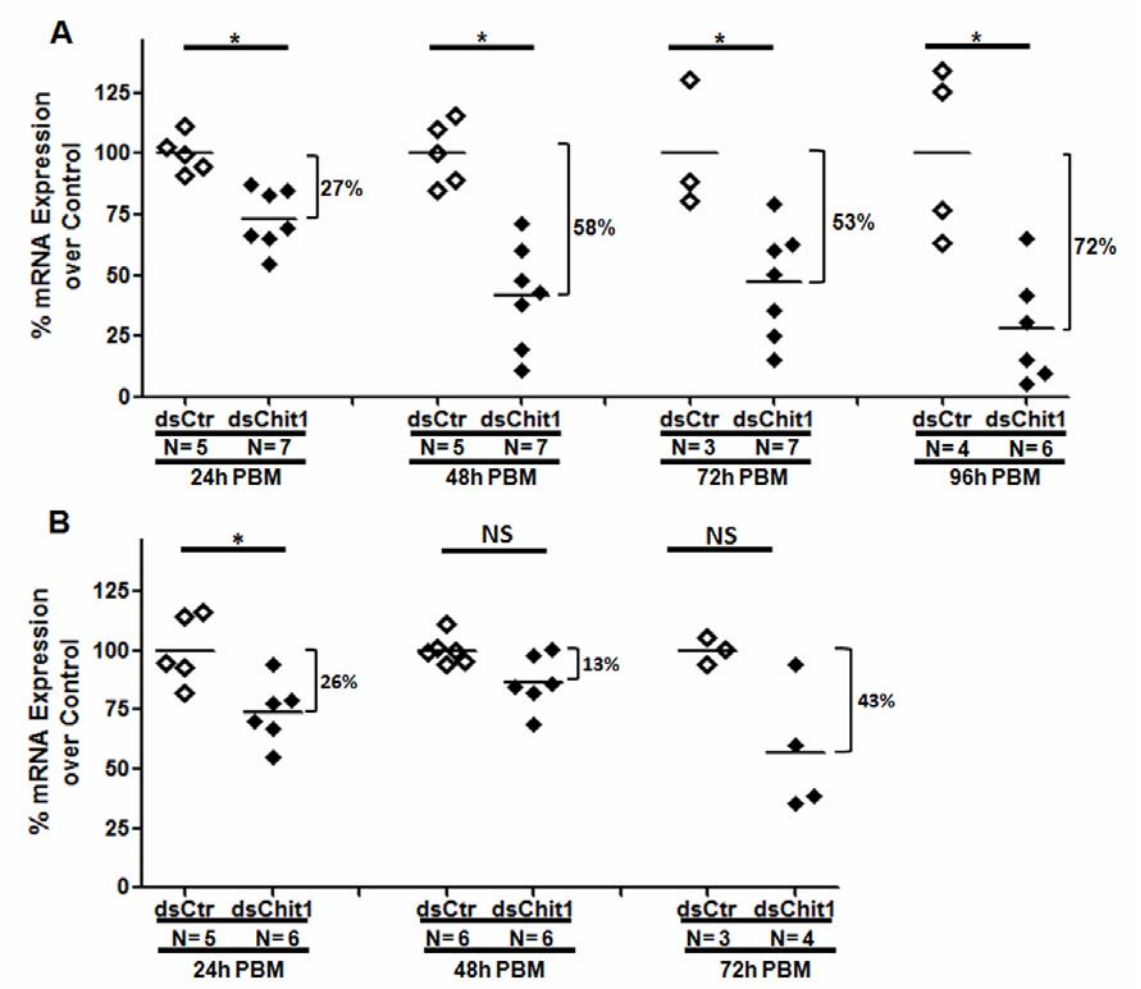

Figure 2.1 dsRNA effect on *PpChit1* RNA levels. Real-Time PCR comparing the mRNA level of *PpChit1* between flies injected with 80.5 ng (A) or 144 ng (B) of dsPpChit1 (dsChit1) or dsControl (dsCtr) double-strand RNAs. Significant *PpChit1* transcript reduction was exhibited by dsPpChit1 injected flies at 24 h (A and B), 48 h, 72 h, and 96 h PBM (A). *PpChit1* mRNA levels were normalized with the S3 housekeeping gene. Results are presented as a percent of *PpChit1* expression levels in dsPpChit1 injected flies over the mean of *PpChit1* expression levels in dsControl injected flies (considered as 100 %) for each time point. The variance in PpChit1 expression in dsControl injected flies is also shown. Each dot represents *PpChit1* RNA levels in a single fly. Horizontal bars indicate mean expression level. *: Statistically significant $p < 0.05$.

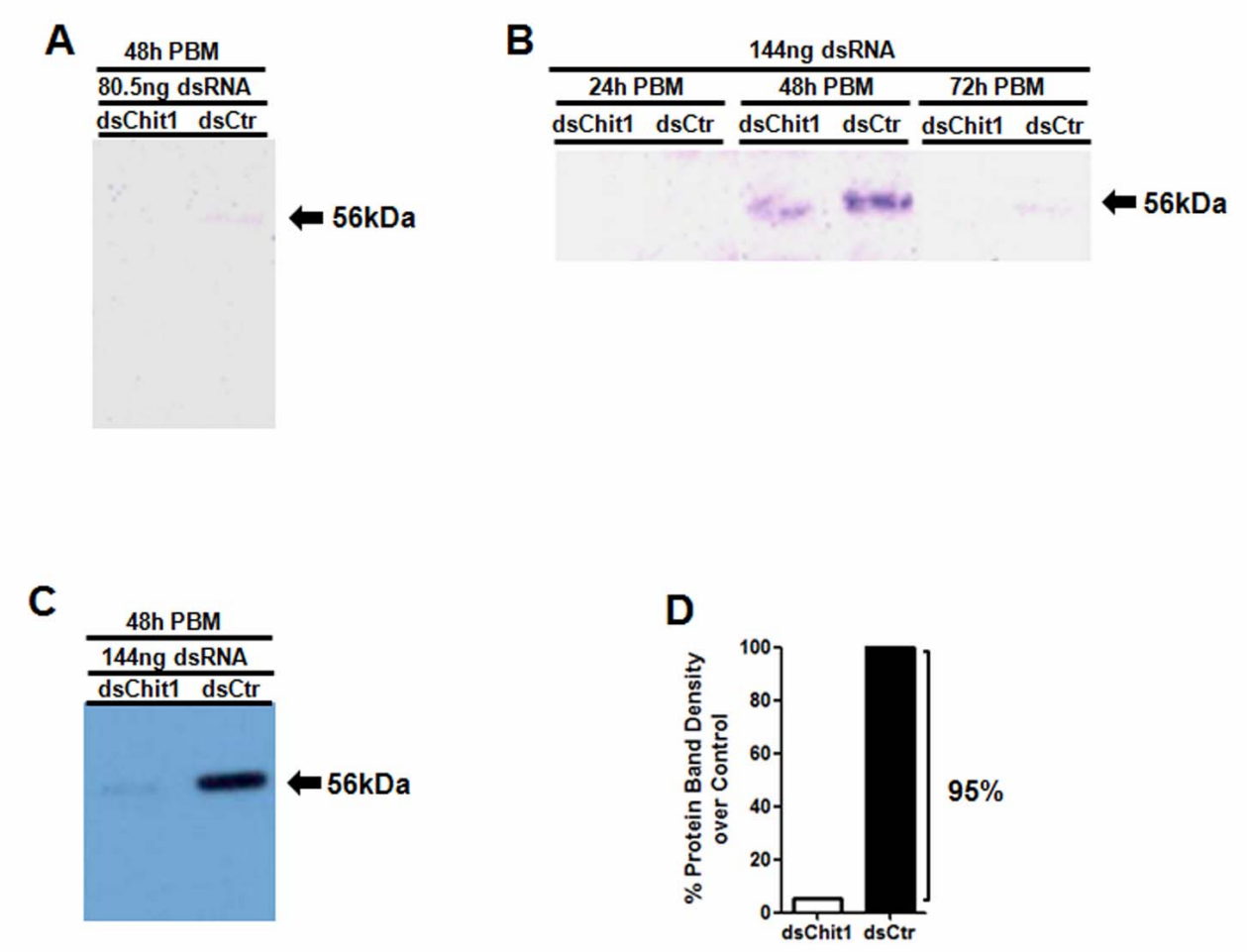

Figure 2.2 dsRNA effect on PpChit1 protein levels. (A). Western blot assay pointing to *PpChit1* knock down in dsPpChit1 injected flies (80.5 ng dsRNA) at 48 h PBM. (B). Midgut extracts from flies injected with 144 ng dsPpChit1 (dsChit1) displayed weaker bands (56 kDa) than dsControl (dsCtr) injected flies at 48 h and 72 h PBM. A-B, Colorimetric development. (C). Western blot assay depicting strong PpChit1 expression reduction in flies injected with 144 ng dsPpChit1 (dsChit1) compared with dsCtr injected ones at 48 h PBM (Chemiluminescence development). (D). Densitometry analysis of PpChit1 protein bands obtained in the chemiluminescence assay revealing 95 % reduction in PpChit1 expression between dsPpChit1 and dsControl injected flies.

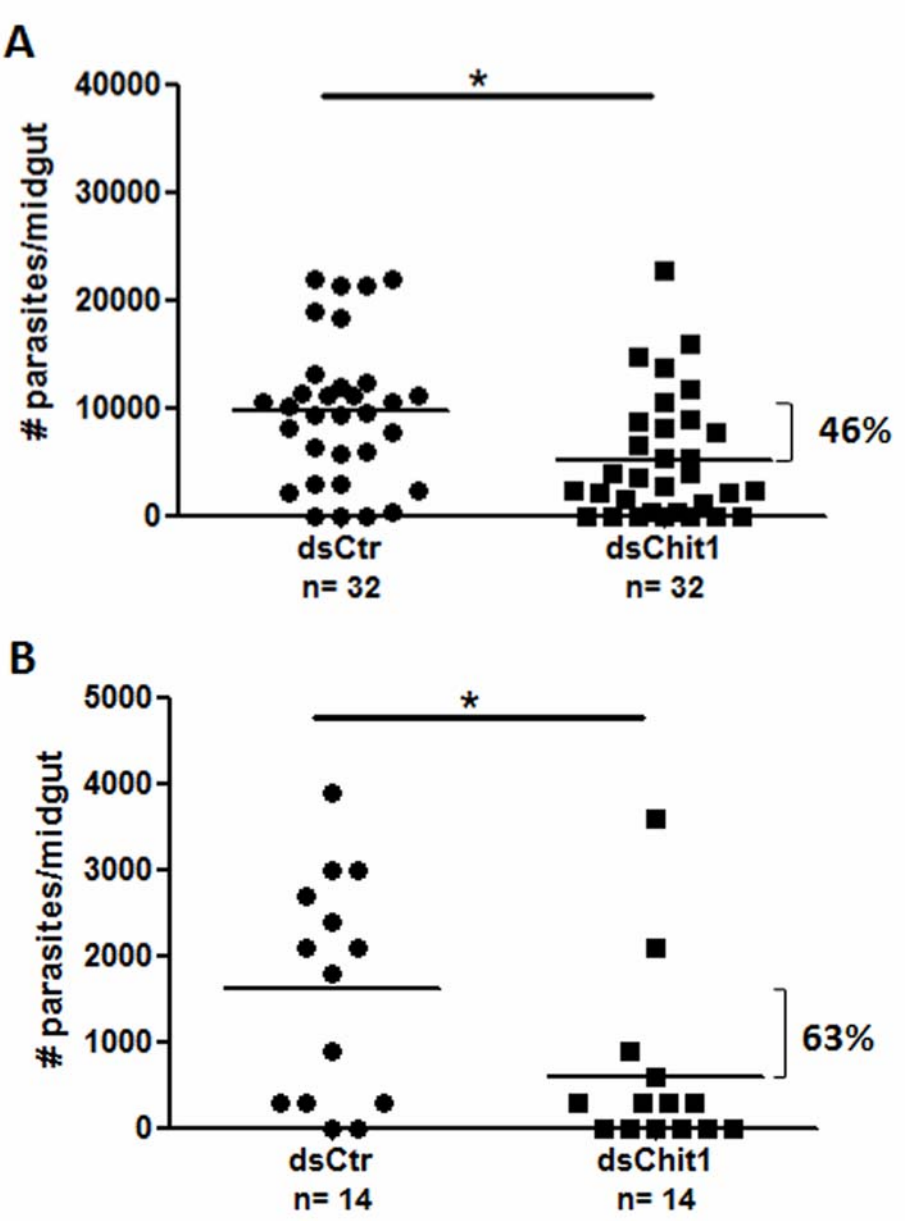

Figure 2.3 dsRNA effect on *Le. major* development. Intra-thoracic injections of dsPpChit1 (80.5 ng) reduce *Le. major* load in *P. papatasi* midgut. (A). At 48 h PBM, *Le. major* density was reduced on average 46 % in dsPpChit1 (dsChit1) injected compared with dsControl (dsCtr) injected. (B). *Le. major* parasites per midgut were further reduced at 120h PBM in dsPpChit1 injected flies, reaching on average 63 % reduction over the dsControl injected ones. Each dot represents parasite number in a single *P. papatasi* midgut. Horizontal bars display mean parasite numbers. n: Number of flies analyzed. *: Statistically significant $p < 0.05$. Graphs represent one similar result of two independent experiments.

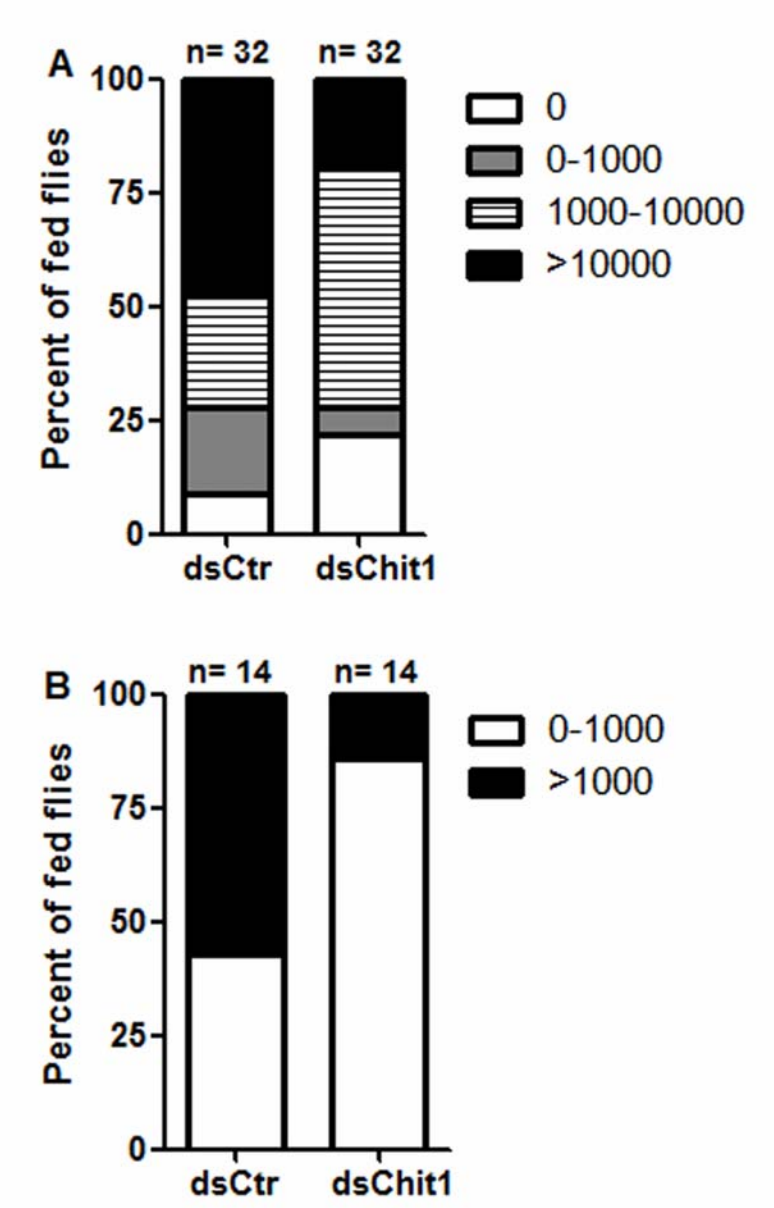

Figure 2.4 Effect of dsRNA injection on *Le. major* infection level in *P. papatasi*. Parasite load was categorized according to the number of *Le. major* per midgut. (A) Percentage of sand flies injected with either dsCtr or dsChit1 exhibiting no infection (0 parasites), as well as light (1-1,000 parasites), moderate (1,000-10,000 parasites), or heavy (>10,000 parasites) infection at 48 h PBM. Differences are statistically significant (Chi-square, p= 0.01). (B) Percentage of sand flies injected with either dsCtr or dsChit1 exhibiting either no parasites or light infection (0-1000 parasites), or moderate infection (>1,000 parasites) at 120 h PBM. Differences are statistically significant (Fisher's exact test, p= 0.04). n: Number of flies dissected.

Chapter 3 - The Role of Peritrophin PpPer1 in PM Formation and *Leishmania major* Escape in *Phlebotomus papatasi*

Abstract

Background: The peritrophic matrix (PM) plays a key role in compartmentalization of the blood meal and as barrier to pathogens in many disease vectors. To establish an infection in sand flies, *Leishmania* must escape from the endoperitrophic space to prevent excretion with remnants of the blood meal digestion. In spite of the role played regarding *Leishmania* survival, little is known about sand fly PM molecular components and structural organization.
Methodology/Principal Findings: We characterized three peritrophins (PpPer1, PpPer2, and PpPer3) from *Phlebotomus papatasi*. 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. *PpPer1* and *PpPer3* expression is regulated by *Le. major* infection and knock down of *PpPer1* led to 45 % reduction in mRNA levels and 44% in protein which resulted in increases of parasite load of 39 % at 48 h and 22 % at 96h post-infection. *Conclusion/Significance*: Our data strongly suggest that PpPer1 is a component of the PM scaffold in *P. papatasi* and may significantly contribute to its overall structure organization and porosity of the matrix as determined by the increase in *Le. major* load following PpPer1 knock down.

Keywords: Sand flies, RNAi, Peritrophic matrix, Peritrophins, *Leishmania*

Author summary

For a successful development within the midgut of the sand fly vector, *Leishmania* must overcome several barriers imposed by the vector that include an early proteolytic attack, the need to escape from the endoperitrophic space, and attachment to the midgut epithelia to prevent excretion with the remnants of the blood meal. That the sand fly PM constitutes an important barrier against *Leishmania* development was demonstrated when *Leishmania* were unable to escape and remained trapped within the endoperitrophic space and the infection was lost as the remnants of the blood meal were passed. These findings were further confirmed by experiments showing that a thicker PM can significantly limit *Leishmania major* development in its natural vector, the sand fly *Phlebotomus papatasi*. Despite its role on *Leishmania* development, characterization of the molecular components of the sand fly PM had not been performed. Our study provides molecular characterization of three peritrophins from *P. papatasi*. Our data indicate that PpPer1 and PpPer2 are likely involved in the organization of the PM. The findings are also supported by temporal and spatial expression analyses. Interestingly, PpPer3 appears to have an additional function, suggested by the presence of an atypical putative CBD domain. Our results also indicate that expression of *PpPer1* and *PpPer3* is regulated by *Le. major*, and that PpPer1 is likely a key component for the *P. papatasi* PM to function as a barrier against *Le. major* infection.

Introduction

Leishmaniasis is a neglected vector-borne disease caused by several different species of *Leishmania* (Herwaldt, 1999, Ramalho-Ortigao et al., 2010). Every year, 500,000 new human cases are diagnosed in 88 countries, and 350 million people are at risk of becoming infected (TDR/WHO, 2002). The DALY (or disability adjusted life years) burden for leishmaniasis is 2 million (TDR/WHO, 2002).

Leishmania are digenetic parasites, developing in a suitable mammalian host and within the sand fly vector (Kamhawi, 2006). Nearly, 35 species of phlebotomine sand flies have been proven or incriminated as vectors of *Leishmania* worldwide (Herwaldt, 1999).

In order to survive and successfully establish an infection in the sand fly, *Leishmania* must overcome many barriers (reviewed by (Ramalho-Ortigao et al., 2010)). First, and following ingestion with the blood meal, transitional stage *Leishmania* amastigotes must survive a proteolytic attack by digestive enzymes (Schlein & Romano, 1986, Borovsky & Schlein, 1987, Pimenta et al., 1997, Secundino et al., 2010). Upon developing into the promastigote stage, parasites (nectomonads) must escape from the endoperitrophic space through the peritrophic matrix (PM) (Pimenta et al., 1997) and attach to the midgut epithelia (Pimenta et al., 1992, Kamhawi et al., 2004), in both cases to prevent excretion following the digestion of the blood meal. As parasites develop into metacyclic promastigotes, they must detach from the midgut and migrate towards the foregut and the cardia (or stomodeal valve area). At the cardia, it has been shown that *Leishmania*-secreted chitinase damages the stomodeal valve preventing its normal function, and forcing the sand fly to regurgitate the contents of the gut as it attempts to blood feed (Schlein et al., 1992, Rogers et al., 2008). It is widely accepted that regurgitation carries *Leishmania* onto the skin of the vertebrate host.

Regarding the sand fly PM, earlier findings suggested that it serves as a barrier against *Leishmania* development (Feng, 1950, Walters et al., 1992). These results were further supported by feeding the chitinase inhibitor allosamidin to *P. papatasi* and showing that *Leishmania major* remained trapped inside a thicker PM (Pimenta et al., 1997). These latter studies also revealed a dual role for the sand fly PM in protecting as well as serving as barrier to *Leishmania*. Altogether, these findings demonstrate that the PM is an important component of sand fly vector competence.

Despite its importance, little is known about the molecular components of the sand fly PM (Ramalho-Ortigao et al., 2007, Jochim et al., 2008), or their roles during infection with *Leishmania*.

Here, we characterized three peritrophins, PpPer1, PpPer2, and PpPer3, previously identified in the midgut of *P. papatasi* (Ramalho-Ortigao et al., 2007). PpPer1 and PpPer2 are likely involved in the formation of the PM scaffold, as suggested by their expression profiles. PpPer3 on the other hand may be involved in mechanisms related to protection of the epithelia, as this peritrophin displays a mucin domain and is expressed in both gut tissues and Malpighian tubules. We also investigated the role of the sand fly PM as a barrier for *Leishmania* development. Our results indicate that reduction of PpPer1 expression levels leads to an increase in *Le. major* load in *P. papatasi*. Thus, we were able to identify PpPer1 as a PM component playing a significant role on *Le. major* development.

Methods

Bioinformatics Analyses

The cDNA sequences of *PpPer1*, *PpPer2*, and *PpPer3* were previously identified in (Ramalho-Ortigao et al., 2007). Predicted isoelectric points and molecular weights of mature proteins were obtained using the Compute pI/Mw tool (Gasteiger et al., 2005). Putative secretory signal peptides were determined using SignalP 3.0 (Nielsen et al., 1997). Prediction of *O*-linked glycosylated amino acids was carried out with NetOGlyc 3.1 (Julenius et al., 2005) while *N*-linked glycosylation site prediction was performed using NetNGlyc 1.0 (http://www.cbs.dtu.dk /services/NetNGlyc/). Protein domains were identified by searching Prosite (http://expasy.org/tools/scanprosite/), Pfam (http://pfam.sanger.ac.uk/search), and CDD (http://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml) domain databases. Chitin binding domain (CBD) classification in type-A ($CX_{13-20}CX_{5-6}CX_{9-19}CX_{10-14}CX_{4-14}C$), type-B ($CX_{12-13}CX_{20-21}CX_{10}CX_{12}CX_{2}CX_{8}CX_{7-12}C$), or type-C ($CX_{8-9}CX_{17-21}CX_{10-11}CX_{12-13}CX_{11}C$) was performed visually, following the Consensus sequences described by Tellam (Tellam et al., 1999). The mucin-like domain amino acid composition was assessed using the GeneRunner software (http://www.generunner.net/). Predicted heme-regulatory motifs (HRM) were visually identified as cysteine-proline dipeptide (Zhang & Guarente, 1995).

Multiple sequence alignment of peritrophin CBDs was performed with the ClustalW tool in the BioEdit package (Hall, 1999). Alignment was adjusted manually to remove some gaps. The CBDs of *P. papatasi* peritrophins were aligned to CBD sequences identified in peritrophins from *Lutzomyia longipalpis* (Dillon et al., 2006, Jochim et al., 2008). Alignment was performed with each CBD sequence located between the first and sixth conserved cysteine residues. The *L. longipalpis* peritrophin cDNA sequence identified in whole body libraries (NSFM-72d06.q1k; (Dillon et al., 2006)), referred to here as LlPer3 is an ortholog of the *P. papatasi* PpPer3. A putative CBD was identified in the PpPer3 N-terminal sequence by visual inspection and named Pp3put. Ll3put was similarly identified within the *L. longipalpis* LlPer3. Peritrophin sequences displaying similarities to Pp3put and Ll3put CBDs were retrieved from GenBank and aligned to the sand fly CBDs.

Phylogenetic analysis was performed using the Maximum Likelihood method based on the Whelan and Goldman model (Whelan & Goldman, 2001). The branch robustness was inferred by 500 bootstrap pseudo-replicates (Felsenstein, 1985). These analyses were carried out with the MEGA5 software (Tamura et al., 2007).

Sand fly samples, RNA isolation and cDNA synthesis

All sand flies used were *P. papatasi* (PPIS strain) reared at Department of Entomology, Kansas State University, as previously described (Coutinho-Abreu et al., 2010). For the adult flies 3-to-5 day-old insects were used in the experiments described below.

Blood feeding of sand flies was performed using two methods: 1) direct feeding on BALB/c mice anesthetized for one hour with a mixture of ketamine/xylazine (100 mg/kg of ketamine; and 4 mg/kg xylazine, diluted 1:5 in PBS); and 2) using glass feeders filled with heat-inactivated mouse blood or heat-inactivated mouse blood mixed with $5x10^6$ *Le. major* amastigotes/ml, as previously described (Coutinho-Abreu et al., 2010). The flies that were fed directly on the anesthetized mouse were used on the RT-PCR assays. The feeding of flies using the glass feeders was for flies used in real time PCR analyses, and for the flies injected with dsRNA. Only fully engorged sand flies were used.

P. papatasi dissections were performed in RNase free 1X PBS (pH 7.4; Fisher Scientific, Pittsburgh, PA) using glass slides and dissecting tools treated with ELIMINase (Fisher Scientific). Dissected tissues were immediately placed into 1.5 ml tubes containing 50 µl

RNAlater (Ambion, Austin, TX). Samples were thoroughly homogenized using a hand-held homogenizer and RNAse free plastic pestles.

RNA isolations and cDNA syntheses were performed as described previously (Coutinho-Abreu et al., 2010). In brief, total RNA was extracted from each sample with the RNAeasy mini kit (Qiagen, Valencia, CA), following the manufacturer's recommendations. RNAs were treated with DNAse (Turbo DNA-free kit, Ambion) to eliminate DNA contamination. cDNAs were synthesized using the SuperScript III Reverse Transcriptase, with all additional reagents supplied by Invitrogen (Carlsbad, CA). All cDNA syntheses were performed using 25 ng total RNA.

Total RNA obtained from various tissues from adult females were dissected and pooled as follows. For midguts, five tissues from sugar fed (0 h) were dissected and combined. Likewise, five blood fed midguts also were dissected and combined at each of the following time points: 6 h, 12 h, 24 h, 36 h, 48 h, 72 h, 96 h, 120 h, and 144 h post blood meal (PBM). Pools of adult carcasses, hindguts, heads plus salivary glands, ovaries, and Malpighian tubules were made from tissues obtained from single sand flies dissected at 0 h, 6 h, 12 h, 24 h, 36 h, 48 h, 72 h, 96 h, 120 h, and 144 h PBM. The pool of fat bodies was made from single flies dissected at 6 h, 12 h, 24 h, and 36 h PBM. RNAs from developmental stages were obtained from pools of 20 eggs, 10 L1 larvae, and five each for stages L2, L3, L4, and five pupae. cDNAs obtained from RNA samples from pooled tissues were used in RT-PCRs described below.

For the real-time PCR (rt-PCR) assays, *P. papatasi* midguts were dissected from flies that fed either on blood or blood plus *Le. major* (glass feeders) at 24 h, 48 h, and 72 h PBM. cDNAs obtained from eight RNA samples representing individual midguts from each of the three time points were used for real time PCRs below.

RT-PCR and real time qRT-PCR

PpPer1, *PpPer2*, *PpPer3*, and *β-tubulin* cDNAs were amplified using primer pairs described in Table 1. The expression profiles of such genes were obtained after 23 amplification cycles for *PpPer2*, 25 cycles for *PpPer1* and *PpPer3*, and 28 cycles for *α-tubulin*. Reactions were performed in 25 µl total volume, containing 12.5 µl GoTaq Master Mix (Promega, Madison, WI), 1 µl cDNA, 0.5 pmoles each primer, and 10.5 µl molecular grade water. Amplification reactions followed the conditions: 94ºC for 3 minutes (min); 23-28 cycles of 94ºC

for 30 seconds (sec), 57-58ºC for 1 min; and 72ºC for 30 sec. A final amplification step at 72ºC for 10 min also was carried out.

Real time quantitative PCRs were performed with a MasterCycler Realplex4 Eppendorf Real-Time PCR (Hamburg, Germany) using BioRad SyBR green (BioRad, Hercules, CA). Reactions were set up as described (Coutinho-Abreu et al., 2010). Amplification conditions and primer pairs used (Table 1) were the same used in RT-PCRs, except that a total of 40 amplification steps were performed. As a housekeeping control, cDNA corresponding to the S3 protein of the 40S ribosomal subunit was amplified (Table 1). Two technical replicates were performed for each PCR.

Knock down of PpPer1

PpPer1 was selected for these studies in light of its mRNA expression profile (midgut-specific and regulated by blood feeding). Double-strand RNAs were synthesized using the Megascript RNAi kit (Ambion). Synthesis and purification of dsRNA as well as injections of sand flies were performed according to (Coutinho-Abreu et al., 2010). The dsRNA targeting PpPer1 (dsPpPer1) was PCR amplified with primers PpPER1T7i_2 forward and reverse (Table 1). dsGFP was used a non gene-specific target control, as described (Arakane et al., 2008). The effects of dsRNA induced knock down were assessed by real time PCR analyses and Western blot.

Next, we assessed the effects of PpPer1 knock down on *Le. major* development within the *P. papatasi* midgut. In that case, 80.5 ng of dsRNA was injected intra-thoracically per sand fly (Coutinho-Abreu et al., 2010). After feeding on an infectious blood meal, midguts were individually dissected at 48 h, 96 h, and 120 h PBM. Midguts were homogenized with a hand-held homogenized and plastic pestle in 30 µl 1X PBS (pH 7.4). Live parasites were counted on a modified Neubauer chamber.

Antisera production and Western blot

Antisera production was performed as described previously (Ramalho-Ortigao et al., 2005, Oliveira et al., 2006, Coutinho-Abreu & Ramalho-Ortigao, 2010). The mature sequence of PpPer1 was amplified from *P. papatasi* midguts (12 h PBM), using the primer pair PpPer1Mat_717F/PpPer1Mat_717R (Table 1). Amplification reaction followed the conditions:

94ºC for 3 min; thirty five cycles of 94ºC for 1 min, 56ºC for 1 min, and 72ºC for 1 min; and 72ºC for 10 min.

To determine the effects of dsRNA injection on the expression of PpPer1 in *P. papatasi* midguts, polyclonal anti-PpPer1 specific antisera (1:50 dilution), as well as Western blot assays were performed according to (Coutinho-Abreu et al., 2010). Densitometry analysis was performed using the TotalLab TL100 software (Nonlinear Dynamics, Durham, NC).

Statistical analyses

Unpaired t-test and Mann-Whitney U test were used to assess for statistically significant differences in expression profiles and parasite counts when data followed or not a normal distribution, respectively. Assessment of distribution pattern was carried out by D'Agostino & Pearson omnibus normality test. Differences were considered statistically significant at $p< 0.05$. All the statistical assessments were performed using GraphPad Prism v.5.0 (GraphPad Software, Inc).

Results

Peritrophins' cDNA sequences and predicted protein organization

The complete cDNA sequences of *P. papatasi PpPer1*, *PpPer2*, and *PpPer3* were previously identified and published in (Ramalho-Ortigao et al., 2007).

PpPer1 open read frame (ORF) is 792 bp long, encoding a protein of 263 amino acids, with a predicted molecular weight of 28 kDa for the mature protein, and an acidic pI (4.84). Predicted *N*- and *O*-linked glycosylation sites at residues N29 and T211, respectively, are expected to add to the molecular weight of the secreted protein. PpPer1 displays a predicted signal peptide (amino acid residues 1-18), suggesting the protein is secreted into the midgut lumen. Four type-A CBDs (PpPer1 CBD consensus sequence: $CX_{13\text{-}19}CX_5CX_{9\text{-}10}CX_{12}CX_7C$) are also present in the mature protein. In addition, two putative HRM were identified at amino acid residues 182-183 in the third CBD (Pp1CBD3 residues 44 and 45 in Figure 3.1), and at residues 209-210 in the fourth CBD (Pp1CBD4 residues 1 and 2 in Figure 3.1).

PpPer2 ORF is 270 bp coding for a predicted 7.8 kDa mature protein with a single type-A CBD (consensus sequence: $CX_{18}CX_5CX_9CX_{12}CX_7C$). Predicted *N*-glycosylation at amino acid residues N19 and N77 are also expected to increase the molecular weight of the protein. PpPer2

is an acidic protein (pI 4.25), with a single putative HRM at residues 22 and 23 of the predicted CBD (corresponding to residues 1 and 2 of Pp2CBD1 in Figure 3.1). The presence of a signal peptide with cleavage site between amino acids A17 and A18 suggests the protein is secreted into the midgut lumen.

PpPer3 is 313 bp with two CBDs. Unlike PpPer1 and PpPer2, PpPer3 has a mucin-like domain rich in serine (18.2 %), threonine (36.4 %), proline (12.1 %), and glutamine (12.1 %) residues in addition to two type-A CBDs (PpPer3 CBD consensus sequence: $CX_{11}CX_5CX_{11\text{-}13}CX_{12}CX_{4\text{-}8}C$). The predicted molecular weight of the mature PpPer3 is 32 kDa. Moreover, this peritrophin has a neutral-to-basic pI (7.75). As a large number of residues (281T, 285T, 286T, 292S, 293T, 294T, 295T, 296T, 300S, 301S, 302S, 303T, 304T, 305T, 306T, 307T, 309S, 310S) within the mucin-like domain are predicted to be *O*-linked glycosylated, PpPer3 molecular mass is expected to be significantly greater. Two additional features of PpPer3 are the presence of a 57-residue long linker between the first (Pp3CBD1) and second (Pp3CBD2) CBDs, and an N-terminal sequence containing eight cysteine residues (Pp3put, in Figure 1). Although the Pp3put sequence displays a type-A CBD signature ($CX_{10}CX_5CX_{11}CX_{14}CX_{10}C$) similar to other CBDs in *P. papatasi* peritrophins, it was not recognized as a *bona fide* CBD by standard bioinformatics' tools. Two predicted HRM were identified at residues 138-139 (corresponding to residues 29 and 30 in Pp3CBD1, Figure 3.1) and 150-151 (residues 44 and 45 in Pp3CBD1, Figure 3.1) in the first PpPer3 CBD while a single HRM was predicted in residues 262-263 (residues 44 and 45 in Pp3CBD2) in the second PpPer3 CBD sequence (Figure 3.1).

According to the multiple sequence alignment between *P. papatasi* and *L. longipalpis* peritrophin CBDs (Figure 3.1), the six conserved cysteine residues characteristic of type-A CBDs are present. Interestingly, the numbers of amino acid residues between the second and third cysteines, and between the fourth and fifth cysteines were the least variable. In addition, aromatic residues Y and F corresponding to positions 25 and 26 between the second and third cysteines, and position 48 between the fourth and fifth cysteines were detected. Regarding the HRM sites, most are co-localized with the first and fourth cysteine residues.

Pp3put, a putative CBD domain present in the N-terminal portion of *P. papatasi* PpPer3 peritrophin, displays two extra cysteine residues at positions 18 and 53, the latter resulted from a dipeptide insertion (Y/F and C) between the fourth and fifth conserved cysteines (Figure 3.1). This putative CBD domain displays neither HRM motifs nor aromatic residues at positions 25,

26, and 48. Interestingly, other insect peritrophins with features similar to Pp3put were also identified by searching the GenBank database against this putative CBD domain from *P. papatasi*.

A phylogenetic analysis suggests a single clade for the Pp3put and Ll3put domains from sand flies (*P. papatasi* and *L. longipalpis*), the Ae45put, Ae52put, and Ae51put from *Ae. aegypti*, the Cq16put from *C. quinquefasciatus*, and the CfPL1put from *C. felis* (Figure 2, blue box). The phylogenetic analysis also highlights the elevated conservancy that exists between the CBD domains in *P. papatasi* and *L. longipalpis* orthologous peritrophins (Figure 3.2).

Peritrophin mRNA expression profiles

The expression profiles of *PpPer1*, *PpPer2*, and *PpPer3* were assessed by semi-quantitative RT-PCR (Figure 3.3). *PpPer1* mRNA expression was adult midgut-specific and blood-induced; transcripts were detected between 12 h and 72 h PBM, with the highest levels at 48 h PBM (Figure 3.3A). *PpPer2* transcripts were expressed in the midgut and in the hindgut. *PpPer2* was constitutively expressed in sugar (0 h) and blood fed guts (Figure 3.3B). *PpPer3* was expressed in the midgut (Figure 3.3B) and in the hindgut and Malpighian tubules (Figure 3.3C). In spite of being expressed in sugar (0 h) and blood fed midguts, *PpPer3* mRNA expression was up-regulated between 12 h and 48 h PBM, somewhat similar to the *PpPer1* expression profile. Among the three *P. papatasi* peritrophins, only *PpPer2* was expressed in larval stages (Figure 3.3C) and, comparatively, also appeared to have the highest expression levels of the three peritrophins, according to Figure 3.3A.

Expression of PpPer1 and PpPer3 is modulated by Le. major

We evaluated the effects of *Le. major* infection on expression of *P. papatasi* peritrophin mRNAs (Figure 4). *PpPer1* and *PpPer3* midgut mRNA levels were differentially expressed in *Le. major*-infected *P. papatasi*, while no difference was observed for *PpPer2* for the three time points assessed (Figure 3.4B). *PpPer1* expression displayed a statistically significant up-regulation (20 %) at 24 h post-infection (Figure 3.4A). However, no statistical difference was observed for *PpPer1* midgut expression at later time points (48 h and 72 h) following *Le. major* infection. For PpPer3, the mucin-like peritrophin, midgut mRNA levels were reduced by 28 % at 24 h and 48 h after *Le. major* infection (Figure 3.4C). No differential *PpPer3* expression was observed in *Le. major* infected midguts at 72 h post-infection.

PpPer1 knock down affects Le. major load within P. papatasi

PpPer1 was selected for the knock down experiments following our assessments of its expression profile (Figure 3.3) and according to the data from chitin binding assays. As PpPer1 is expressed exclusively in the midgut after blood feeding and binds chitin, we reasoned it was involved in PM formation. *PpPer2* expression could also be knocked down (not shown), but PpPer3 knock down has yet to be performed. Intra thoracic injections of *P. papatasi* females with 80.5 ng of double-strand RNA specific for *PpPer1* (dsPpPer1) were performed to assess the role, if any, of PpPer1 protein on *Le. major* development. First we determined whether injection of dsPer1 was able to reduce mRNA and protein levels. As shown in Figure 3.5, injection of the dsPpPer1 led to 45 % and 30 % reduction in mRNA expression levels at 24 h and 48 h PBM, respectively (Figure 3.5A), and to a corresponding reduction of 44 % in protein levels at 24 h PBM (Figure 3.5B and C).

We then proceeded to investigate whether PpPer1 knock down achieved by the exogenous RNAi pathway would produce a phenotype in infected flies. Interestingly, the knock down of PpPer1 led to an increase in *Le. major* load within *P. papatasi* midguts of 39 % at 48 h and 22 % at 96 h post-infection, as shown in Figures 3.6A and 3.6B.

Discussion

Chitin, proteins, and proteoglycans are the major components of the PM in insects (Tellam et al., 1999, Devenport & Jacobs-Lorena, 2005). Chitin, a linear polymer of β-(1,4)-*N*-acetyl-D-glucosamine (GlcNAc), is thought to be an important structural component of the PM, providing the scaffold onto which proteins and other components attach (Devenport & Jacobs-Lorena, 2005). The proteoglycans make up roughly 30 % of the PM and, along with chitin microfibrils, account for the mechanical strength and permeability (Lehane, 1997). The majority of the PM proteins are known as peritrophins (Tellam et al., 1999), and a common feature among these proteins is the presence of cysteine-containing domains forming internal disulfide bonds. They also display conserved aromatic amino acids between the cysteines predicted to bind to chitin as well as some potential *N*-linked and/or *O*-linked glycosylation sites (Tellam et al., 1999).

Two types of PM are found in hematophagous insects. Type 1 (or PM1) is synthesized in adult hematophagous insects by midgut epithelial cells, forming a sac-like structure that envelops

the blood meal. The PM1 is approximately 1-20 μm thick, and synthesized, following a blood meal, in adult female mosquitoes and sand flies. Type 2, or PM2, is produced by the cardia, a specialized organ located at the junction of the cuticle-lined foregut and midgut. The PM2 is an open-ended sleeve-like structure that lines both the midgut and hindgut. The PM2 is significantly thinner than the PM1, with only 0.1-2 μm. In mosquitoes and sand flies, the PM2 is only produced during larval stages (Devenport & Jacobs-Lorena, 2005). More importantly, for blood feeding insects the PM1 represents a barrier against pathogens including viruses, bacteria, fungi, protozoa, and nematodes (Peters, 1991, Devenport & Jacobs-Lorena, 2005).

Here, we characterized three peritrophins of the sand fly *P. papatasi* believed to be involved in the formation of the PM in adult females. In addition, as the PM is an important component of vector competence in sand flies (Feng, 1950, Walters et al., 1992, Pimenta et al., 1997, Coutinho-Abreu et al., 2010), we assessed the role of PpPer1 as a molecular barrier against *Le. major*.

Of the three peritrophins, PpPer1 displays four CBDs and shares 57 % amino acid identity with LuloPer1, a 4-CBD putative peritrophin identified in the New World sand fly *L. longipalpis* (Jochim et al., 2008). PpPer2 displays a single CBD and is 63 % and 57 % identical to LuloPer3 and LuloPer2 (Jochim et al., 2008), respectively. PpPer3 shares 59 % amino acid identity with the predicted protein sequence of the *L. longipalpis* peritrophin cDNA sequence LlPer3 (Cluster 6; NSFM-72d06.q1k) identified in whole body libraries (Dillon et al., 2006). The predicted function for multi-domain peritrophins is to cross-link chitin fibrils while single CBD peritrophins are expected to bind and protect the tips of chitin fibrils from exochitinolytic activity (Shi et al., 2004).

Different than PpPer1 and PpPer2, PpPer3 is a mucin-like peritrophin with two CBDs and a mucin-like domain rich in serine, threonine, glutamine, and proline residues. Mucin-like domains are predicted to be heavily *O*-linked glycosylated that contributes to a gel-like consistency for the PM, critical to their role in protecting the midgut epithelia from abrasion, hydrolytic enzymes, heavy metals, and pathogens (Wang & Granados, 1997, Sarauer et al., 2003).

In addition to their role in PM formation, peritrophins are also known to participate in detoxification (Devenport et al., 2006). In the mosquito *A. aegypti*, the mucin-like peritrophin AeIMUC was shown to bind heme *in vitro* via heme-regulatory motifs (HRM), while it also

bound chitin (Rayms-Keller et al., 2000, Devenport et al., 2006). HRMs are predicted for all three *P. papatasi* as well as the *L. longipalpis* peritrophins, suggesting a role for these proteins in heme binding and detoxification in sand flies.

We identified the CBDs present in the three peritrophins from *P. papatasi* and those identified in *L. longipalpis* sequence databases as type-A CBDs displaying the molecular hallmarks required for chitin binding (Shen & Jacobs-Lorena, 1999, Tellam et al., 1999, Hegedus et al., 2009). Chitin binding hallmarks include six conserved cysteine residues (with a conserved number of residues between each conserved cysteines matching the consensus sequence for type-A CBDs) (Tellam et al., 1999), and conserved aromatic amino acids residues predicted to interact with chitin fibrils (Shen & Jacobs-Lorena, 1999, Tellam et al., 1999).

Although the two putative CBDs Pp3put and Ll3put display the six conserved cysteines interspaced by the characteristic length expected for type-A CBDs (Tellam et al., 1999), amino acids other than aromatics are found in these putative domains. In addition, the Pp3put and Ll3put CBDs display two extra cysteines at residues 20 and 54, and have an unusual two-peptide insertion between the fourth and fifth conserved cysteines. Such features are in contrast with all the other *bona fide* sand fly type-A CBDs that display a conserved number of amino acids between the fourth and fifth cysteines (12 residues), and lack the extra cysteines present in the putative domains. Thus, it is tempting to speculate that Pp3put and Ll3put CBD domains underwent some type of neo-functionalization (Hahn, 2009), and that such change might also have occurred in peritrophins of other insects, such as the cat flea and mosquitoes. This hypothesis is to some extent supported by our findings that PpPer3 (which has the Pp3put domain) also is expressed in the Malpighian tubules, and by the lack of binding of rPpPer3 to chitin (not shown). Although the latter may be due to other factors, the expression in tissues other than the sand fly gut is suggestive of an additional function for this protein. Future functional characterization of these putative domains, both in *P. papatasi* and in *L. longipalpis*, will shed light on their functions.

Our assessment of temporal and spatial expression of the *P. papatasi* peritrophins demonstrated that *PpPer1* expression is midgut-specific and blood-induced, resembling the transcriptional profile of *PpChit1*, a midgut specific *P. papatasi* chitinase (Ramalho-Ortigao et al., 2005). The PpPer1 protein appears to be secreted in the midgut at an earlier time point (24 h PBM) following a blood meal than PpChit1, whose activity peaks between 48 h and 72 h PBM

(Coutinho-Abreu et al., 2010). Nonetheless, these patterns of protein expression are consistent with the functional roles of PpPer1 and PpChit1 in PM formation and degradation, respectively.

Contrasting the expression profile detected for *PpPer1*, *PpPer2* and *PpPer3* mRNAs are expressed not only before blood feeding (constitutively), but also in the hindgut and/or Malpighian tubules. Although peritrophin expression in hindguts and/or Malpighian tubules have also been detected in the cat flea *Ctenocephalides felis* (Gaines et al., 2003) and the fruit fly *Drosophila melanogaster* (Beyenbach et al., 2010), the physiological roles of peritrophins in these tissues have not been determined. Regarding the constitutive expression patterns of *PpPer2* and *PpPer3*, the corresponding proteins might not be translated in the same fashion. In *Aedes aegypti*, the peritrophin *AeIMUCI* is constitutively expressed, and yet the protein is only detected in blood fed midguts and up to 24 h PBM (Devenport et al., 2006). *PpPer2* also is expressed in larval stages, similar to *AeIMUCI* (Rayms-Keller et al., 2000).

To assess whether or not *Le. major* infection is capable of modulating *P. papatasi* peritrophin gene expression, we compared *PpPer1*, *PpPer2*, and *PpPer3* mRNA levels between *Le. major* infected and blood fed midguts. Although *Le. major* infection was not able to modulate *PpPer2* expression profile, *PpPer1* and *PpPer3* expression levels changed significantly upon infection. Regulation of peritrophins was suggested by previous transcriptome analyses studies of both *P. papatasi* and *L. longipalpis* (Ramalho-Ortigao et al., 2007, Jochim et al., 2008, Telleria et al.). The expression of *PpPer1* was up-regulated at 24 h post-infection whereas *PpPer3* mRNA levels were reduced at 24 h and 48 h post-infection. Up-regulation of *PpPer1* by *Le. major* may assist in protecting the parasite against proteolytic enzymes (parasite advantage), or may be a response by the sand fly in order to possibly reduce permeability of the PM (disadvantageous to the parasite). Whether one or multiple signals secreted by the parasite or present in the infected blood are involved in this regulation still needs to be determined. Similarly, regarding *PpPer3*, whether differential gene expression in infected midguts was parasite-mediated, or a vector-induced defensive response against infection, needs to be further investigated.

The role of the *P. papatasi* peritrophin PpPer1 on *Le. major* development in *P. papatasi* midgut was assessed via RNA interference (RNAi). PpPer1 was chosen for RNAi experiments because it was shown to be expressed exclusively in the midgut, and only after blood feeding.

The sand fly PM is thought to fulfill two apparently opposing roles (protection and barrier) when it comes to *Leishmania* infection (Pimenta et al., 1997). That sand fly PM protects *Le. major* against digestive enzymes early in infection (Pimenta et al., 1997) initially suggested to us that a potential alteration of the PM scaffold, increasing its permeability by the knock down or removal of one or more peritrophins, would lead to killing of parasites. The injection of dsPpPer1 into *P. papatasi* thorax reduced *PpPer1* mRNA and PpPer1 protein levels by 45 %. At 48 h post-infection, PpPer1 knock down led to a significant increase (39 %) in *Le. major* load in *P. papatasi* midguts. We envision at least three possible scenarios. First, upon dsPpPer1 injection and reduced PpPer1 protein availability to associate with chitin, a significantly more permeable PM might have been assembled, allowing *Le. major* to escape towards the ectoperitrophic space earlier than in control-injected flies, and at the same time avoid the harmful action of midgut digestive proteases (Pimenta et al., 1997). Alternatively, a PM with increased permeability would allow a greater influx of digestive enzymes to the endoperitrophic space, turning blood meal digestion faster and making nutrients more readily available for *Le. major* multiplication. Finally, a greater number of *Le. major* escape from the endoperitrophic space and a higher digestive enzyme influx in dsPer1-injected flies might have contributed to the overall increase *Le. major* load observed.

Although increased *Le. major* load in *PpPer1* knock down also was noted at 96 h post-infection (22 %), this increase was not statistically significant. The lower increase detected in *Le. major* load in dsPpPer1-injected flies 96 h post-infection also coincides with the point in time when the PM is significantly degraded by chitinolytic activities within the midgut. Thus, 96 h post feeding (or infection) might be too late in the digestive process for PpPer1 to maintain the PM structure, and its role as a barrier to *Le. major* infection. Alternatively, in dsPpPer1-injected flies a conceivably faster digestion of the blood meal (with beneficial effects for *Le. major* replication) would be less evident when the PM is degraded and the blood meal has already been fully digested. In this case (e.g., by 96 h PBM) the nutrients may be equally available to parasites in either dsPpPer1- and dsGFP-injected flies. Further studies on the events taking place during the *Leishmania* escape through the PM will clarify the mechanism leading to greater *Le. major* load after PpPer1 knock down.

Overall, in this work we characterized three peritrophins from *P. papatasi* (PpPer1, PpPer2 and PpPer3). We also described an overlooked conserved sequence present in insect

peritrophins that is similar to CBDs, but with potential novel or distinct physiological function(s). We also showed the differential expression of two peritrophin transcripts following *Le. major* infection, and demonstrated for the first time a role for PpPer1 on *Le. major* development. The data suggest that PpPer1 is a component of the PM, and may also be key player in the PM's function as a barrier for *Leishmania* development within the *P. papatasi* midgut.

Ethics Statement

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

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Table 3.1 List of primers. Primer name, sequence, and annealing temperatures for the primer pairs used in dsRNA synthesis and real time PCR analyses

Primer Name	Nucleotide Sequence (5' to 3')	Annealing Temperature
PpPER1T7i_2_F	TAATACGACTCACTATAGGGAGAATGAAGAACGTTGCAGTGAT	55°C/65°C
PpPER1T7i_2_R	TAATACGACTCACTATAGGGAGATTAGTGGTCGAAGCAGACTG	
PpPer1_122F	CTCATGAAGAGTTTTGCATG	57°C
PpPer1_122R	GAAACCGTCTTCACAGCTC	
PpPer2_168F	TGCCTGGTTTTCCTGTTC	58°C
PpPer2_168R	TCCTTGCACGAAAGTTCCC	
PpPer3_121F	ATCTGCCCAGGACCATTAC	58°C
PpPer3_121R	AGTCGACTGTAGCGCAATC	
PpTub_148F	GCGATGACTCCTTCAACAC	57°C
PpTub_148R	GTGATCAATTGTTCGGGATG	
Pp40S_S3_136F	GGACAGAAATCATCATCATG	57°C
Pp40S_S3_136R	CCTTTTCAGCGTACAGCTC	
PpPer1Mat_717F	GCTCATGAAGAGTTTTGCATG	56°C
PpPer1Mat_717R	TTAGTGGTCGAAGCAGACTG	

Figure 3.1 Multiple sequence alignment. Multiple sequence alignment was performed with individual CBD domains identified from peritrophins from *P. papatasi* and *L. longipalpis*. Pp1CBD1, Pp1CBD2, Pp1CBD3, and Pp1CBD4 are PpPer1 CBDs (Gen Bank accession number: EU031912). Pp2CBD1 is the single CBD in PpPer2 (EU047543). Pp3CBD1 and Pp3CBD2 are the two CBDs identified in PpPer3 (EU045354). Lulo1CBD1, Lulo1CBD2, Lulo1CBD3, and Lulo1CBD4 are the four CBDs in LuloPer1 (EU124588). Lulo2CBD1 and Lulo3CBD1 are the single CBDs in LuloPer2 (EU124602) and in LuloPer3 (EU124607), respectively. Ll3CBD1 and Ll3CBD2 are CBDs in LlPer3 (AM093395). Pp3put and Ll3put are putative domains similar to CBDs identified in *P. papatasi* PpPer3 and in *L. longipalpis* Ll3Per3. Such putative domain sequences also were identified in the N-terminal region of *C. felis* PL1 (AAM21354) - CfPl1put; *C. quinquefasciatus* conserved hypothetical protein (XP_001864216) - Cq16put; and *A. aegypti* AaeL_AAEL012651 (XP_001662775) -Ae51put, AaeL_AAEL012645 (XP_001662776) - Ae45put, AaeL_AAEL012652 (XP_001662772) - Ae52put. The six conserved cysteines are highlighted in grey with the conserved aromatic amino acids predicted to bind chitin shown in white with black highlight; HRM motifs are underlined. Conserved amino acid residues displayed exclusively by the putative CBD domain sequences are shown in red, and the additional cysteine residues are indicated by asterisk (*).

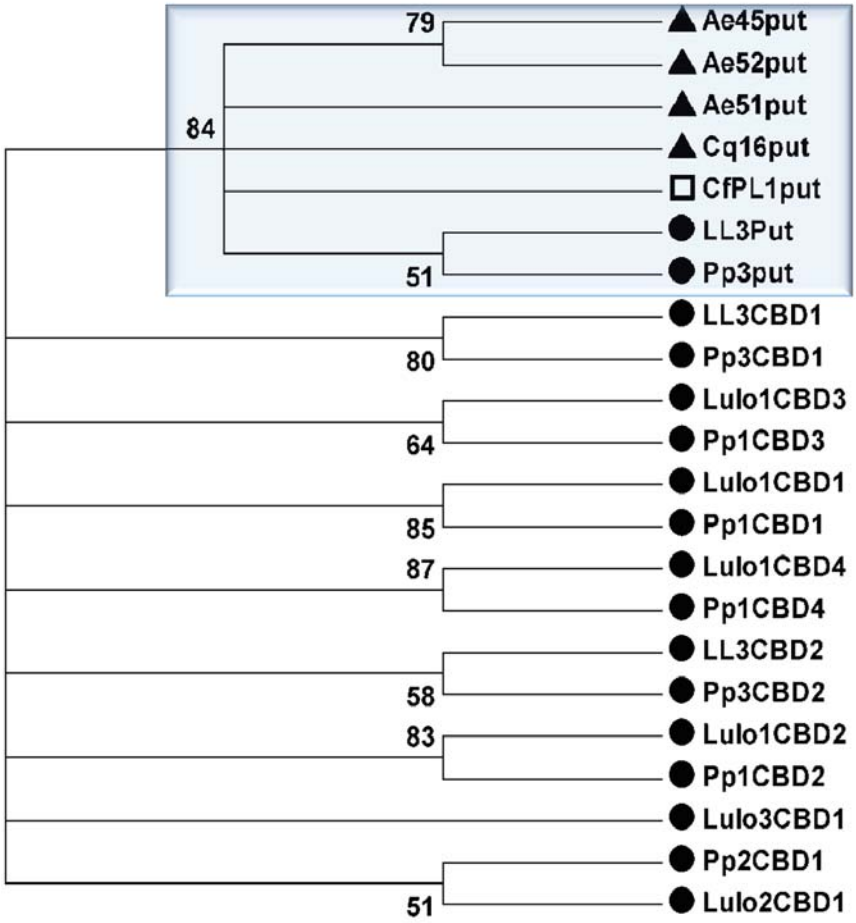

Figure 3.2 Phylogenetic comparison. Condensed tree depicts all the putative CBD domains in a single branch (blue shadow box), displaying strong bootstrap support (84 %). All other branches are CBDs found in orthologs of sand fly peritrophins. Filled circles, filled triangles, and open squares indicate sand fly, mosquito, and flea peritrophins, respectively.

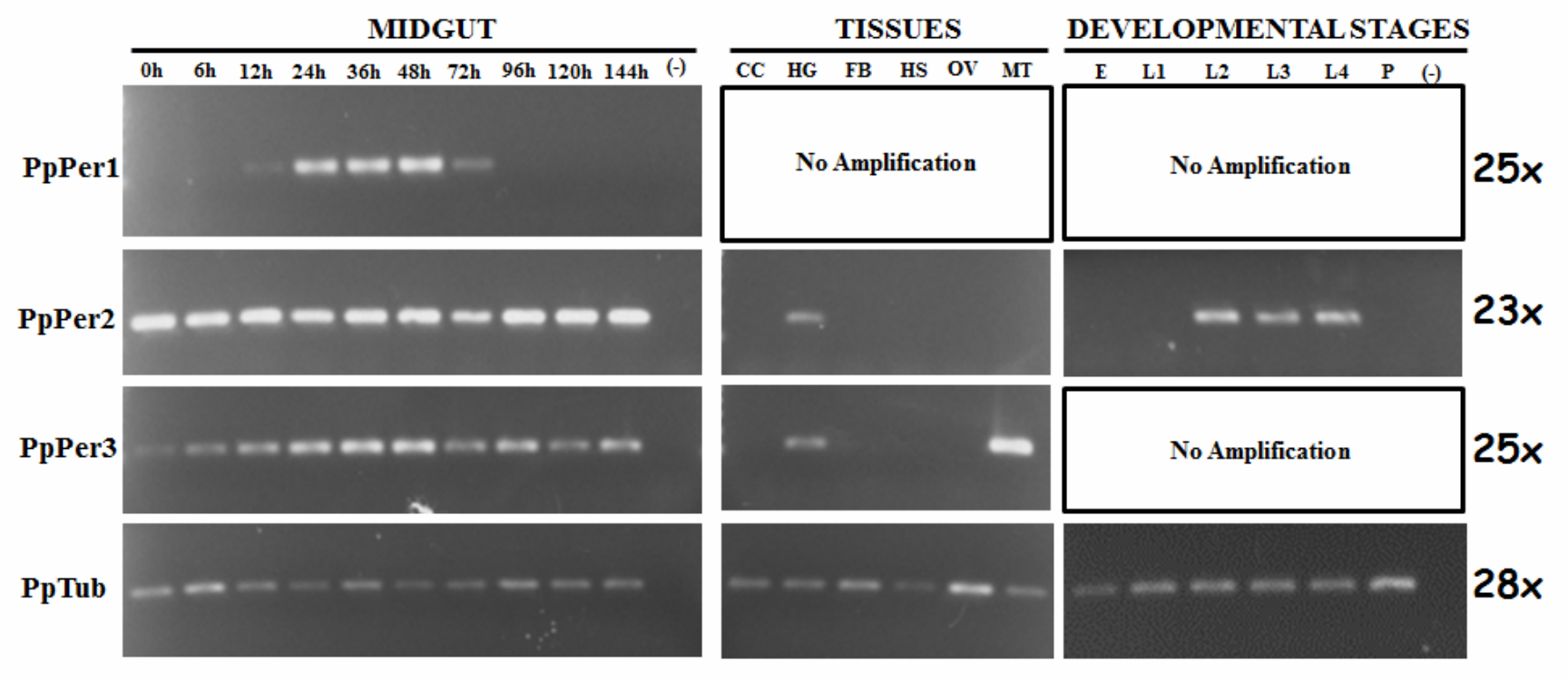

Figure 3.3 Peritrophin mRNA expression profiles. Expression of *PpPer1*, *PpPer2*, and *PpPer3* mRNAs was assessed by 23-25 RT-PCR cycles in *P. papatasi* midguts of adult females dissected at different time points before and after blood feeding (0-144 hours PBM), pools of other adult tissues, and developmental stages (eggs or whole body). α-Tub was used as the housekeeping control gene. CC: Carcass. HG: Hindgut. FB: Fat Body. HS: Head along with salivary glands. OV: Ovaries. MT: Malpighian Tubules. E: Eggs. L1: Larval stage 1. L2: Larval stage 2. L3: Larval stage 3. L4: Larval stage 4. P: Pupa. (-): Negative control.

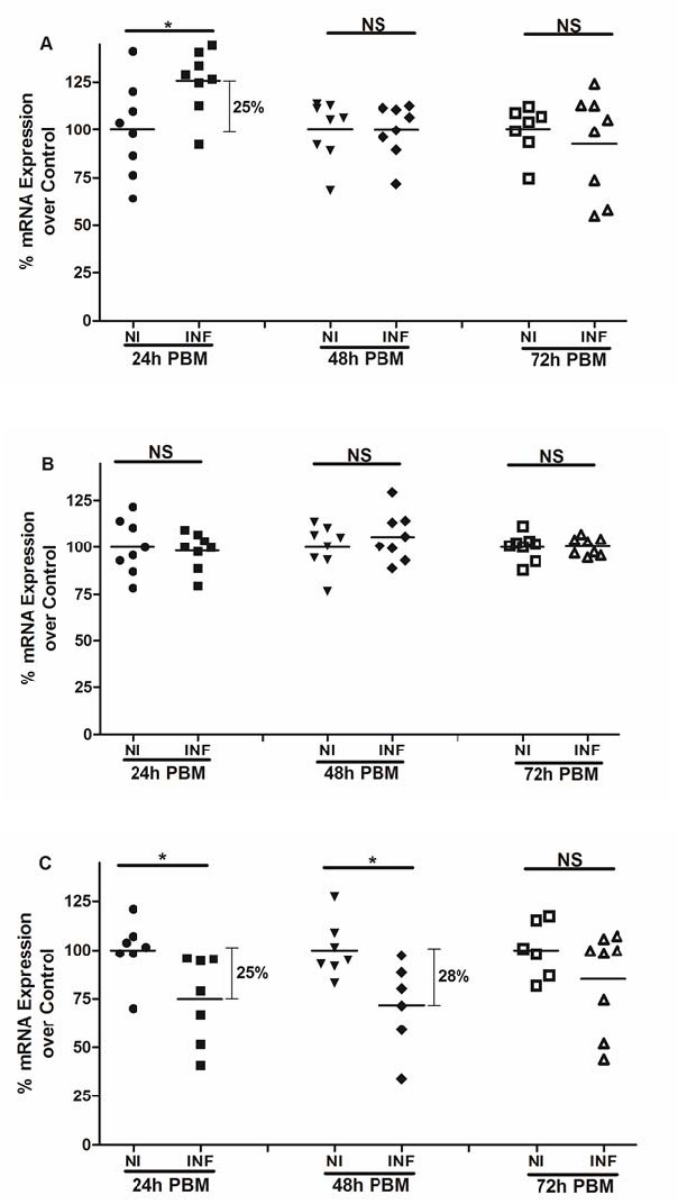

Figure 3.4 Modulation of peritrophin mRNA expression upon *Le. major* infection. qRT-PCR assays depicting differences in PpPer1 (A), PpPer2 (B), and PpPer3 (C) mRNA levels between non-infected and *Le. major* infected midguts dissected at 24 h, 48 h, and 72 h PBM. Each dot (symbol) represents the mRNA expression levels in a single midgut whereas horizontal bars indicate mean expression levels. The cDNA encoding the S3 protein of the 40S ribosomal subunit was used as the housekeeping control gene. The mean expression of non-infected midguts was used as a standard (100 %) for comparisons to the percentage of mRNA expression of *Le. major* infected midguts for each time point. NI: Non-infected. INF: *Le. major* infected. NS: Not significant. *: Statistically significant $p < 0.05$.

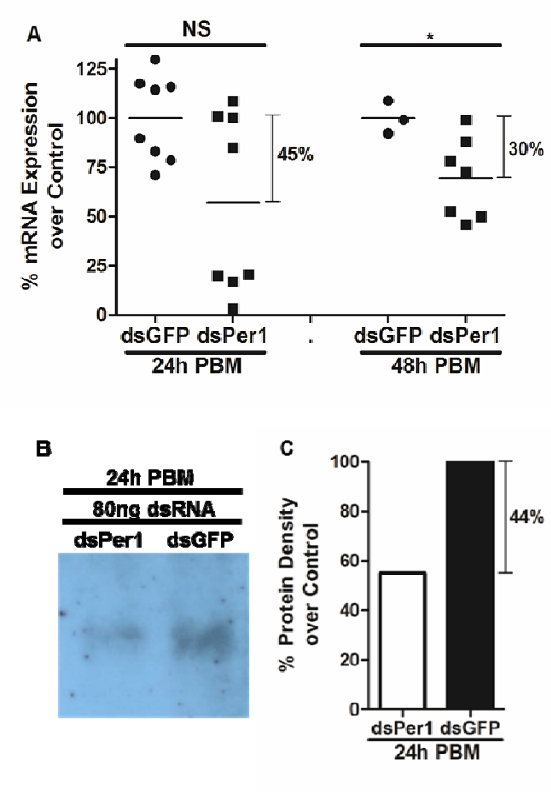

Figure 3.5 *PpPer1* knock down at mRNA and protein levels. (A) qRT-PCR assay showing PpPer1 mRNA level reduction in PpPer1 dsRNA-injected (dsPer1) *P. papatasi* compared to GFP dsRNA-injected ones. Knock down effects in PpPer1 mRNA expression were assessed at 24 h (45 % average reduction) and 48 h PBM (30 % average reduction), which corresponds to 72 h and 96 h after dsRNA injection. Each symbol represents the mRNA expression levels in a single midgut. Horizontal bars indicate mean expression levels. The S3 gene was used as the housekeeping control gene. The mean expression of *PpPer1* in dsGFP-injected specimens was used as a standard (100 %) for comparisons to the percentage of PpPer1 mRNA expression of dsPer1-injected ones. NS: Not significant. *: Statistically significant $p < 0.05$ (Unpaired t-test). (B) Western blot assay depicting reduction in PpPer1 protein levels at 24 h PBM (72 h after dsRNA injection) in dsPer1-injected flies compared to dsGFP-injected ones (chemiluminescence development). (C) Densitometry assay showing 44 % reduction in the intensity of PpPer1 protein band obtained after chemiluminescence development compared to the PpPer1 band intensity of dsGFP-injected flies. For all *PpPer1* knock down assays, 80 ng of dsRNA was injected intrathoraxically into three to five days old *P. papatasi* females fed on 30 % sucrose solution *ad libitum*.

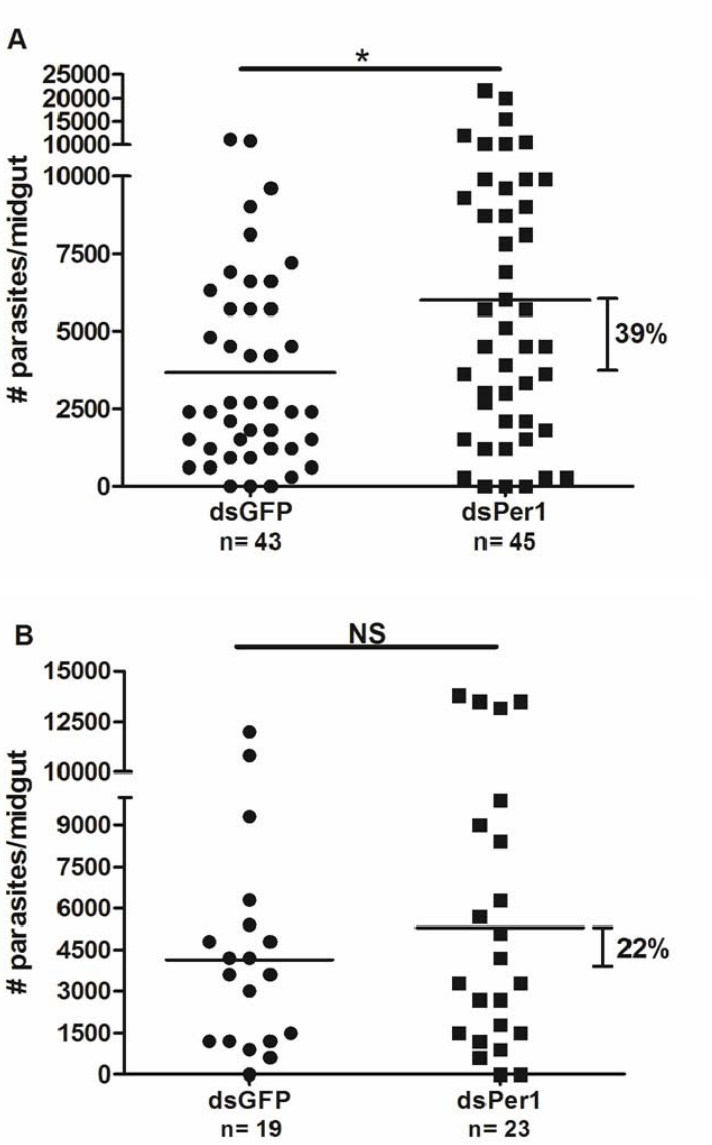

Figure 3.6 Effects of PpPer1 knock down on *Le. major* infection. Knocking down PpPer1 leads to greater *Le. major* load in the midgut of *P. papatasi*. At 48 h post-infection (A), dsPpPer1 (dsPer1) injection caused an increased (39 %; $p< 0.05$ Mann-Whitney U test) in *Le. major* load compared to dsGFP-injected *P. papatasi*. Pool data of two independent experiments. (B) Although not statistically significant, *PpPer1* knock down led to 22 % increase in *Le. major* load in *P. papatasi* midguts at 96 h post-infection when compared to dsGFP-injected ones (Data of one experiment). Each dot (filled circle or square) represents *Le. major* counting in a single midgut whereas horizontal bars indicate mean parasite countings. *P. papatasi* was infected with $5x10^6$ *Le. major* amastigotes/ml in heat-inactivated mouse blood. N: Number of *P. papatasi* midguts dissected. NS: Not significant. *: Statistically significant $p< 0.05$.

Chapter 4 - Introduction II: Ecological genomics of sand fly salivary gland genes: an overview

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Abstract

Sand fly saliva contains an array of bioactive molecules that facilitate blood feeding and also function as modulators of the vertebrate immune response. Such complex of biologically active molecules was shown to be both conserved and divergent among sand fly species. Likely, expression of sand fly salivary molecules can be modulated by environmental factors, both biotic and abiotic, that ultimately dictate the quality, and possibly quantity of the saliva been secreted. Carbohydrates are an integral part of the sand fly diet, and sugar-sources found in natural habitats are potentially involved in defining the profile of sand fly saliva, and may influence vectorial capacity. Saliva can drive the outcome of *Leishmania* infection in animal models, and salivary molecules are potential targets for development of vaccines to control *Leishmania* infection. Thus, identifying what environmental factors effectively modulate sand fly saliva in the field is a critical step towards the development of meaningful protection strategies against leishmaniasis that are based on salivary compounds from sand fly vectors.

Keyword Index: Sand flies, saliva, gene expression, ecological genetics

Introduction

Sand fly saliva is a strong modulator of the host immune response and capable of enhancing transmission of *Leishmania* (Titus & Ribeiro, 1988, Belkaid et al., 2000, Kamhawi et al., 2000). The enhancing effects of sand fly saliva are correlated with its ability to inhibit macrophage functions, such as antigen presentation and nitric oxide (NO) synthesis by the infected cells (Belkaid et al., 2000, Titus et al., 2006). In contrast, immunity to salivary components can prevent establishment of infection (Kamhawi et al., 2000, Valenzuela et al., 2001, Gomes et al., 2008, Collin et al., 2009). Due to the crucial role played by sand fly saliva during transmission of *Leishmania* to vertebrate hosts, and to the possibility of using saliva or salivary components to protect against these parasites, understanding the role of individual salivary proteins is of great importance for the development of salivary components-based vaccines (Oliveira et al., 2006).

The salivary transcriptomes of several sand fly species have been identified providing an insight on the types of molecules inoculated into host skin by these vectors. Further, at least for some salivary secreted proteins, their roles in protecting animals against challenge with *Leishmania* have been ascertained (Kamhawi et al., 2000, Valenzuela et al., 2001, Gomes et al., 2008, Collin et al., 2009). In spite of the efforts to identify salivary proteins in sand fly saliva, few studies have focused on the variability of saliva and salivary components in sand flies (Lanzaro et al., 1999, Elnaiem et al., 2005, Anderson et al., 2006, Kato et al., 2006), and none of which has focused on potential effects of environmental factors.

In this review, we discuss some of the underlying environmental factors that may be associated with the gene expression plasticity in sand flies and outline how such plasticity can influence aspects of sand fly physiology. In addition, the potential involvement of seasonal expression profiles of salivary gland genes on pathogen transmission and epidemiology of leishmaniasis also is exploited.

Sand fly saliva

Since the seminal study by Titus and Ribeiro (Titus & Ribeiro, 1988) demonstrating the effect of sand fly saliva exacerbating lesion development in mice injected with *Le. major*, many studies have focused on the role of saliva and salivary molecules in pathogen transmission. For sand fly-transmitted leishmaniasis, it became evident that the strong immunomodulatory effects

of the vector saliva contribute to the establishment of the parasite and the onset of disease. However, pre-exposure to saliva and salivary gland homogenates can lead to protection against challenge with *Leishmania*, supporting the assumption that saliva may be used in vaccinations strategies.

For *P. papatasi,* the principal vector of *Le. major* in North Africa and in the Middle East, transcriptome analyses of salivary glands of female sand flies revealed a total of roughly 35 predicted secreted salivary proteins. One such protein, PpSP15 was shown to confer protection to mice against challenge with *Le. major* (Valenzuela et al., 2001). This protection was observed following natural (infected sand fly bites) or artificial (needle injection) challenge with *Le. major* (Valenzuela et al., 2001). However, the protection conferred by PpSP15 has to date only been confirmed for mice, as this effect was not observed for this molecule when Rhesus monkeys were used (Valenzuela, pers. comm.). Thus, different salivary molecules are associated with protection against *Leishmania* in different vertebrate hosts (Gomes et al., 2008, Collin et al., 2009). Interestingly, SP15 constitute a family of proteins present in other species of sand flies, including *P. duboscqi*, *P. ariasi*, and *P. arabicus* (Anderson et al., 2006, Kato et al., 2006, Oliveira et al., 2006, Hostomska et al., 2009), and a SP15-like molecule also was found in *Lu. longipalpis* (Charlab et al., 1999, Valenzuela et al., 2004).

For humans, no single *P. papatasi* (or for that matter from other sand fly species) salivary molecule has been identified that might lead to protection. However, in line with the argument that salivary components may be targets for vaccine development against *Leishmania* and due to the fact that the majority of studies undertaken relied on long term laboratory-reared sand fly colonies, Elnaiem et al. (2005) investigated the variability of SP15 in natural and colonized populations of *P. papatasi*. The results obtained suggested some degree of variability for PpSP15 in which wild-caught *P. papatasi* displays higher genetic levels of variation than colonized flies (Elnaiem et al., 2005).

Over the last twenty years, several investigators have relied on colonized sand flies to investigate the effect of saliva on transmission of *Leishmania* to animal models. Now, recent data suggest that some of the effects detected might have been due to changes in saliva following the colonization process. In one set of studies the effects of salivary gland homogenate (SGH) from wild-caught *Lu. longipalpis* were shown to vary greatly from those produced by laboratory-reared flies (Laurenti et al., 2009a, Laurenti et al., 2009b). Mice inoculated in the ear or foot pad

with 10^6 *Le. amazonensis* with SGH from wild-caught flies displayed smaller lesions, and macrophages infected by parasites were fewer than those isolated from mice inoculated with SGH from laboratory-reared flies (Laurenti et al., 2009b). In addition, the immunomodulatory effects of these two SGH also were different: wild-caught SGH led to lower production of IL-4 and IL-10 but higher IL-12 levels compared with laboratory-reared SGH (Laurenti et al., 2009a). In separate studies involving *P. papatasi*-transmitted *Le. major*, pre-immunization of mice with SGH obtained from long-term laboratory colonies (F29) induced protection against *Le. major* co-inoculated with the same type of SGH, yet it did not confer protection against inoculation with SGH of wild-caught specimens (Ben Hadj Ahmed et al., 2009). Moreover, pre-immunization of mice with SGH of wild-caught female *P. papatasi* did not confer protection against *Le. major* co-inoculated with the same type of SGH (Ahmed et al., 2010).

In addition to the effects of SGH described above, protein sequence polymorphisms as well as differences in the amounts of salivary proteins also were tentatively associated with different disease outcomes (Warburg et al., 1994, Lanzaro et al., 1999, Yin et al., 2000, Morris et al., 2001, Milleron et al., 2004). Specifically, differential expression of maxadilan transcripts between cryptic species of the New World sand fly *Lu. longipalpis* is believed to be involved in visceralization of *Le. infantum chagasi* transmitted by this sand fly as it correlates with the different clinical manifestations of leishmaniasis in Central and South America (Warburg et al., 1994, Lanzaro et al., 1999, Yin et al., 2000). While in Brazil and Colombia *Lu. longipalpis*-transmitted *Le. i. chagasi* causes visceral leishmaniasis, in Costa Rica, Nicaragua, and Honduras it leads to atypical cutaneous lesions (Zeledon et al., 1984, Zeledon et al., 1989, Warburg et al., 1994, Carrasco et al., 1998, Belli et al., 1999). Experimentally, lesions obtained by needle injection of *Leishmania* in mice also correlated with amounts of co-injected maxadilan (Morris et al., 2001).

Together, these data underscore the significant role played by sand fly genetic divergence in modulating the quantity of sand fly saliva inoculated into host skin, leading to different disease outcomes.

Genetic structure of P. papatasi

In spite of data suggesting that *P. papatasi* is not a species complex (Hamarsheh et al., 2007, Depaquit et al., 2008), previous studies demonstrated physiological, behavioral, and

genetic differences between *P. papatasi* populations from different geographic localities (Wu & Tesh, 1990, Hanafi et al., 1998). Susceptibility to infection with *Le. major* also was shown to differ between colonized flies originally from Israel, India, and Egypt, whereby the Israeli strain (PPIS) displayed the highest rate of infection (Wu & Tesh, 1990). Distinct infection levels also were detected in the laboratory with *P. papatasi* from different locations in Egypt (Hanafi et al., 1998).

Recent analysis of polymorphisms in the cytochrome b gene (*cyt b*) from colonized and natural populations of *P. papatasi* revealed moderate genetic differentiation between populations from Egypt and Middle East (Hamarsheh et al., 2007). These results contrasted those obtained for the internal transcribed spacer 2 (*ITS2*) and NAD dehydrogenase subunit 4 (*ND4*) genes that pointed to a lack of genetic structuring across the *P. papatasi* geographical range (Depaquit et al., 2008). Thus, despite the levels of genetic similarities among *P. papatasi* populations, specific trait differences (physiological and/or behavioral) exist, which might have been shaped by environmental pressures.

The vectorial capacity of *P. papatasi* was previously associated with hunger tolerance (Schlein & Jacobson, 2001, Schlein & Jacobson, 2002). Thus, selective pressure from ecological factors can have a significant impact on the evolution of *P. papatasi* genes associated with vectorial capacity, such as salivary gland- and midgut-expressed genes. Much is known about the ecology of *P. papatasi* in the Middle East and North Africa (Schlein et al., 1982a, Schlein et al., 1982b, Yuval & Schlein, 1986, Schlein & Yuval, 1987, Yuval et al., 1988, Yuval, 1991, Schlein & Jacobson, 1994, Janini et al., 1995, Schlein & Jacobson, 1999, Schlein & Jacobson, 2000, Schlein & Jacobson, 2001, Schlein & Jacobson, 2002, Chelbi et al., 2007, Zhioua et al., 2007, Chelbi et al., 2009); nevertheless, how the environment influences gene expression in this sand fly is vastly unexplored.

Environmental effects on P. papatasi gene expression

Ecological genomics seeks to uncover the genetic mechanism that respond to environmental changes (Ungerer et al., 2008). The effects of the environment on the gene expression are frequently referred to as genotype-by-environment interaction, and the responses displayed by organisms to such environmental change are named phenotypic plasticity (Gibson, 2008). Differential gene expression or gene expression plasticity can be triggered by biotic or

abiotic factors (Hodgins-Davis & Townsend, 2009), and gene expression can be more strongly correlated with the environment than with genetic divergence (Whitehead & Crawford, 2006). Thus, selection by environment can fine-tune gene expression for higher fitness (Hodgins-Davis & Townsend, 2009).

Ecological studies on *P. papatasi* have addressed important questions about which environmental factors can influence *P. papatasi* vector competence (Schlein & Jacobson, 1994, Schlein & Jacobson, 2001, Schlein & Jacobson, 2002). It seems that sugar sources and water availability in natural habitats (which modulates photosynthesis and in turn plant sugar levels) can modulate expression of vector competence-associated genes. So far, only the influences of the latter factor on sand fly gene expression have been exploited.

P. papatasi caught in arid and irrigated habitats, and at different periods of the *P. papatasi* season, exhibited different rates of activity for glycosidases and chitinases (Jacobson et al., 2007), two enzymes possibly involved in vector competence. Our own studies suggest similar seasonal variations for salivary gland transcripts in *P. papatasi* collected in the Middle East. For sand flies collected in a natural habitat (a site without any irrigation system), a gradual increase in the abundance of these mRNAs was detected, with the highest levels assessed late in the sand fly season – September (Coutinho-Abreu et al, unpubl. data). This upregulation in salivary gland gene expression, as well as in glycosidase and chitinase enzymatic activities (Jacobson et al., 2007), coincided with the fact that this non-irrigated area becomes even dryer late in the season and with scarce sugar sources for sugar feeding insects, such as sand flies (Schlein & Jacobson, 2000).

The finding that sap from plants in dry and irrigated habitats varies in sugar concentration (Schlein & Jacobson, 2002) suggests that availability of sugar sources is one of the factors responsible for the differential expression of salivary gland genes exhibited throughout the season. On the other hand, other biotic (and abiotic) factors might also influence the expression profile of *P. papatasi* salivary gland genes, such as senescence and pregnancy. Phenology studies demonstrated that *P. papatasi* populations from different habitats can exhibit differences in the percentages of gravid or engorged females, as observed in the Jordan Valley (Yuval, 1991, Janini et al., 1995). Additionally, *P. papatasi* parous rate also may vary (Yuval, 1991, Hanafi et al., 2007), suggesting that sand flies are older late in the season. However, our results from a study on the influences of aging and gonotrophic stage on the expression of salivary gland genes in

colonized *P. papatasi* do not broadly support this hypothesis. In our analyses, *PpSP44* was the only transcript amongst the ten most abundant salivary transcripts investigated that was influenced by such factors (Coutinho-Abreu et al., 2010).

P. papatasi gene expression plasticity and Leishmania transmission

For some endemic sites of transmission of zoonotic cutaneous leishmaniasis (ZCL), such as Tunisia, the entomological risk index (ERI), associating the infection rate of *P. papatasi* with *Le. major* and the abundance of the vector, is related for the most part with the geographical distribution of the disease (Chelbi et al., 2007). However, such correlation between ERI and ZCL is not necessarily found at all ZCL endemic sites (Chelbi et al., 2009). Several reasons may explain this epidemiological behavior for the disease, including greater rate of infectious bites shown to be highest in the fall (Chelbi et al., 2009). Nevertheless, another potential explanation for such large incidence of parasites, both in terms of high number of infected reservoir as well as human cases, may be associated with changes in the salivary gland profile of sand flies. Higher dose of saliva inoculated into the host skin may modulate *Leishmania* development, as observed in animal model in laboratory (Morris et al., 2001).

Concluding remarks

In our view, sand flies are an important model for studies focusing on the influence of the environment on vectorial capacity of disease vectors. *P. papatasi* simple ecotone in Middle East deserts, where inter-specific competition and trophic level interactions are limited, makes this vector a good choice for studies focused on ecological genomics, sand fly behavior, and impact on epidemiology of ZCL. Pioneer work revealing the influences of biotic and abiotic components on *P. papatasi* vectorial capacity (Yosef Schlein, Raymond Jacobson, etc.) provides the basis for the ecological genomics hypotheses addressing the influences of ecological factors on genes associated with sand fly vectorial capacity. In regards to *P. papatasi* salivary gland genes, expression levels are likely fine-tuned based upon the levels of water available in the environment. Although we still do not know the molecular basis for the *P. papatasi* gene expression differences (and different levels of enzymatic activity) in seasonally changing environments, plant carbohydrates change according to water availability. Accordingly, sand flies would need to adjust their saliva in order to obtain more nutrients from such plants

(improving their fitness). Nevertheless, the role of sand fly saliva on sugar feeding remains vastly unexplored (Calvo et al., 2006).

Overall, the assessment of *P. papatasi* salivary gland gene expression in a changing environment has significant impact for the sand fly saliva-based vaccine studies. In addition, it opens new avenues for future studies of environment influences on the expression of other genes related with vectorial capacity, such as genes affecting host and plant-seeking behavior as well as vector-parasite interactions.

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Specific Aims and Significance

Sand fly saliva and salivary molecules can drive the outcome of *Leishmania* infection in animal models (Titus & Ribeiro, 1988, Belkaid et al., 2000, Kamhawi et al., 2000), and have been postulated as components in multi-component vaccines against leishmaniasis (Kamhawi et al., 2000, Valenzuela et al., 2001, Gomes et al., 2008, Collin et al., 2009). For blood and sugar feeding insects such as the sand fly *P. papatasi*, sugar-sources found in natural habitats modulate the activity of proteins involved in meal digestion and possibly influence vectorial capacity (Jacobson et al., 2007). While the activity of some midgut proteins from *P. papatasi* were shown to be influenced by the quality of their sugar meal (Jacobson et al., 2007), to date only a handful of studies has focused on the variability of sand fly saliva (Lanzaro et al., 1999, Elnaiem et al., 2005, Anderson et al., 2006, Kato et al., 2006), none of which as a function of environmental factors in natural habitats. Assessing geographic and seasonal variations in *P. papatasi* salivary gland gene expression can shed light on how environmental factors fine-tune gene expression. Moreover, differential expression of salivary gland secreted genes reflects variation in the amount of salivary antigen inoculated into host skin, which in turn may interfere with sand fly saliva-based vaccine efficacy because dose is an important component of immune responses (Lee et al., 2002, Yao et al., 2002, Dhar et al., 2003). In this section, the specific aims are:

(1) Assess the expression profiles of selected *P. papatasi* salivary gland genes (*SP12*, *SP14*, *SP28*, *SP29*, *SP30*, *SP32*, *SP36*, *SP42*, and *SP44*) in females collected in three different geographic habitats in North Africa and Middle East as well as in different periods of the sand fly activity season (June, August, and September)

(2) Associate differential expression of *P. papatasi* salivary gland genes with ecological factors

(3) Assess the effects of senescence and diet on the expression profiles of selected *P. papatasi* salivary gland genes (*SP12*, *SP14*, *SP15*, *SP28*, *SP29*, *SP30*, *SP32*, *SP36*, *SP42*, and *SP44*) in colonized specimens

Regarding sand fly salivary gland-based vaccines, knowledge of seasonal pattern of salivary gland gene expression will define how much antigen will be delivered to people living in endemic regions.

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Chapter 5 - Gene Expression Plasticity of *Phlebotomus papatasi* Salivary Gland Genes in Distinct Ecotopes throughout the Sand fly Season

Abstract

Sand fly saliva can drive the outcome of *Leishmania* infection in animal models, and salivary components have been postulated as vaccine candidates against leishmaniasis. In the sand fly *Phlebotomus papatasi*, natural sugar-sources modulate the activity of proteins involved in meal digestion, and possibly influence vectorial capacity. However, only a handful of studies have assessed the variability of salivary components in sand flies, focusing on the effects of environmental factors in natural habitats. In order to better understand such interactions, we compared the expression profiles of nine *P. papatasi* salivary gland genes of specimens inhabiting different ecological habitats in Egypt (Aswan and North Sinai) and Jordan (Swaymeh) and throughout the sand fly season in each habitat. Expression levels of most of the genes were up-regulated in specimens from Swaymeh late in the season, when the availability of sugar sources is reduced due to water deprivation. On the other hand, the up-regulation of these genes at the end of the season was not evident in specimens collected in Aswan, which is an irrigated area less susceptible to drought effects. Therefore, expression plasticity of genes involved with vectorial capacity in disease vectors may play an important epidemiological role in the establishment of diseases in natural habitats.

Keywords: Sand fly; *Phlebotomus papatasi*; Saliva; Gene expression plasticity; Gene-by-environment interactions

Introduction

Many studies have demonstrated an environmental role in gene expression. Differential gene expression can be caused by biotic (e.g., infections with viruses and menopause in humans) or abiotic factors (e.g., arsenic poisoning and exposure to diesel in humans; or temperature variation in worms and in plants). Effects of the environment on gene expression are referred to as gene-by-environment-interactions, and the response displayed by organisms to such environmental change, phenotypic plasticity (Gibson, 2008). Although much is known about the ecology of the sand fly *Phlebotomus papatasi* (Yuval & Schlein, 1986, Schlein & Yuval, 1987, Yuval et al., 1988, Schlein & Jacobson, 1994, Schlein & Jacobson, 1999, Schlein & Jacobson, 2000, Schlein & Jacobson, 2001, Schlein & Jacobson, 2002), how the environment influences gene expression in this insect remains largely unexplored (Jacobson et al., 2007).

P. papatasi is the primary vector of *Leishmania major* in Northern Africa and Middle East (Schlein et al., 1982a, Fryauff et al., 1993). The behavior of this sand fly species is well documented with regards to resting places (Schlein et al., 1982a), blood sources (Schlein et al., 1982b) and dispersal ability (Yuval et al., 1988). In addition to blood, sugar also constitutes a key component of the sand fly life cycle, and several plant species are able to attract sand flies. In the Middle East, *Prosopis farcta*, *Capparis spinosa*, *Ricinus communis*, *Solanum nigrum*, and *Rochia indica* are some of the most attractive plants for *P. papatasi* (Schlein & Yuval, 1987). Analyses of sugar contents in the gut of field caught flies revealed that 15.5 % of the flies were fed on some type of sugar, and 22.5 % of them presented cellulose shreds within their guts. Starch also is an important nutrient for *P. papatasi*, as demonstrated by the finding that 50% of the field collected flies had ingested this carbohydrate (Schlein & Jacobson, 1999). Starch likely is obtained from the sap of the succulent plant *Atriplex halimus* (Schlein & Jacobson, 2000), which is frequently associated with burrows of the *L. major* reservoir host, the fat sand rat *Psammomys obesus* (Schlein & Yuval, 1987).

Previous studies have demonstrated that sugar appears to influence many aspects of sand fly physiology, including longevity (Yuval, 1991, Schlein & Jacobson, 1999). The gonotrophic cycle of *P. papatasi* collected in non-irrigated areas in the Jordan Valley ranges from six to eight days, with 2.6 % of the females being older than 8 days (Schlein & Jacobson, 1999). On the

other hand, in irrigated regions, where rich-sugar sources are available, longevity of flies is much greater, averaging 33 days (Schlein & Jacobson, 2000). Accordingly, estimates of sugar content in three species of plants inhabiting non-irrigated areas are more than 3 fold lower than in the same species from irrigated habitats (Schlein & Jacobson, 2002).

The interaction of sugars with various aspects of sand fly physiology and *Leishmania* development is not yet fully understood. Recent studies have contributed to our understanding of some of these complex interactions. Although a sugar-rich diet is associated with a greater number of gonotrophic cycles for *P. papatasi* (i.e., up to 5 cycles), thus, increasing the chance for *Leishmania* transmission (Schlein & Jacobson, 2000), feeding on plants, such as *R. communis*, *C. spinosa*, and *S. luteum*, can actually decrease the number of *Leishmania* in *P. papatasi* by 45 % (Schlein & Jacobson, 1994). In addition, an eight fold decrease in *Leishmania* load in the gut of flies was found when they fed on *Malva nicaeensis* compared to *A. halimus* (Schlein & Jacobson, 2001, Schlein & Jacobson, 2002).

The quality of the sugar meals also is believed to influence sugar-feeding behavior of *P. papatasi* (Schlein & Jacobson, 2000). Such effects appear to be driven by the rate of photosynthesis and the quality of the sugars produced by a given plant species. These data are supported by the observation that *P. papatasi* prefers feeding on branches of the Syrian mesquite *P. farcta* collected from a humid habitat rather than on branches of the same plant collected from a dry and salty soil (Schlein & Jacobson, 2000).

The expression of some sand fly genes involved in digestion and nutrient acquisition are also modulated by sugar-meals. Chitinases of the sand flies *P. papatasi* and *L. longipalpis* s.l. are not expressed after a sugar meal (Ramalho-Ortigao et al., 2005), whereas the trypsin encoding gene, *Lltryp2*, is up-regulated when *L. longipalpis* s.l. is sugar-fed (Telleria et al., 2007). Likewise, *L. longipalpis* s.l. salivary protein content also is increased after a sugar-meal (Prates et al., 2008).

Saliva components have been identified for several insect species including sand flies (Valenzuela et al., 2001, Valenzuela, 2004, Anderson et al., 2006, Kato et al., 2006, Oliveira et al., 2006). Like many other blood sucking insects, sand fly saliva was shown to play roles in vasodilation as well as in inhibiting blood clotting and platelet aggregation (Valenzuela, 2004), though the molecules utilized for such tasks may vary between different sand fly genera. For instance, in the New World sand fly *L. longipalpis s.l.* a 6 KDa peptide named maxadilan is

responsible for the vasodilatory effect (Valenzuela, 2004). In *P. papatasi*, this is accomplished by adenosine and 5`AMP (Valenzuela, 2004). Other functions, however, are performed by molecules conserved between the two genera (Valenzuela, 2004).

Sand fly saliva also is essential for the success of *Leishmania* transmission as it is essential for successful blood feeding. Interestingly, *P. papatasi* saliva exacerbates *L. major* infection in mice (Belkaid et al., 2000); however, pre-exposure to sand fly saliva or non-infected sand fly bites confers protection against lesion development in the same animals (Kamhawi et al., 2000), suggesting that sand fly saliva could potentially serve as a component in a vaccine against leishmanial disease. Moreover, protection in mice can be achieved by pre-vaccination of animals with a plasmid encoding a 15 kDa protein (*SP15*) present in the sialome of *P. papatasi* (Valenzuela et al., 2001), though protection is mediated by different antigens in different hosts (Kamhawi et al., 2000, Valenzuela et al., 2001, Gomes et al., 2008, Collin et al., 2009). Before sand fly saliva can be fully exploited as a vaccine target, the genetic and expression variability of salivary proteins must first be assessed in the field.

The most highly expressed proteins in the sialoma from *P. papatasi* have been identified, and those encompass the products of 12 genes (Valenzuela et al., 2001); however, no complete transcriptome data is available. Recently, it was suggested that various enzyme activities associated with *P. papatasi* vectorial capacity are differentially modulated in distinct ecological habitats (Jacobson et al., 2007). Here, we analyzed the gene expression plasticity of nine *P. papatasi* salivary gland genes across specimens collected in distinct ecotopes and obtained during different periods along the sand fly season. Our results indicate that the pattern of salivary gland gene expression exhibited is more associated with the distinct environmental conditions presented in natural habitats than with the geographic origins of the specimens. Additionally, the data presented support the notion that expression plasticity of sand fly salivary gland genes exhibited in distinct ecological habitats may have epidemiological consequences and may also affect the immunogenicity of a sand fly salivary gland protein-based vaccine.

Material and Methods

Sand flies

P. papatasi used in this study were either obtained from field collections or from a colony (Israeli strain - PPIS) maintained at the University of Notre Dame. These PPIS specimens are from a colony originally established in the mid 1970's that went through several bottlenecks, the most current of which was in July, 2007. Thus, the PPIS colony essentially displays very low levels of genetic polymorphisms. For field samples, sand flies were collected at 3 locations: Aswan (GPS coordinates N 24°10, E 32°52), in a village adjacent to the Nile River (Baharif - Southern Egypt); Northern Sinai (GPS coordinates N 30°50', E 34°10'), in a Bedouin village (Om Shikhan – Northeastern Egypt); and Swaymeh (GPS coordinates N 31°48', E 35°35'), near the Dead Sea, in Jordan.

The collection site in Aswan, Baharif village, is located on the east margin of the Nile. This village is typically cultivated with date palms (*Phoenix dactylifera*), mangoes (*Mangifera indica*), wheat (*Triticum aestivum*), corn (*Zea mays*), and clover (*Trifolium* spp.) under artificial irrigation. The human population of approximately 400 and is stocked with domestic animals including cattle, dogs, and goats. Daily temperatures typically range from 24°C to 45°C, and it seldom rains in this locality (the village received no rainfall during 2006). This site was chosen for our study because of the large number of sand flies present in the area as observed by Naval Medical Research Unit No. 3 (NAMRU-3) researchers over the previous 15 years, for the absence of *Leishmania*-infected flies (Hoel et al., 2007), and for it is an irrigated area.

In North Sinai, sand flies were collected in Om Shikhan, located approximately 340 km east of Cairo, 80 km inland from the Mediterranean coast, and 30 km west of the Israeli border in North Sinai, Egypt. The area terrain is typical rolling sand desert with sufficient rainfall and humidity to permit cultivation of fruit trees, melon, and millet by the local Bedouin population. This area is unique in having a heightened water table, produced by the nearby (3 km distant) El Ruafa Dam, on Wadi El Arish. Uncultivated areas around the reservoir are variably covered by low desert brush, with *Artemisia, Panicum, Salicornia*, Tamarisk and *Thymelaea* predominating (Hanafi et al., 2007). Climatic conditions produce a mean precipitation of 87mm per year, with summer mean daily maximum temperature of 33.5°C, and winter mean daily minimum of 6°C. This collection site is an endemic site for *L. major* infections.

Swaymeh, Jordan, is an area of low elevation at approximately 350 m below sea level. The climate is considered Saharan Mediterranean with temperatures ranging from a minimum of 8-12°C in the winter and a maximum of 35-40°C in the summer. Mean rainfall is <50 mm, and rain falls entirely during November-April. Area soil is mostly sandy or sandy hammada with granite fragments and saline soils, with tropical and halophytic vegetation (chenopods such as *Atriplex halimu*s and *Suaeda* spp.) as the natural flora. Swaymeh also is an endemic area for *L. major*.

Whenever possible, sand fly trappings were carried out three times a year, early (June), middle (August) and late (September) for years 2006 and 2007. While in Aswan and Swaymeh we performed 3 trappings (late 06, early and middle 07); for the sites in North Sinai only 2 trappings took place: early and middle 07 in North Sinai. For each of the 3 locations *P. papatasi* represents approximately 95% of the *Phlebotomus* species (Janini et al., 1995, Hoel et al., 2007). Sand flies were trapped using CDC-style light traps between 18:00 and 06:00. Traps were either baited with CO_2 (dry ice) (for trappings done in Aswan and North Sinai), or non-baited (Swaymeh). Sand flies were transferred from collection bags and maintained alive until dissected. Flies were euthanized in water and detergent just prior to dissection. *P. papatasi* were identified by microscopic examination of female spermateca according to Lane (Lane, 1986); their heads along with the salivary glands were pulled off from the bodies, transferred to 50 µl of RNAlater™ solution (Ambion, Austin, TX, USA), homogenized, and stored at -20°C.

RNA extraction and cDNA synthesis

The RNA was extracted from the dissected tissues (head and salivary glands) of all the *P. papatasi* individually using the RNAeasy Mini Kit (Qiagen, Valencia, CA, USA) according to instructions and stored at -80°C.

cDNAs were synthesized using Invitrogen reagents (Invitrogen, Carlsbad, CA, USA) and following the manufacturer's instructions. In brief, 12 µl RNA from each sample were added with 2.5 µM Oligo $(dT)_{20}$ primer and 0.5 µM dNTPs (10mM), incubated at 65°C for 5 minutes (min) and kept in ice for at least 1 min; 4 µl 5X SuperScript™ III Reverse Transcriptase First-Strand Buffer, 5mM DTT (0.1M), 20 Units of RNase OUT, and 200 Units of SuperScript™ III Reverse Transcriptase (200 u/µl) were added to the reaction. The mixture was incubated for one hour at 50°C and stored at -20°C.

Real-time polymerase chain reactions (RT-PCR)

RT-PCRs were set up with 10 µl SYBR Green reagent (Applied Biosystems, Foster City, CA, USA), 0.6 µl each forward and reverse primer (0.3 µM final concentration) (Table 1), 0.5 µl each cDNA sample, and 8.3 µl Ultra Pure DNase/RNase-Free Water (Invitrogen). Reactions were analyzed in 96-well plate format using a 7900HT Fast Real Time PCR System (Applied Biosystems) under the following conditions: initial incubation at 50°C for 2 min and 95°C for 10 min; followed by 40 cycles of 95°C for 15 sec, 55°C for 1 min; ending with a dissociation step of 95°C for 15 sec, 55°C for 15 sec, and 95°C for 15 sec.

The 9 *P. papatasi* salivary gland genes assayed for expression in this study are SP12, SP14, SP28, SP29, SP30, SP32, SP36, SP42, and SP44. The primer used in the RT-PCRs were published elsewhere (Coutinho-Abreu et al., 2010). α-tubulin was used as a housekeeping load control (Ramalho-Ortigao et al., 2007). A total of 20 *P. papatasi* specimens from each field catch were used for individual RT-PCR, and the expression profile for all 9 cDNAs was assessed using the same 20 field-caught samples. Each reaction was repeated four times for each gene (twice in two different plates) for a total of 80 reactions per sand fly trapping. Since the expression analyses of nine genes in eight different sand fly trappings were performed, a total of 5760 RT-PCRs were performed during the completion of this study.

Differential expression results for each salivary protein gene were displayed as fold changes over a control, using the $2^{-\Delta\Delta CT}$ method (Livak & Schmittgen, 2001). The fold changes were calculated by the expression $2^{-\Delta\Delta CT}$, where $\Delta\Delta C_T = \Delta C_T(\text{sample}) - \Delta C_T(\text{calibrator})$, $\Delta C_T = \Delta C_T(\text{sample}) - \Delta C_T(\text{alpha tubulin gene})$, C_T = cycle at which a statistically significant increase in the emission intensity over the background. The calibrator was represented by the average expression (mean ΔC_T) of the seven non-fed samples (PPIS) dissected 24 hours after emerging (Coutinho-Abreu et al., 2010). Fold changes were calculated for each sample.

Statistical analysis

Statistical analyses were carried out using the software GraphPad Prism v. 5.01 (GraphPad Software, Inc). The statistical tests used were the non-parametric Kruskal-Wallis tests, for comparisons among more than two data sets, and/or Mann-Whitney tests, for pairwise comparisons between data sets when the results for Kruskal-Wallis test were statistically significant, or for comparisons when only two data sets were present. The level of significance

was adjusted for multiple comparisons using Bonferroni's correction. Differences were considered statistically significant at $\alpha = 0.0006$. The values of fold change in Tables 5.2 and 5.3 are based on the ratio of the expression medians between the time point up-regulated (+) over the time point down-regulated (-).

Results

Specimens of the sand fly *P. papatasi* were collected in three distinct geographic locations. In Egypt, samples were collected in Aswan, Southern Egypt, as well as in the Northern Sinai Peninsula, Northeastern Egypt. *P. papatasi* also were collected in Swaymeh, Jordan. Swaymeh and North Sinai are ecologically similar habitats, both of which are wetter early in the season than the middle of the season and become dry late in the season. Aswan, on the other hand, is an irrigated area adjacent to the Nile River and less influenced by drought effects late in the season. For each location involved in this study, sand fly trappings were carried out during different periods of *P. papatasi* activity season (Hanafi et al., 2007). The sand fly season was defined as the period of the year when this sand fly is not overwintering. In Aswan and Swaymeh, trappings were performed in September (late in the sand fly season), 2006, and in June (early) and August (middle) of 2007. In North Sinai, due to security concerns all trappings were performed in June and August of 2007. We analyzed the expression of nine out of the 12 most expressed *P. papatasi* salivary gland genes in 20 individual *P. papatasi* collected from each location at each period of the season (early, middle, or late). Two types of analyses were carried out. In the seasonal analyses, expression levels of each of the genes in specimens collected at different time points of the season in each habitat were compared (Figs. 5.1-9, A-C). In the geographic analyses, we compared the expression levels of each gene in specimens collected in different geographic locations but at the same period of the season (Figs. 5.1-9, D-E). Following is a description of the results obtained regarding the expression profile of all nine genes.

Seasonal and geographic analyses

Quantitative data of seasonal and geographic analyses are summarized in Tables 5.1 and 5.2. For sand flies from Aswan, only the *SP30* gene is differentially regulated throughout the sand fly season (Table 5.1; Fig. 5.5). In sand flies from Swaymeh, five out of the nine genes analyzed (*SP12*, *SP29*, *SP36*, *SP42*, and *SP44*) were differentially regulated along the season

(Table 5.1; Figs. 5.1, 5.4, 5.7, 5.8, and 5.9). For all 5 genes, we detected an up-regulation of expression towards the driest period of the season (i.e., late in the season; Table 5.1). For sand flies from North Sinai, none of the nine genes were differentially expressed towards the late period of the season (Table 5.1).

Following the comparisons between sand flies collected in different habitats early in the season, only one gene (*SP12*) displayed significantly different levels of expression between the populations of Aswan and North Sinai (Table 5.2; Fig. 5.1), being expressed to higher levels in the latter. Between the populations from Swaymeh and North Sinai, or Swaymeh and Aswan, all the genes analyzed exhibited similar levels of expression (Table 5.2; Figs. 5.1-9). At mid season, three genes were differentially expressed between the populations of Aswan and Swaymeh. Two were up-regulated in flies from Aswan (*SP28* and *SP30*) and one (*SP32*) in individuals from Swaymeh (Table 5.2; Figs. 5.3, 5.5, and 5.6). Between the populations of Aswan and North Sinai, only one gene (*SP30*) was up-regulated in flies from Aswan. Likewise, between Swaymeh and North Sinai one gene was differentially expressed: *SP32* was up-regulated in Swaymeh (Table 5.2; Fig. 5.6). Late in the season, analyses of genes expression revealed four genes (*SP29*, *SP32*, *SP36*, and *SP42*) were up-regulated in Swaymeh in comparison with Aswan (Table 5.2; Figs. 5.4, 5.6, 5.7, and 5.8). As indicated previously, no data was obtained from sand flies in North Sinai late in 2006 due to safety concerns (Table 5.2).

Discussion

The saliva of hematophagous insects plays an essential role in blood feeding. Salivary molecules secreted by blood-sucking arthropods into a vertebrate host overcome the hemostatic system of vertebrates, maintaining blood flow at the site of the bite as well as inhibiting the blood coagulation cascade (Valenzuela, 2004). In the case of sand flies, saliva also plays a role in the establishment of *Leishmania* infection (Belkaid et al., 2000), and salivary proteins are potential vaccine candidates (Kamhawi et al., 2000, Valenzuela et al., 2001, Oliveira et al., 2006, Gomes et al., 2008, Oliveira et al., 2008, Collin et al., 2009).

A comparative analysis of salivary protein polymorphisms in four species of sand flies vectors of visceral leishmaniasis suggested that the development of a vaccine derived from a protein of one species is unlikely to protect against *Leishmania* transmitted by species of a different genera (Anderson et al., 2006). However, protection may be achieved after

immunization with salivary proteins of sand flies of the same genus or same species, due to the high degree of similarity exhibited between the salivary proteins between these species (Anderson et al., 2006). Studies of intra-specific genetic variability further support this hypothesis; low levels of genetic differentiation were displayed between the salivary protein encoding genes of two *P. duboscqi* populations. The similarities between protein sequences ranged from 84-100 % while the similarities between their predicted MHC class II binding regions were between 75 % and 100 % (Kato et al., 2006). Furthermore, Elnaiem *et al.* (Elnaiem et al., 2005) suggested that *P. papatasi* SP15 may be used in a vaccination strategy as their data pointed to a high degree of similarity between different populations, and that the gene was under no selective pressure.

In addition to genetic variability, expression level differences could influence vaccine efficacy. Our study is the first to investigate differences in the expression profiles of geographically distinct field-collected *P. papatasi* populations and the potential effect of different environments on such profiles. Nine salivary transcripts were analyzed and compared between three different *P. papatasi* populations from the Middle East through three time points during the sand fly season: early (June), middle (August) and late (September). Our study involved the assessment by real time quantitative PCR of 160 individuals and was based on comparisons made to colonized, water-fed only flies. Significant differences in expression levels were found between distinct ecotopes and periods of the season.

Expression differences of a sand fly salivary gland gene were noted in *L. longipalpis s.l.* (Yin et al., 2000). Such differences were used to explain erythema sizes caused by bites of different *L. longipalpis s.l.* (Warburg et al., 1994). However, unlike *L. longipalpis s.l.*, *P. papatasi* is not a species complex. Nevertheless, several studies have shown physiological, behavioral, and genetic differences between *P. papatasi* populations from different geographic localities (Wu & Tesh, 1990, Hanafi et al., 1998, Hanafi et al., 1999, Hamarsheh et al., 2007, Depaquit et al., 2008). Studying colonized sand fly, Wu and Tesh (Wu & Tesh, 1990) demonstrated that the rate of infection of *P. papatasi* Israeli strain was higher than sand flies from India or Egypt for infection with *L. major*. Likewise, comparing *P. papatasi* colonies originating from three different populations in Egypt, Hanafi *et al.* (Hanafi et al., 1998) indicated that flies from Sinai were more susceptible to *L. major* infection than flies from Aswan or the Nile Delta. Additionally, the feeding rate on mice of *P. papatasi* from Aswan was lower than the

rate obtained for the other two populations (Hanafi et al., 1999). In addition to the physiological and behavioral differences, genetic analysis of *P. papatasi* from several colonized and natural populations based on polymorphisms on the *Cytochrome b* (*cyt b*) haplotypes demonstrated moderate genetic differentiation between populations from Egypt and Middle East (Hamarsheh et al., 2007). Contrasting to previous studies, analyses of polymorphisms on *P. papatasi Internal Transcribed Spacer 2* (*ITS2*) and *NAD dehydrogenase subunit 4* gene (*ND4*) indicated absence of genetic structuring across the *P. papatasi* geographical range (Depaquit et al., 2008). Despite the contrasting information about the genetic structuring of *P. papatasi* populations, gene expression can present stronger correlation with ecological habitat than with genetic distance (Whitehead & Crawford, 2006). Therefore, *P. papatasi* salivary gland gene expression from field collected sand flies needs to be thoroughly assessed so that differences observed in flies collected in different ecological habitats can be correlated with the geographic origin of the populations, with seasonal factors present in each habitat, or with both.

To assess whether differences in *P. papatasi* salivary gland gene expression are driven by factors associated with the geographic origin of the populations or by environmental factors (or perhaps both) we performed two types of gene expression comparisons: geographic analyses between specimens collected in different ecological habitats during the same period of the season; and seasonal analyses between specimens collected in the same habitat and in different periods of the season. From our analyses, three types of results may be expected: (1) a population displaying higher or lower levels of expression of a given gene for the three geographic comparisons made, but no seasonal differences, indicating that only factors associated with the geographic origin of the populations were responsible for the differences observed in regards to the expression of that specific gene; (2) a population exhibiting higher (or lower) expression medians of a specific gene in the three geographic comparisons as well as in the seasonal analysis, suggesting that both geographic origin-related and seasonal factors drove the expression of that gene; and (3) a population displaying only significant seasonal differences in the expression of a gene, indicating that seasonal environmental factors played a major role in controlling the expression of that gene.

Our geographic analyses indicate that salivary gland genes displayed expression variability between *P. papatasi* populations from different ecological habitats. Although most of the salivary gland genes from *P. papatasi* collected early in the season exhibited similar levels of

expression (Table 5.2), and only three genes were differentially expressed in the middle of the season (*SP28* and *SP30*, highly expressed in Aswan; *SP32*, in Swaymeh, Table 5.2; Figs. 5.3, 5.5, and 5.6), late in the season four out of the nine salivary gland genes analyzed displayed greater levels of expression in flies from Swaymeh than from Aswan (Table 5.2). As none of these populations displayed predominantly higher or lower levels of expression throughout the whole season for all of the genes analyzed, our data suggest that the expression differences of salivary gland genes between *P. papatasi* populations are more influenced by environmental changes during the season in the three localities than by factors associated with the geographic origin the populations studied. Moreover, the higher expression levels of four *P. papatasi* salivary gland genes in flies from a dryer habitat (Swaymeh) than in flies from an irrigated area (Aswan) late in the season were similar to the pattern of midgut glycosidase activities observed in extracts of colonized *P. papatasi* originated from different ecological habitats (Jacobson et al., 2007).

Phenology studies demonstrated that *P. papatasi* populations from different habitats can exhibit differences in the percentages of gravid or engorged females, as observed in the Jordan Valley (Yuval, 1991, Janini et al., 1995). However, our own results using laboratory-reared flies indicate that for the genes studied, gravid and engorged females do not exhibit higher levels of expression than sugar fed flies (Coutinho-Abreu et al., 2010).

The seasonal analyses results exhibited here also are similar to *P. papatasi* glycosidase activity patterns presented elsewhere (Jacobson et al., 2007). Five out of nine *P. papatasi* salivary gland genes (*SP12*, *SP29*, *SP36*, *SP42*, and *SP44*) also are differentially regulated during the season (Table 5.1; Figs, 5.1, 5.4, 5.7, 5.8, and 5.9). Expression of these *P. papatasi* salivary gland genes was gradually up-regulated reaching highest levels of expression late in the season in Swaymeh, when the environment is dryer and the sugar sources are scarce (Table 5.1). In contrast, in a well irrigated area such as Aswan, where drought has little influence on the availability of sugar-sources, no late season effect was detected (Table 5.1). Late season up-regulation effects could not be determined for North Sinai, as sand fly trapping was not possible due to security concerns. Thus, validation of this effect still needs to be demonstrated for sand flies from North Sinai.

Taken together, our data suggest that environmental factors play a major role in the expression profiles of *P. papatasi* salivary gland genes. Sap of plants from dry habitats and

irrigated areas varies in sugar concentration (Schlein & Jacobson, 2002), suggesting that availability of sugar sources is possibly one of the principal factors responsible for the differential expression of salivary gland genes exhibited throughout the season.

Schlein and Jacobson (Schlein & Jacobson, 2001) demonstrated that *P. papatasi* vectorial capacity in the Middle East deserts is linked to hunger tolerance, which is under natural selection. Thus, sand flies from a dry habitat can exhibit greater vectorial capacities than those from an irrigated area (Schlein & Jacobson, 2001). Salivary gland proteins also play a role in *P. papatasi* vectorial capacity as these proteins participate in the establishment of *Leishmania* infection in the vertebrate host (Belkaid et al., 2000, Kamhawi et al., 2000, Valenzuela et al., 2001). Accordingly, late in the season, when the expression of some *P. papatasi* salivary gland genes in flies from Swaymeh is in their highest level, the number of human cases of cutaneous leishmaniasis (CL) is also higher than early in the season (Janini et al., 1995). In 1992, the number of cases of CL increased from 27 between early and the middle of the season, to 205 cases late in the season (Janini et al., 1995). However, in Aswan, where no current cases of CL have been reported, and 93 % of the *P. papatasi* population is autogenous (Hanafi et al., 1998), none of the *P. papatasi* salivary gland genes analyzed was up-regulated late in the season. In this population only one gene (*SP30*) exhibited up-regulation, displayed in the middle of the season. As the incubation period for CL caused by *L. major* ranges between two and eight weeks only (Beach et al., 1984, Amaral et al., 2001), these data further support the notion that the differential gene expression of salivary gland genes exhibited by *P. papatasi* specimens throughout the season in natural habitats may contribute to the increase in the number of cases of *L. major*-caused CL.

The use of saliva or salivary components in a multi-components vaccination strategy is a viable option (Belkaid et al., 2000, Kamhawi et al., 2000, Valenzuela et al., 2001). However, the geographic and seasonal variations in salivary gland gene expression displayed in this study must be considered in the development of a sand fly salivary protein-based vaccine, as antigen dosage is an important component in the modulation of immune responses and can interfere with T-cell activation (Henrickson et al., 2008), Th1/Th2 balance (Yoshida et al., 2002, Dhar et al., 2003), T_{reg} cell activity (George et al., 2003) and immunogenicity and specificity of vaccines (Lee et al., 2002, Yao et al., 2002, Dhar et al., 2003).

The genetic plasticity of genes involved with *P. papatasi* vectorial capacity to transmit *L. major*, a parasite responsible for cases of cutaneous leishmaniasis, is evident in field caught specimens, as demonstrated in this work. Moreover, more than half of the salivary gland genes are up-regulated at the end of the sand fly season, when availability of sugar is scarce and disease transmission is increased. Therefore, gene-by-environment interactions also can be an important factor in transmission of pathogens in natural habitats.

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Table 5.1 *P. papatasi* salivary gland gene expression differences throughout the season. Only statistically significant ($p < 0.0006$) differences are shown. Values of fold change are derived from the comparison between expression medians. Regulation of expression levels up (+) or down (-) are indicated.

Genes	Regulation of expression			p-values	Fold change
Aswan	Early	x	Middle		
SP30	-		+	$p < 0.0001$	9.35
	Middle	x	Late		
SP30	+		-	$p < 0.0001$	5.54
Genes	Regulation of expression			p-values	Fold change
Swaymeh	Early	x	Middle		
SP36	-		+	$p = 0.0005$	3.33
	Early	x	Late		
SP12	-		+	$p < 0.0001$	2.86
SP29	-		+	$p < 0.0001$	3.87
SP36	-		+	$p < 0.0001$	5.51
SP42	-		+	$p < 0.0001$	4.78
SP44	-		+	$p = 0.0004$	5.48
	Middle	x	Late		
SP29	-		+	$p = 0.0005$	2.45
SP42	-		+	$p < 0.0001$	4.58
SP44	-		+	$p < 0.0001$	6.33

Table 5.2 Geographic comparisons of *P. papatasi* salivary gland cDNA expression. Only statistically significant ($p < 0.0006$) differences are shown. Values of fold change are derived from the comparison between expression medians. Regulation of expression levels up (+) or down (-) are indicated.

Genes	Regulation of expression		p-values	Fold change
Early	Aswan x North Sinai			
SP12	-	+	$p = 0.0005$	2.64
Genes	Regulation of expression		p-values	Fold change
Middle	Aswan x Swaymeh			
SP28	+	-	$p = 0.0001$	15.40
SP30	+	-	$p = 0.0002$	2.65
SP32	-	+	$p < 0.0001$	24.90
	Aswan x North Sinai			
SP30	+	-	$p < 0.0001$	3.90
	Swaymeh x North Sinai			
SP32	+	-	$p < 0.0001$	8.46
Genes	Regulation of expression		p-values	Fold change
Late	Aswan x Swaymeh			
SP29	-	+	$p < 0.0001$	5.33
SP32	-	+	$p = 0.0005$	7.59
SP36	-	+	$p < 0.0001$	6.31
SP42	-	+	$p < 0.0001$	6.73

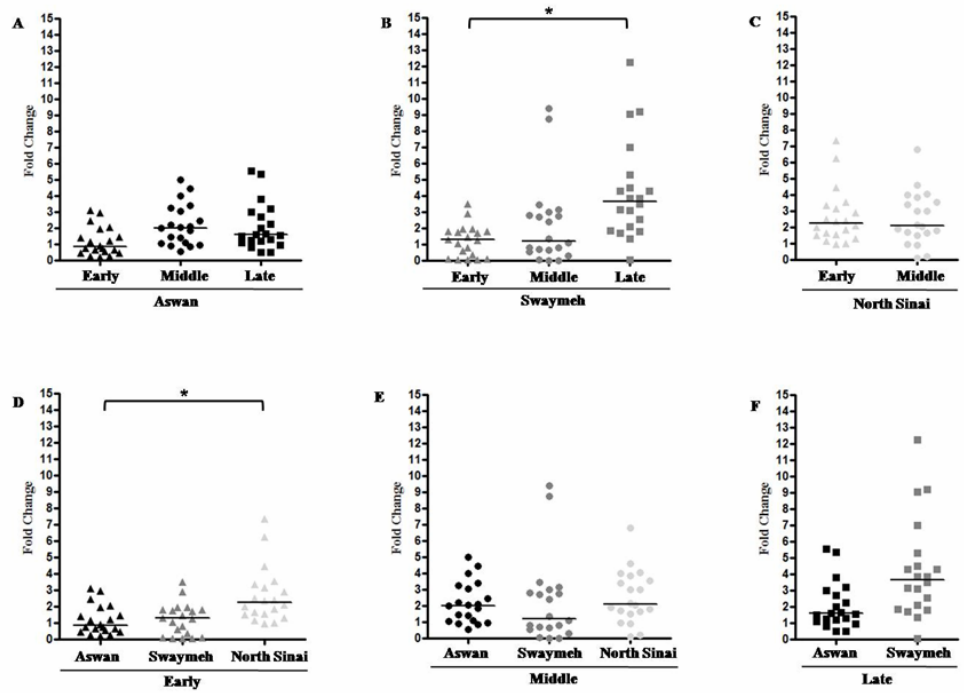

Figure 5.1 *SP12* expression . Expression profiles were assessed as fold changes (Y axis) over the control non-sugar fed, and colony-maintained *P. papatasi* using the $2^{-\Delta\Delta CT}$ method. Seasonal analyses are displayed in (A), (B) and (C), representing the populations of Aswan, Swaymeh and North Sinai, respectively. *P. papatasi* were collected at different periods during the sand fly activity season. Graphs (D), (E) and (F) display the geographic comparisons between expression profiles of *P. papatasi* collected in June (early in the season) and August (middle) 2007, and September (late) 2006, respectively. Horizontal bars represent the expression mean values between the samples and each sample represents an individual fly. Asterisk (*) indicates statistically significant differences ($p< 0.0006$) between every two groups analyzed. Aswan (black), Swaymeh (dark gray) and North Sinai (light gray) color schemes are shown. Triangle, circle and square represent expression levels of sand flies collected early, in the middle and late in the season, respectively.

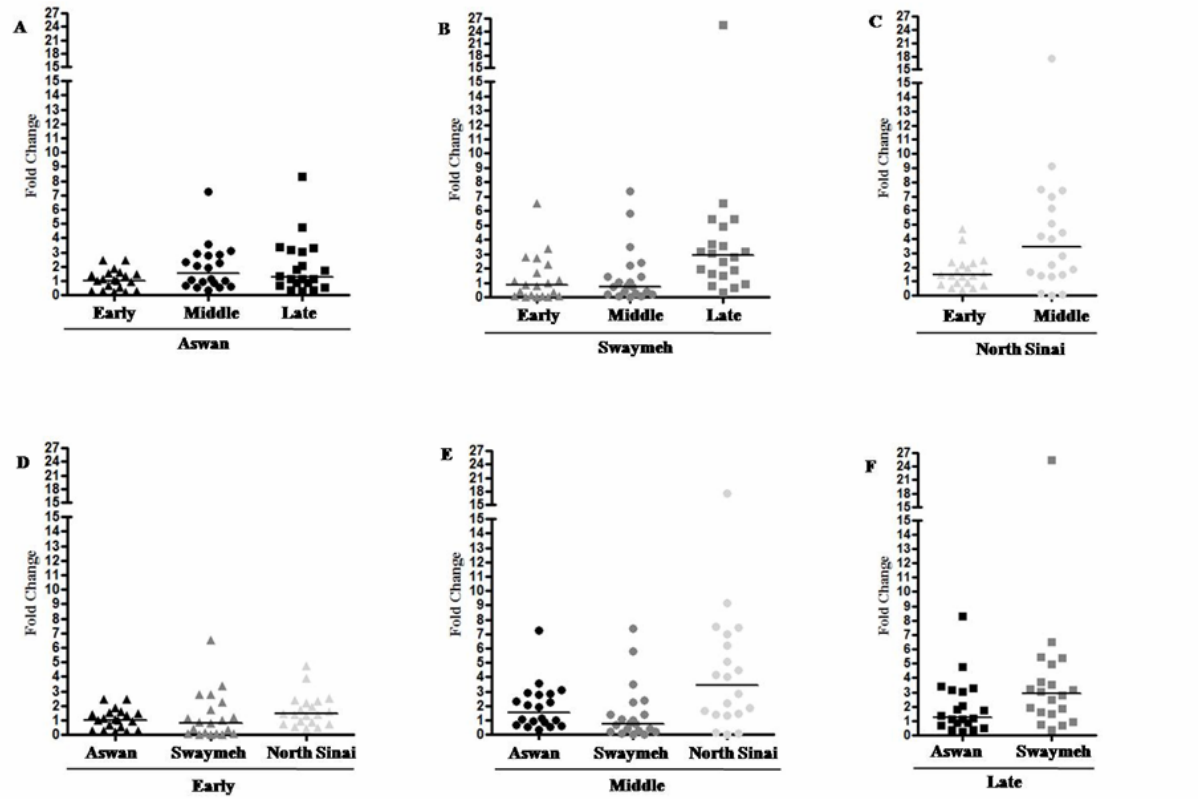

Figure 5.2 *SP14* expression. Expression profiles were assessed as in Figure 1. Seasonal analyses are displayed in (A), (B) and (C), representing the populations of Aswan, Swaymeh and North Sinai, respectively. *P. papatasi* were collected at different periods during the sand fly activity season. Graphs (D), (E) and (F) display the geographic comparisons between expression profiles of *P. papatasi* collected in June (early in the season) and August (middle) 2007, and September (late) 2006, respectively. Horizontal bars represent the expression mean values between the samples, and each sample represents an individual fly. Asterisk (*) indicates statistically significant differences ($p < 0.0006$) between every two groups analyzed. Aswan (black), Swaymeh (dark gray) and North Sinai (light gray) color schemes are shown. Triangle, circle and square represent expression levels of sand flies collected early, in the middle and late in the season, respectively.

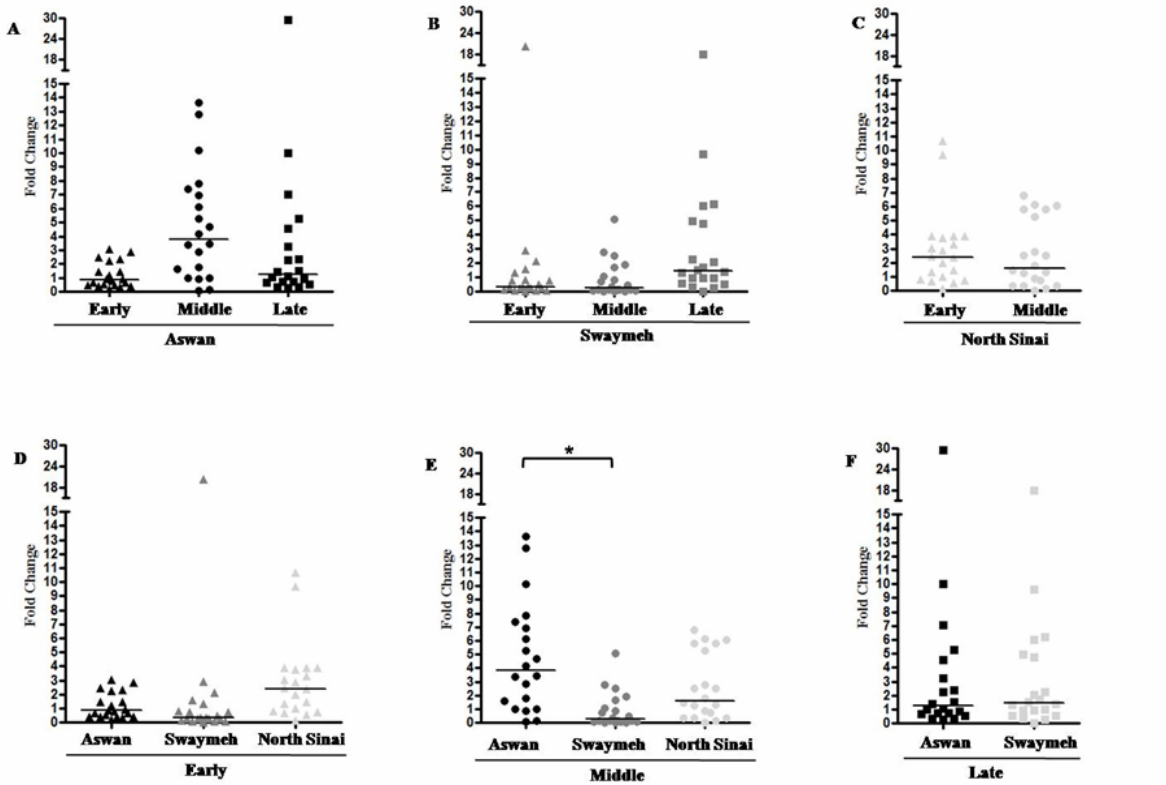

Figure 5.3 *SP28* expression. Expression profiles were assessed as in Figure 1. Seasonal analyses are displayed in (A), (B) and (C), representing the populations of Aswan, Swaymeh and North Sinai, respectively. *P. papatasi* were collected at different periods during the sand fly activity season. Graphs (D), (E) and (F) display the geographic comparisons between expression profiles of *P. papatasi* collected in June (early in the season) and August (middle) 2007, and September (late) 2006, respectively. Horizontal bars represent the expression mean values between the samples and each sample represents an individual fly. Asterisk (*) indicates statistically significant differences ($p < 0.0006$) between every two groups analyzed. Aswan (black), Swaymeh (dark gray) and North Sinai (light gray) color schemes are shown. Triangle, circle and square represent expression levels of sand flies collected early, in the middle and late in the season, respectively.

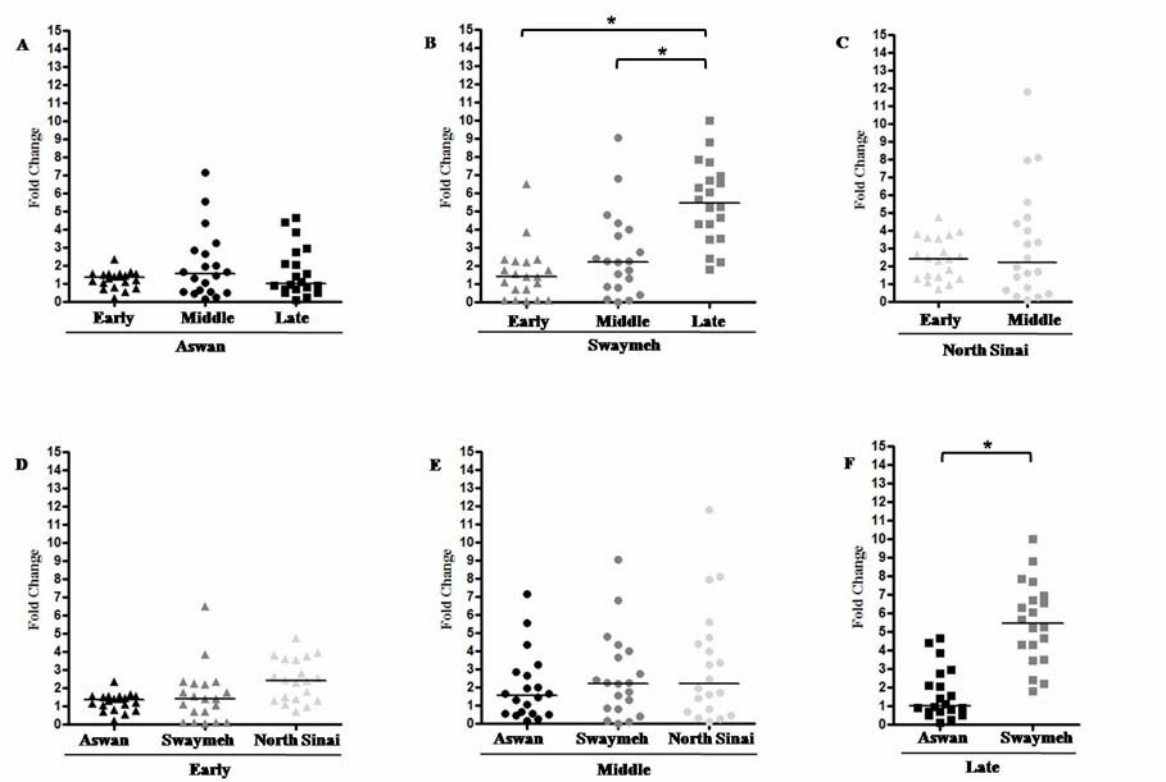

Figure 5.4 *SP29* expression. Expression profiles were assessed as in Figure 1. Seasonal analyses are displayed in (A), (B) and (C), representing the populations of Aswan, Swaymeh and North Sinai, respectively. *P. papatasi* were collected at different periods during the sand fly activity season. Graphs (D), (E) and (F) display the geographic comparisons between expression profiles of *P. papatasi* collected in June (early in the season) and August (middle) 2007, and September (late) 2006, respectively. Horizontal bars represent the expression mean values between the samples and each sample represents an individual fly. Asterisk (*) indicates statistically significant differences ($p < 0.0006$) between every two groups analyzed. Aswan (black), Swaymeh (dark gray) and North Sinai (light gray) color schemes are shown. Triangle, circle and square represent expression levels of sand flies collected early, in the middle and late in the season, respectively.

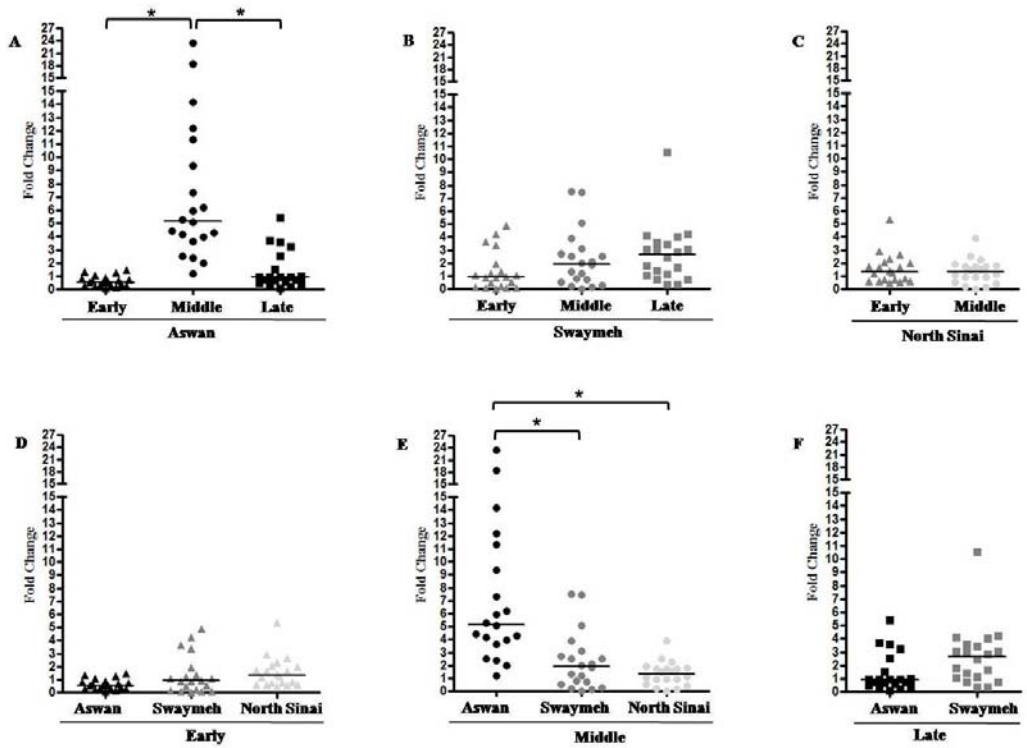

Figure 5.5 *SP30* expression. Expression profiles were assessed as in Figure 1. Seasonal analyses are displayed in (A), (B) and (C), representing the populations of Aswan, Swaymeh and North Sinai, respectively. *P. papatasi* were collected at different periods during the sand fly activity season. Graphs (D), (E) and (F) display the geographic comparisons between expression profiles of *P. papatasi* collected in June (early in the season) and August (middle) 2007, and September (late) 2006, respectively. Horizontal bars represent the expression mean values between the samples and each sample represents an individual fly. Asterisk (*) indicates statistically significant differences ($p < 0.0006$) between every two groups analyzed. Aswan (black), Swaymeh (dark gray) and North Sinai (light gray) color schemes are shown. Triangle, circle and square represent expression levels of sand flies collected early, in the middle and late in the season, respectively.

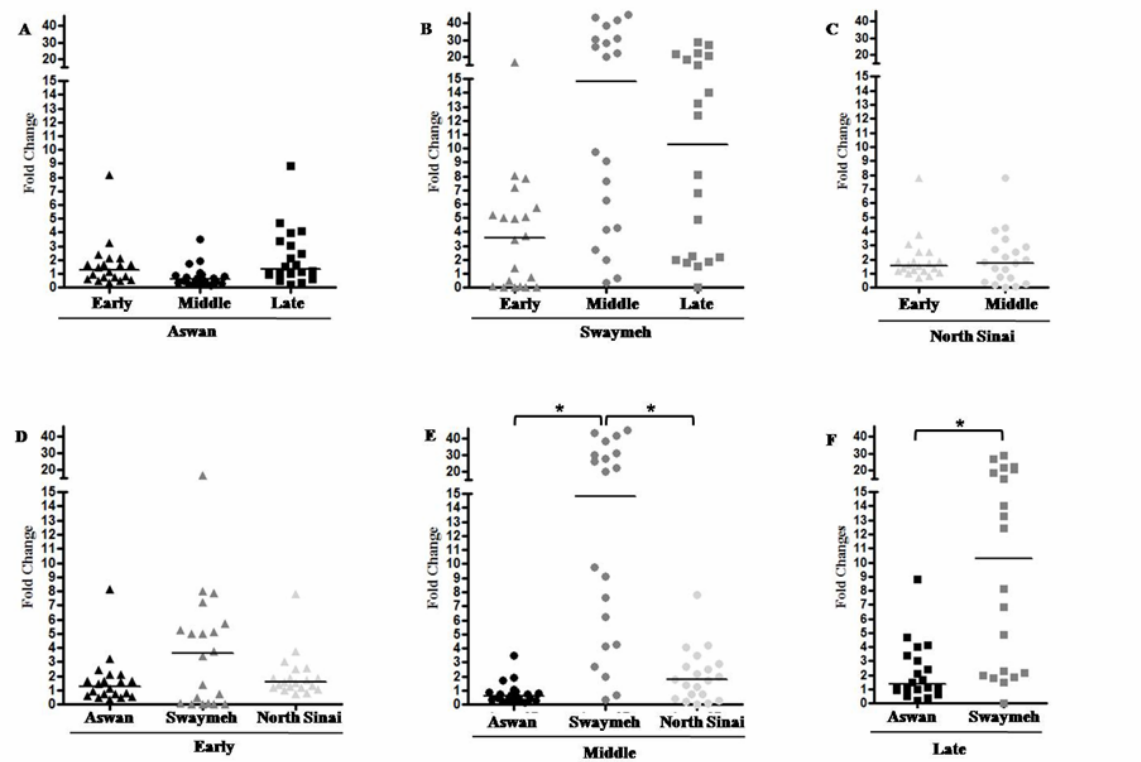

Figure 5.6 *SP32* expression. Expression profiles were assessed as in Figure 1. Seasonal analyses are displayed in (A), (B) and (C), representing the populations of Aswan, Swaymeh and North Sinai, respectively. *P. papatasi* were collected at different periods during the sand fly activity season. Graphs (D), (E) and (F) display the geographic comparisons between expression profiles of *P. papatasi* collected in June (early in the season) and August (middle) 2007, and September (late) 2006, respectively. Horizontal bars represent the expression mean values between the samples and each sample represents an individual fly. Asterisk (*) indicates statistically significant differences ($p < 0.0006$) between every two groups analyzed. Aswan (black), Swaymeh (dark gray) and North Sinai (light gray) color schemes are shown. Triangle, circle and square represent expression levels of sand flies collected early, in the middle and late in the season, respectively.

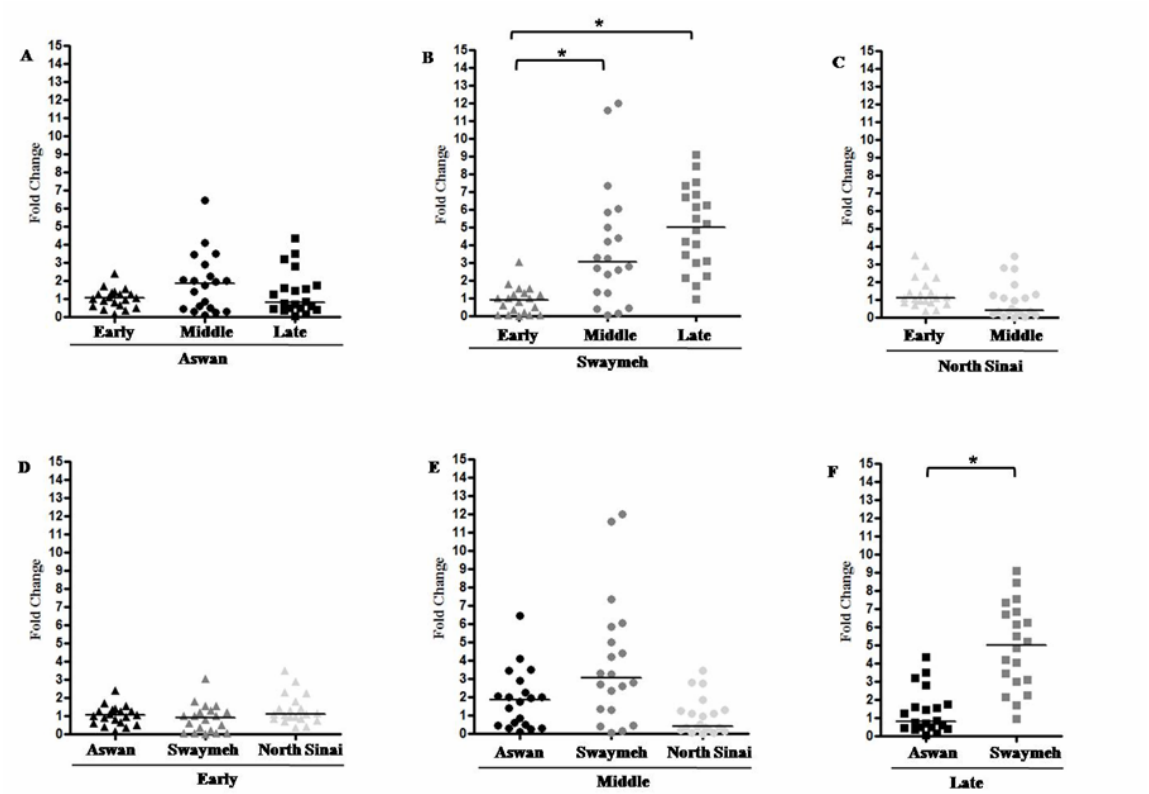

Figure 5.7 *SP36* expression. Expression profiles were assessed as in Figure 1. Seasonal analyses are displayed in (A), (B) and (C), representing the populations of Aswan, Swaymeh and North Sinai, respectively. *P. papatasi* were collected at different periods during the sand fly activity season. Graphs (D), (E) and (F) display the geographic comparisons between expression profiles of *P. papatasi* collected in June (early in the season) and August (middle) 2007, and September (late) 2006, respectively. Horizontal bars represent the expression median values between the samples and each sample represents an individual fly. Asterisk (*) indicates statistically significant differences ($p < 0.0006$) between every two groups analyzed. Aswan (black), Swaymeh (dark gray) and North Sinai (light gray) color schemes are shown. Triangle, circle and square represent expression levels of sand flies collected early, in the middle and late in the season, respectively.

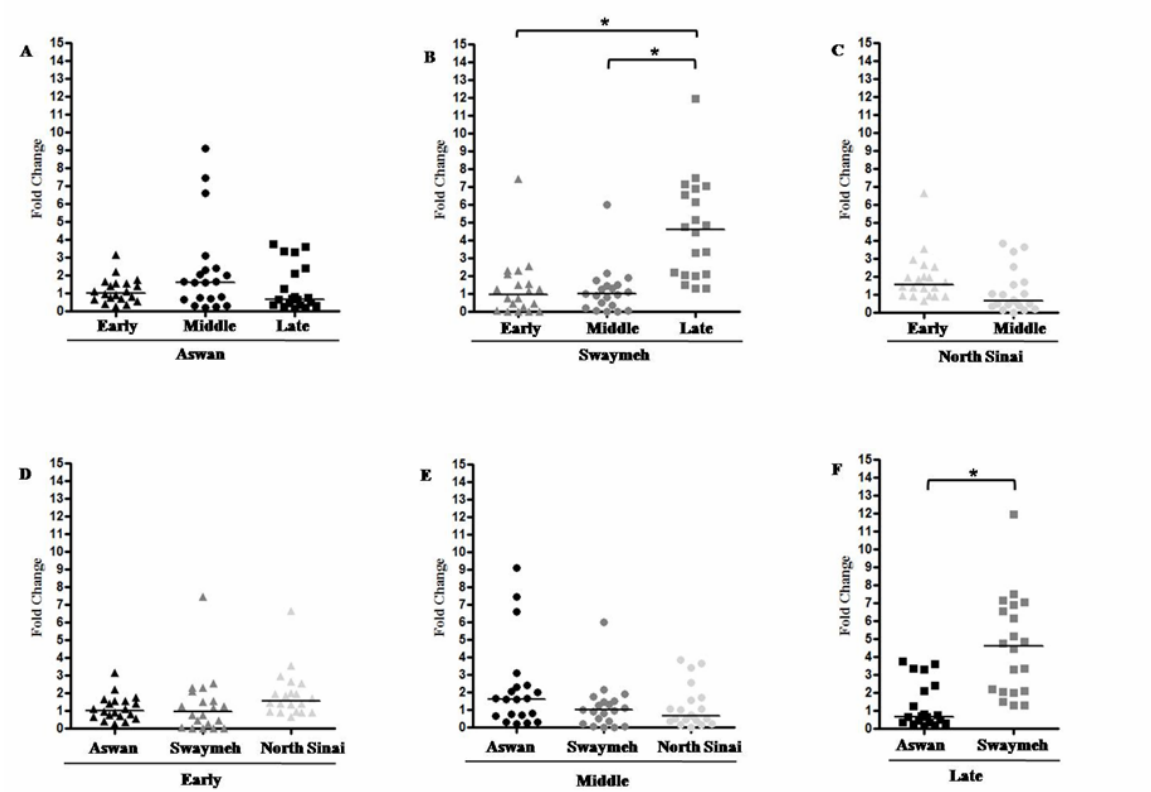

Figure 5.8 *SP42* expression. Expression profiles were assessed as in Figure 1. Seasonal analyses are displayed in (A), (B) and (C), representing the populations of Aswan, Swaymeh and North Sinai, respectively. *P. papatasi* were collected at different periods during the sand fly activity season. Graphs (D), (E) and (F) display the geographic comparisons between expression profiles of *P. papatasi* collected in June (early in the season) and August (middle) 2007, and September (late) 2006, respectively. Horizontal bars represent the expression mean values between the samples and each sample represents an individual fly. Asterisk (*) indicates statistically significant differences ($p < 0.0006$) between every two groups analyzed. Aswan (black), Swaymeh (dark gray) and North Sinai (light gray) color schemes are shown. Triangle, circle and square represent expression levels of sand flies collected early, in the middle and late in the season, respectively.

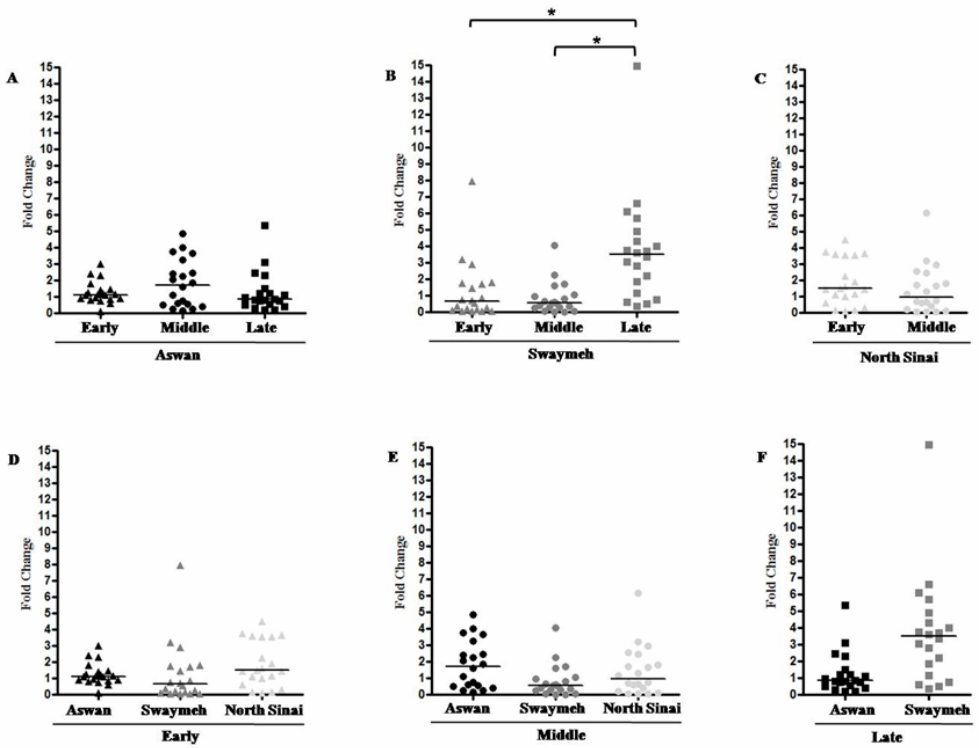

Figure 5.9 *SP44* expression. Expression profiles were assessed as in Figure 1. Seasonal analyses are displayed in (A), (B) and (C), representing the populations of Aswan, Swaymeh and North Sinai, respectively. *P. papatasi* were collected at different periods during the sand fly activity season. Graphs (D), (E) and (F) display the geographic comparisons between expression profiles of *P. papatasi* collected in June (early in the season) and August (middle) 2007, and September (late) 2006, respectively. Horizontal bars represent the expression mean values between the samples and each sample represents an individual fly. Asterisk (*) indicates statistically significant differences ($p < 0.0006$) between every two groups analyzed. Aswan (black), Swaymeh (dark gray) and North Sinai (light gray) color schemes are shown. Triangle, circle and square represent expression levels of sand flies collected early, in the middle and late in the season, respectively.

Chapter 6 - Differential Expression of Salivary Gland-encoding Genes in the Female Sand Fly *Phlebotomus papatasi*

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Abstract

Saliva from blood-sucking arthropods modulates host homeostasis and immunity, making salivary components potential candidates to be used against pathogens transmitted by these biting insects. Functional characterization of salivary molecules is fundamental in order to gain a better understanding into their roles during blood feeding and to determine under which conditions such molecules are expressed in the insect saliva. In the present study, we investigated the expression profile of 10 salivary genes from the sand fly *Phlebotomus papatasi*, a principal vector of *Leishmania major*. Our analyses using quantitative PCR were aimed at defining whether diet or senescence influences the expression of *P. papatasi* salivary gland-expressed genes in laboratory reared female sand flies. Our results demonstrate that at least one of the most abundant salivary transcripts, *SP44*, is consistently modulated by either senescence or diet. In contrast, another abundant transcript, *SP32*, was expressed without any influence from the diet received or the age of the sand fly. Differential expression of the other eight transcripts was not consistently regulated by either diet or age, suggesting that other factors may have a greater influence on differential expression of these salivary gland proteins.

Keywords: Sand fly; *Phlebotomus papatasi*; Saliva; Gene expression; Senescence; Diet

Introduction

The saliva of blood-sucking arthropods plays an essential role in blood vessel location (Ribeiro et al., 1984), influences probing time (Ribeiro et al., 1985), and modulates host hemostatic, inflammatory, and immunomodulatory systems (Kamhawi et al., 2000, Anderson et al., 2006, Kato et al., 2006, Oliveira et al., 2006, Gomes et al., 2008, Oliveira et al., 2008). Pre-exposure to saliva, through non-infected sand fly or mosquito bites, can confer protection against *Leishmania major* and *Plasmodium berghei* development in mice, respectively (Ribeiro, 1995, Belkaid et al., 1998, Kamhawi et al., 2000, Donovan et al., 2007). Additionally, many studies have demonstrated the potential of salivary proteins as vaccine candidates against vector transmitted diseases (Valenzuela et al., 2001a, Oliveira et al., 2006, Donovan et al., 2007, Gomes et al., 2008, Kotsyfakis et al., 2008, Collin et al., 2009).

A critical step in the development of a saliva-based vaccine is the identification of the genes expressed in the insect salivary glands (Valenzuela et al., 2001a, Oliveira et al., 2006). Transcriptome studies of salivary glands, or sialome characterization, have been carried out on a variety of blood-sucking insects including mosquitoes (Calvo et al., 2004, Arca et al., 2005, Calvo et al., 2006, Almeras et al., 2009), ticks (Chmelar et al., 2008, Francischetti et al., 2008, Aljamali et al., 2009), a reduviid (Assumpcao et al., 2008), black flies (Andersen et al., 2009), and various species of sand flies (Anderson et al., 2006, Kato et al., 2006, Oliveira et al., 2006, Hostomska et al., 2009). Transcriptome analyses also have been conducted to distinguish between salivary gland transcripts putatively involved in sugar versus blood feeding processes (Calvo et al., 2006, Marinotti et al., 2006, Thangamani & Wikel, 2009). These analyses have a significant impact in understanding biological functions of genes and provide clues about regulatory mechanisms as well as physiological responses to environmental stimuli (Lockhart & Winzeler, 2000). Results from transcriptome studies of various organisms suggest that abiotic factors can be more important in modulating gene expression than genetic divergence (Oleksiak et al., 2002, Whitehead & Crawford, 2006) and that gene expression can be influenced by genotype, sex, or aging (Jin et al., 2001, Marinotti et al., 2006).

Sand fly salivary components are candidates for anti-*Leishmania* vaccines (Morris, 2001, Valenzuela et al., 2001a, Oliveira et al., 2006, Gomes et al., 2008, Collin et al., 2009). Crucial for the development of such a vaccine is the identification of the most immunogenic salivary

components (Valenzuela et al., 2001a, Oliveira et al., 2006); thus, assessing potential differences in salivary gland gene expression and polymorphisms is a critical step towards this effort. Sand fly salivary protein polymorphisms previously were evaluated in different sand fly populations to identify possible antigen variability (Elnaiem et al., 2005, Kato et al., 2006). Additionally, expression level differences of sand fly salivary components between laboratory-reared and field caught specimens appear to influence the development of *Leishmania* in mice (Laurenti et al., 2009). Such differences may interfere with the sand fly saliva-mediated protection against *Leishmania* (Kamhawi et al., 2000, Gomes et al., 2008) and consequently may prevent development of a sand fly saliva-based vaccine (Laurenti et al., 2009).

Using a quantitative real-time PCR approach, the present study was designed to assess the expression profile of the most abundantly expressed *P. papatasi* salivary gland transcripts (Valenzuela et al., 2001a) and focused on differential expression induced by senescence and diet. Our results demonstrate that some salivary gene expression can be regulated by senescence and diet, while some genes are constitutively expressed. Additional transcripts are regulated, but not by either age or diet, raising the possibility of other factors controlling the expression of these genes.

Materials and Methods

Sand flies

To avoid confounding effects due to genetic polymorphisms *P. papatasi* Israeli strain (PPIS) specimens used in this study were obtained from a laboratory colony maintained at the University of Notre Dame (UND). This colony derives from an original colony established in the early 1970's from sand flies caught in Israel, transferred to Walter Reed Army Institute for Research (WRAIR), Silver Springs, MD in 1983 and subsequently to UND in 2006.

Three groups of female PPIS sand flies were used during the study: non-fed, consisting of flies with access only to water; sugar-fed, consisting of flies with unlimited access to sugar-embedded cotton; and blood-fed, consisting of flies that fed once on an anesthetized mouse and subsequently had unlimited access to sugar-embedded cotton. Non-fed female sand flies were dissected 2, 3, 5, and 7 days after emergence; significant mortality was observed >7 days post emergence in the non-fed group. Sugar feeding began on day 2 because many newly emerged

flies do not feed and therefore, sugar-fed females were dissected at 3, 5, 7, and 18 days after emergence. For blood feeding, 3 day old non-fed PPIS females were fed on BALB/c mice anesthetized with a mixture of ketamine/xylazine for one hour. Fly dissections were carried out at 6, 48, 96, and 144 hours post blood meal (PBM), respectively 3, 5, 7, and 9 days after emergence; blood meals were fully digested and excreted by 96 hours PBM. Sucrose (30 %)-embedded cotton was provided as sugar meal to the sugar and blood-fed flies *ad libitum*. Similarly, water-embedded cotton was offered to non-fed flies during the experiment. Following sugar or blood feeding, only flies that clearly presented sugar-filled (distended) crops or blood-filled midguts were used in the analyses.

PPIS females were anesthetized at -20^{0}C, washed in 10 % Alconox solution in water, transferred to small Petri dishes with 1X PBS, and individually dissected on RNAse free (cleaned with ELIMINase, Fisher Scientific) glass slides using dissecting needles under an Olympus SZ61 steroscope microscope. Salivary glands, still attached to the heads, were transferred to clean 1.5 ml microcentrifuge tubes containing 50 µl of RNAlater™ (Ambion, Austin, TX, USA), thoroughly homogenized using a hand held tissue homogenizer and RNAse-free pestle, and stored at -80^{0}C.

RNA extraction and cDNA synthesis

RNA was extracted from the dissected tissues using the RNAeasy Mini Kit (Qiagen, Valencia, CA, USA) and stored at -80^{0}C. A total of seven PPIS females from each time point were used for RNA extraction. The cDNAs were synthesized using Invitrogen's reagent (Invitrogen, Carlsbad, CA, USA), following the manufacturer's instructions. In brief, 12 µl of RNA of each sample was added to a reaction mix containing 2.5 µM of Oligo $(dT)_{20}$ primers and 0.5 µM of dNTPs. This mixture was incubated at 65^{0}C for five minutes and left in ice for at least one minute. Subsequently, a mix of 4µl of First-Strand Buffer, 5mM of DTT (0.1M), 20 Units of RNaseOUT, and 200 Units of SuperScript^{TM} III Reverse Transcriptase was added to each reaction. Reactions were incubated for cDNA syntheses for one hour at 50^0 C and stored at -20^{0}C.

Quantitative real-time polymerase chain reactions (qRT-PCR)

The qRT-PCRs were set up using 10µl of 2X SYBR Green (Applied Biosystems, Foster City, CA, USA) along with 0.6µl of each forward and reverse primer (0.3 µM final

concentration) (Integrated DNA Technologies, Inc., Coraville, IA, USA), 0.5 µl of cDNA, and 8.3 µl of Ultra Pure Water (Invitrogen). Reactions were analyzed using the 7900HT Real Time PCR System (Applied Biosystems). The qRT-PCR cycle encompassed one incubation step at 50^0C for two minutes, followed by a step at 95^0C for 10 minutes, and 40 cycles of 95^0C for 15 seconds and 55^0C for one minute. A dissociation curve was also obtained following the cycle: 15 seconds at 95^0C, 15 seconds at 55^0C, and 95^0C for 15 seconds.

Expression of 10 of the most abundant *P. papatasi* salivary gland-expressed genes (*SP12*, *SP14*, *SP15*, *SP28*, *SP29*, *SP30*, *SP32*, *SP36*, *SP42*, and *SP44*) was assessed for each of the seven sand fly specimens analyzed per time point. Each reaction was repeated four times for each gene, twice in two different plates. For qRT-PCR, primer sequences targeting all salivary cDNAs used in this study are presented in Table 6.1. Sequences for the α-tubulin housekeeping control were previously published (Ramalho-Ortigao et al., 2005).

Data analyses

We employed the comparative threshold cycle method to determine relative differential expression for each salivary protein-encoding gene (Livak & Schmittgen, 2001). After generation of C_T values (the cycle number at which the reaction crosses the threshold), relative copy number was determined according to the following equation: number of copies = $2^{-\Delta\Delta}C_T$, where $\Delta\Delta C_T = \Delta C_T$(sample) – ΔC_T(calibrator), $\Delta C_T = C_T$(sample) – C_T(alpha tubulin gene), C_T = cycle at which a statistically significant increase in the emission intensity over the background. The calibrator was represented by the average expression (mean ΔC_T) of the seven non-fed samples dissected 24 hours after drinking water, and was calculated for each gene. Fold change (or expression level) was calculated for each gene of individual specimens.

Expression level comparisons were carried out to assess whether diet or senescence influences *P. papatasi* salivary gland gene expression. Effects from diet were assessed via 2-way data set comparisons of expression levels between specimens of the same age (3, 5, or 7 day-old) and fed on different diets (Table 6.3). Likewise, effects of age (senescence) on expression profiles of salivary genes were assessed by 2-way data set comparisons between specimens fed on the same diet [no food, sucrose, or blood (plus sucrose)] and dissected at different time points (Table 6.4).

Statistical analyses

Statistical analyses were carried out using the software GraphPad Prism v. 5.01 (GraphPad Software, Inc). The statistical tests used were the non-parametric Kruskal-Wallis for comparisons among more than two data sets, and/or Mann-Whitney for comparisons between data sets within a group when the results for Kruskal-Wallis tests were statistically significant, or for comparisons when only two data sets were present. The levels of significance were adjusted for multiple comparisons using Bonferroni's correction. Differences were considered statistically significant at $\alpha = 0.0008$ when the expression levels of specimens of different ages (2 days, 3 days, 18 days, etc.) fed on water only (non-fed), sugar, or blood were compared (60 2-way data set comparisons in each group). When the influence of meal type on the expression levels of flies in the same age was assessed, the comparisons were statistically significant at $\alpha = 0.0016$ for 3, 5, or 7 day old flies (30 2-way data set comparisons). Fold change values in Tables 6.3 and 6.4 are based on the ratio of the expression medians between the time point up-regulated (+) over the time point down-regulated (-).

Results and discussion

In this study, we assessed the effects of diet or senescence (aging) on the expression profiles of 10 of the most abundant transcripts found in the salivary glands of female *P. papatasi* sand flies (Valenzuela et al., 2001a). All sand flies used in the study were obtained from a laboratory-reared colony originally established in the 1970's. Gene expression value medians and ranges for each age and diet are presented in Table 6.2. Despite using levels of significance as low as 0.0008 after Bonferroni's correction (a value close to those used in microarray studies – 0.0001, (Jin et al., 2001)), even small differences of fold change ratio, such as of 1.65 and 1.80, were considered statistically significant. Fold change ratios ranged from 1.65 to 17.16 (Table 6.3) and 1.80 to 11.61 (Table 6.4) when the effects of either diet or senescence on gene expression were analyzed, respectively. Roughly, 73.6 % of the fold change ratios were lower than five-fold, whereas 17.6 % and 8.8 % of the fold change ratios accounted for differences between five- and ten-fold, and greater than tenfold, respectively. One salivary transcript, *SP32*, was constitutively expressed without modulation by diet or senescence (Fig. 6.1). In contrast, another abundantly expressed gene, *SP44*, was consistently regulated (Fig. 6.2). Expression of

the remaining eight transcripts was not consistently influenced by either food source or sand fly age.

Diet effect on salivary gland gene expression

As the components of insect meal can influence hormonal levels in blood-sucking insects (Hagedorn, 2004), and consequently gene expression (Raikhel, 2004, Marinotti et al., 2006), we verified whether or not the different components in the sugar or blood diet can modulate expression of *P. papatasi* salivary gland genes. For 3 day-old sand flies, seven out of ten salivary gland genes were up-regulated in sugar-fed compared with non-fed (given only water) sand flies (Table 6.3). Six out of these seven transcripts also are up-regulated in 3 day-old blood-fed (6h PBM) compared to non-fed flies (Table 6.3). The distinction between the two data set comparisons is transcripts *SP28*, up-regulated in the sugar-fed group, and *SP14*, up-regulated by blood feeding (Table 6.3). This induction of a significant number of genes following feeding of sand flies on substrates containing sugar and/or protein in comparison to water is suggestive of the important role played by saliva (and salivation) during feeding. Greater mRNA expression in sugar-fed and blood-fed flies is possibly associated with a subsequent need for renewal of the protein levels in the saliva following a meal. This observation is in accordance with previous findings that total protein levels in the salivary glands of mosquitoes and sand flies significantly decrease after a sugar or blood meal (Marinotti et al., 1990, Golenda et al., 1995, Prates et al., 2008). In contrast, non-fed flies at 3 days old are ready to take their first blood meal. Comparing sugar versus blood-fed groups, only a single transcript, *SP44* (Fig. 6.2), was up regulated in the former. Thus, lower expression levels in non-fed flies may indicate that at this age the sand fly has already accumulated enough salivary proteins that can be used during the feeding process, either for sugar or blood feeding, down-regulating salivary gene transcription.

At 5 days post emergence, the trend of gene up-regulation detected for 3 day-old sand flies fed sugar or blood is no longer observed. Forty-eight hours PBM, *SP15*, *SP28* (Table 6.3), and *SP44* (Table 6.3; Fig. 6.2) are down regulated in comparison to sugar-fed flies. Down-regulation of salivary gland gene expression after a blood meal also has been detected for *Anopheles gambiae* (Marinotti et al., 2005). It is plausible that down-regulation detected for the three transcripts in the salivary glands of the 5 day-old blood-fed flies (48 h PBM) was due to their turn-over following protein synthesis. The time lapse for *de novo* salivary protein synthesis

following a blood meal varies from protein to protein in mosquitoes (Marinotti et al., 1990); a similar event may be present here for *P. papatasi* salivary proteins, supporting a delay in restoring mRNA levels of *SP15*, *SP28*, and *SP44* by 48 hours PBM to the levels detected for the 5 day-old sugar-fed flies.

The first gonotrophic cycle for *P. papatasi* is completed in 6 days (Magnarelli et al., 1984, Schlein & Jacobson, 1999), and blood meal digestion is accomplished by 72 h PBM (Yuval & Schlein, 1986). In regards to the effects of diet on salivary gland gene expression late in the *P. papatasi* gonotrophic cycle (96 h PBM), three genes (*SP30*, *SP36*, and *SP42*) were up-regulated in the blood-fed compared with 7 day-old non-fed flies. Similarly, late in the gonotrophic cycle *Aedes aegypti* (Raikhel, 2004) exhibited greater total protein levels of α-glucosidase and apyrase than non-fed mosquitoes (Marinotti et al., 1990). The salivary gland specific apyrase of *Ae. aegypti* is a member of the 5'-nucleotidase family of apyrases (Champagne et al., 1995), while the *P. papatasi* apyrase (SP36) belongs to the novel Cimex family of apyrases (Valenzuela et al., 2001b). Interestingly, in spite of belonging to different apyrase families, both genes may be regulated by similar physiological conditions in these two vector species. Moreover, total *L. longipalpis* protein levels also are greater late in the vitellogenic period (Magnarelli et al., 1984, Prates et al., 2008).

SP44 is unique in that it is strongly influenced by diet as this gene is up-regulated after a sugar meal (when compared to water or blood meal) in 3, 5, and 7 day old specimens (Table 6.3, Fig. 6.2). Diet has no effect on two of the ten genes analyzed, *SP29* (Table 6.3) and *SP32* (Table 6.3, Fig. 6.1) and only influences expression of *SP12* and *SP14* in 3 day old flies (Table 6.3).

Senescence effects on salivary gland gene expression

Another biotic factor influencing gene expression profiles is senescence; however, such effects are not thought to be as significant as genotype or sex in insects (Jin et al., 2001). In regards to salivary gland gene expression in *P. papatasi*, only *SP44* (Table 6.4; Fig. 6.2) appears to be consistently influenced by senescence. Among the sugar-fed flies, 18 day-old flies exhibited lower *SP44* expression levels compared to the younger groups (i.e., 3, 5, or 7 day-old flies; Table 6.4). Interestingly, senescence influences expression of only 1 % of *Drosophila melanogaster* and 5 % of *Anopheles gambiae* genes (Jin et al., 2001, Marinotti et al., 2006). In all other comparisons, age difference levels of expression were smaller, and differential

expression could be associated with physiological changes (possibly hormonal) related to the feeding process or meal digestion.

Differential gene expression exhibited by non-fed flies at different ages appears unrelated to senescence as differential expression only was exhibited between specimens differing in age between 1-2 days. Rather, these changes seem to be related to sand fly preparation for a first meal. Our results indicate a down regulation of four transcripts (*SP12*, *SP30*, *SP42*, and *SP44*) 3 days after emergence, followed by an up-regulation of three of the four transcripts in addition to another four transcripts in 5 day-old flies (*SP14*, *SP15*, *SP29*, and *SP36*) (Table 6.4). One day after emergence mosquitoes cannot take a meal because cuticle is neither thick nor hard enough to allow insects to pierce the host skin with their proboscis (Lehane, 2005). Similarly, sand flies will usually not take a blood meal until at least 2-3 days following emergence. In our study, we only observed sugar-filled crops in *P. papatasi* specimens 2 to 3 day-old (sugar-filled crops also were observed in older flies), and only 3 day-old flies were capable of taking a blood meal. As indicated above, lower expression levels in 3 day-old non-fed flies possibly indicate that the sand flies have accumulated sufficient salivary proteins to be used during the feeding process, either for sugar or blood feeding.

Among blood-fed samples, *SP14* and *SP15* gene expression levels were significantly different between 5 and 9 day-old flies (Table 6.4). Five day-old (48 h PBM) flies were engorged whereas 9 day-old (144 h PBM) were gravid and had already passed all the digested blood meal. Although the physiological processes that may lead to such differences in salivary gland gene expression between engorged and gravid females are not known, these findings are important for the elucidation of the mechanisms involved in the differential expression of such genes in field collected flies. Interestingly, as with diet, *SP32* are not regulated by age (Table 6.4).

Summary

Our results indicate that *SP44* is the only abundant transcript from the *P. papatasi* sialome that is strongly modulated by either senescence or diet whereas *SP32* was the only gene that is not influenced by either of the parameters analyzed, thus behaving as a constitutive gene. Both SP44 and SP32 belong to protein families present in sand flies. SP44 is similar to the yellow protein family (major royal jelly protein – MJRP) from *Apis mellifera* (Geyer et al., 1986). This family also includes the sequence-related yellow protein of *Drosophila* which

controls pigmentation of the adult cuticle and larval mouth parts. However, the function of SP44 in sand fly saliva currently is unknown. The SP32 family belongs to the silk-related and collagen-like protein in sand flies (Valenzuela et al., 2001a). This protein has not been described in other blood feeding arthropods, yet it is present in sand flies, including specimens from Old (*Phlebotomus* spp.) and New World (*Lutzomyia* spp.) (Valenzuela et al., 2001a).

Although the current study was not intended to precisely identify the environmental factors involved in regulating salivary gland gene expression in natural populations, these data can inform studies assessing gene expression in field samples. Phenology studies have demonstrated that *P. papatasi* populations from different habitats display different percentages of engorged or gravid females (Yuval, 1991, Janini et al., 1995, Hanafi et al., 2007). If salivary gland gene expression levels of gravid or blood-engorged female sand flies were significantly modulated in this analysis, these biotic or physiological factors may account for differential expression observed in field collected flies specimens. However, our results suggest that biotic factors such as blood-feeding or gonotrophic state appear unlikely to be responsible for differential expression of salivary gland genes observed in field collected sand flies, supporting a role for environmental factors in modulating expression of salivary gland genes in natural *P. papatasi* sand flies.

In natural populations of *P. papatasi*, the proportion of gravid females is reported to increase late in the season (Yuval, 1991, Janini et al., 1995, Hanafi et al., 2007), a characteristic believed to be associated with older sand flies (Hanafi et al., 2007). Thus, population senescence could have been argued as an important factor influencing salivary gland gene expression profiles in natural habitats. Although senescence influences *SP44* gene expression, our data indicate that older laboratory reared flies display lower levels of *SP44* expression than younger ones (Table 6.4; Fig. 6.2). This contrasts with our data obtained with field caught *P. papatasi* (Coutinho-Abreu et al., unpublished results), which exhibit greater *SP44* expression late in the season, when sand fly populations are thought to be older. Taken together, these results suggest that senescence is likely not a major factor modulating *P. papatasi* salivary gland gene expression in natural habitats.

The rate of photosynthesis, and the quality of the sugars produced, is known to influence sugar-feeding behavior of *P. papatasi* (Schlein & Jacobson, 2000), raising the possibility that sugar availability throughout the sand fly season may be an important abiotic factor in regulating

salivary gland gene expression in natural populations of *P. papatasi*. In our laboratory conditions, feeding 30 % sucrose to sand flies altered the expression levels of 7 out of 10 salivary genes 3 days post emergence (Table 6.3), indicating that availability of sugar also may modulate *P. papatasi* salivary gland gene expression of young flies in natural habitats.

Uncovering *P. papatasi* salivary gland gene expression in laboratory-reared specimens is important to understand the factors, such as senescence and diet, capable of modulating the expression of these genes, indicating what biotic and abiotic factors are indeed involved in modulating sand fly saliva levels in natural habitats. Such findings are an important part of developing saliva-based vaccines as immunogenicity and efficacy of vaccines are modulated by the antigen challenge dose (Lee et al., 2002, Yao et al., 2002, Dhar et al., 2003).

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Table 6.1 Genes, protein products, and respective primer pairs used in the Real-Time PCRs.

Gene#	Protein	Function	Primers
SP12	12kDa	Unknown	PPSP12_116F: 5` – TTGGAGAATCTTTTAAGAGG – 3` PPSP12_116R: 5` – TTGATCAATTTTTGATCAGC – 3`
SP14	14kDa	Unknown	PPSP14_122F: 5` - ATGAAGTACTTGTTCGCTTT – 3` PPSP14_122R: 5` - AGAATGCACTCAAAATCAGT – 3`
SP15	15kDa	Unknown	PPSP15_152F: 5'- GGACAAAAGCCTGAAAGCAG – 3` PPSP15_152R: 5'- GAGGTCCAATTCGTTTGTCG– 3`
SP28	28kDa	D7 family	PPSP28_104F: 5` - CTTCGAACCTATACACTTTAG- 3` PPSP28_104R: 5` – ACCTTTGGCATCCTTGACC – 3`
SP29	29kDa	Antigen-5	PPSP29_131F: 5` – TTTGCGCATGATAAATGTCG – 3` PPSP29_131R: 5` - CGGAACCACTCCCTCGTGAT – 3`
SP30	30kDa	D7 family	PpSP30_128F: 5` - GAGAAGAGTCAGATAAGTGG – 3` PpSP30_128R: 5` - AAGACTACATGCACGTCTGC – 3`
SP32	32kDa	Unknown	PpSP32_110F: 5` - GGACATATTTTAACAGTGGG – 3` PpSP32_110R: 5` - GGAACAGGAAAATCTTTTCC – 3`
SP36	36kDa	Apyrase*	PpSP36_129F: 5` - ATGGAAACTTTACACTTTTG – 3` PpSP36_129R: 5` - CATTCTGCCTTAAAGCCATC – 3`
SP42	42kDa	Yellow	PpSP42_162F: 5` - AGTGGGAGTTAGGCCAAATG – 3` PpSP42_162R: 5` - TCAGCTGAATTGTAACTACG – 3`
SP44	44kDa	Yellow	PPSP44_135F: 5` – TGTGCCAAATCCGATGAAAC – 3` PPSP44_135R: 5` – TACGGACTTCCCTGGTTCTG – 3`
α-tubulin	Tubulin	Structural	PPTUB24hF : 5` – GCGATGACTCCTTCAACAC – 3` PPTUB24hR : 5` – TCAGCCAGCTTGCGAATAC – 3`

[#] Salivary gland predicted gene functions were based on genetic similarities (Lee et al., 2002, Yao et al., 2002, Dhar et al., 2003).

[*] Apyrase is the only protein whose function is known (Valenzuela et al., 2001b).

Table 6.2 *P. papatasi* salivary gland gene expression. Median gene expression values for each age and diet are presented. Ranges are indicated between parentheses.

Non-Fed	2 days[#]	3 days	5 days	7 days
SP12	1.09 (0.64-1.46)	0.35 (0.23-0.47)	0.78 (0.55-2.43)	0.78 (0.29-1.52)
SP14	1.25 (0.29-1.75)	0.34 (0.14-0.42)	1.36 (0.76-2.36)	0.87 (0.29-1.38)
SP15	1.00 (0.47-1.72)	0.35 (0.26-0.50)	1.31 (0.85-2.37)	0.67 (0.37-1.83)
SP28	1.20 (0.60-1.90)	0.30 (0.07-0.66)	1.53 (0.45-3.71)	0.74 (0.16-2.35)
SP29	1.05 (0.46-2.59)	0.41 (0.31-0.55)	1.11 (0.68-2.59)	0.67 (0.38-1.40)
SP30	1.22 (0.35-1.77)	0.22 (0.16-0.28)	0.63 (0.16-0.28)	0.28 (0.15-0.81)
SP32	1.73 (0.33-2.71)	0.44 (0.09-0.85)	0.70 (0.53-4.81)	0.59 (0.28-1.48)
SP36	1.31 (0.26-2.09)	0.23 (0.15-0.32)	0.60 (0.48-1.14)	0.38 (0.19-0.61)
SP42	0.90 (0.54-1.92)	0.27 (0.21-0.30)	0.79 (0.56-1.08)	0.61 (0.29-0.94)
SP44	0.89 (0.41-1.94)	0.19 (0.14-0.30)	0.42 (0.22-1.17)	0.22 (0.10-0.52)
Sugar-Fed	3 days	5 days	7 days	18 days
SP12	0.77 (0.50-1.04)	1.23 (0.57-1.89)	0.73 (0.32-1.15)	1.64 (0.71-2.57)
SP14	1.03 (0.35-1.44)	1.38 (0.88-3.11)	0.69 (0.27-1.66)	1.23 (0.52-2.58)
SP15	1.08 (0.91-1.44)	1.43 (0.81-1.92)	0.53 (0.41-1.44)	1.17 (0.71-2.30)
SP28	1.85 (0.72-2.87)	2.40 (1.16-3.51)	0.91 (0.55-1.46)	2.41 (0.75-4.86)
SP29	1.42 (0.86-1.82)	1.62 (0.75-2.17)	0.83 (0.58-2.19)	1.03 (0.61-1.97)
SP30	1.30 (0.95-1.97)	1.89 (0.89-3.76)	0.93 (0.47-1.34)	0.82 (0.36-1.22)
SP32	0.86 (0.50-4.52)	1.38 (0.45-3.22)	1.39 (0.43-3.62)	0.68 (0.27-1.39)
SP36	1.11 (0.81-2.41)	1.61 (0.77-2.70)	0.69 (0.41-1.59)	0.98 (0.46-2.10)
SP42	1.28 (0.89-2.35)	1.31 (1.02-2.46)	0.85 (0.41-1.89)	1.31 (0.90-2.34)
SP44	3.34 (2.82-6.51)	2.76 (1.49-4.96)	0.88 (0.58-2.85)	0.29 (0.17-0.40)
Blood-Fed	3 days (6h)[*]	5 days (48h)	7 days (96h)	9 days (144h)
SP12	1.39 (0.56-1.39)	1.43 (0.87-2.54)	0.95 (0.48-2.32)	1.15 (0.76-2.57)
SP14	1.03 (0.46-2.61)	0.95 (0.51-1.19)	2.52 (0.74-5.13)	1.72 (1.35-4.55)
SP15	1.52 (0.63-2.49)	0.44 (0.27-0.59)	0.82 (0.26-1.69)	1.21 (1.14-4.04)
SP28	1.18 (0.10-2.55)	0.87 (0.57-1.10)	3.40 (0.63-4.97)	3.01 (0.31-6.18)
SP29	0.97 (0.43-1.71)	1.41 (0.83-1.91)	1.93 (1.04-3.59)	1.76 (0.96-4.14)
SP30	1.02 (0.59-1.53)	1.12 (0.61-1.35)	1.89 (0.72-3.10)	1.80 (1.19-4.86)
SP32	1.26 (0.35-6.99)	0.69 (0.34-3.13)	0.62 (0.32-3.55)	1.85 (0.65-1.37)
SP36	0.86 (0.39-1.76)	0.84 (0.40-1.36)	1.40 (0.68-2.29)	1.72 (0.96-3.08)
SP42	1.10 (0.49-1.67)	2.29 (0.80-3.02)	3.34 (1.11-4.92)	1.90 (1.02-4.24)
SP44	1.23 (0.49-1.98)	1.06 (0.21-1.34)	0.69 (0.29-1.25)	0.69 (0.33-2.59)

[#] "Days" indicates insect age (days after emergence).

[*] Time points between parentheses indicate time after feeding in hours.

Table 6.3 *P. papatasi* salivary gland gene expression differences between specimens at the same age and fed on different diets. Only statistically significant (Mann-Whitney with Bonferonni's adjustment; $p < 0.0016$) differences between data sets are shown. Values of fold change displayed are derived from a ratio between the mean fold change expression values of the up- over the down-regulated groups. Regulation of expression levels up (+) or down (-) are indicated.

Genes	Regulation of expression	p-values	Fold change
3 days old	Non-fed x Sugar-fed		
SP12	- +	p= 0.0006	2.18
SP15	- +	p= 0.0006	3.10
SP28	- +	p= 0.0006	6.23
SP30	- +	p= 0.0006	5.97
SP36	- +	p= 0.0006	4.86
SP42	- +	p= 0.0006	4.76
SP44	- +	p= 0.0006	17.16
	Non-fed x Blood-fed		
SP12	- +	p= 0.0006	3.95
SP14	- +	p= 0.0006	3.05
SP15	- +	p= 0.0006	4.37
SP30	- +	p= 0.0006	4.67
SP36	- +	p= 0.0006	3.77
SP42	- +	p= 0.0006	4.12
SP44	- +	p= 0.0006	6.32
	Sugar-fed x Blood-fed		
SP44	+ -	p= 0.0006	2.71
5 days old	Non-fed x Sugar-fed		
SP42	- +	p= 0.0012	1.65
SP44	- +	p= 0.0006	6.59
	Non-fed x Blood-fed		
SP15	+ -	p= 0.0006	2.96
	Sugar-fed x Blood-fed		
SP15	+ -	p= 0.0006	3.22
SP28	+ -	p= 0.0006	2.74
SP44	+ -	p= 0.0006	2.59
7 days old	Non-fed x Sugar-fed		
SP44	- +	p= 0.0006	3.97
	Non-fed x Blood-fed		
SP30	- +	p= 0.0012	6.65
SP36	- +	p= 0.0006	3.65
SP42	- +	p= 0.0006	5.49

Table 6.4 *P. papatasi* salivary gland gene expression differences between specimens at different ages. Only statistically significant (Mann-Whitney with Bonferonni's adjustment; p< 0.0008) differences are shown. Values of fold change displayed are derived from the ratios between the fold changes of expression profiles up over down regulated ones. Regulation of expression levels up (+) or down (-) are indicated.

Genes	Regulation of expression		p-values	Fold change
Non-fed	2 days[#]	3 days		
SP12	+	-	p= 0.0006	3.09
SP30	+	-	p= 0.0006	5.58
SP42	+	-	p= 0.0006	3.35
SP44	+	-	p= 0.0006	4.56
	3 days	5 days		
SP12	-	+	p= 0.0006	2.22
SP14	-	+	p= 0.0006	4.02
SP15	-	+	p= 0.0006	3.77
SP29	-	+	p= 0.0006	2.70
SP30	-	+	p= 0.0006	2.89
SP36	-	+	p= 0.0006	2.63
SP42	-	+	p= 0.0006	2.96
Genes	Regulation of expression		p-values	Fold change
Sugar-fed	3 days	18 days		
SP44	+	-	p= 0.0006	11.61
	5 days	18 days		
SP44	+	-	p= 0.0006	9.59
	7 days	18 days		
SP44	+	-	p= 0.0006	3.07
Genes	Regulation of expression		p-values	Fold change
Blood-fed	3 days (6h)[*]	5 days (48h)		
SP15	+	-	p= 0.0006	3.43
	5 days (48h)	9 days (144h)		
SP14	-	+	p= 0.0006	1.80
SP15	-	+	p= 0.0006	2.73

[#] "Days" indicates insect age (days after emergence).

[*] Time points between parentheses indicate time after feeding in hours.

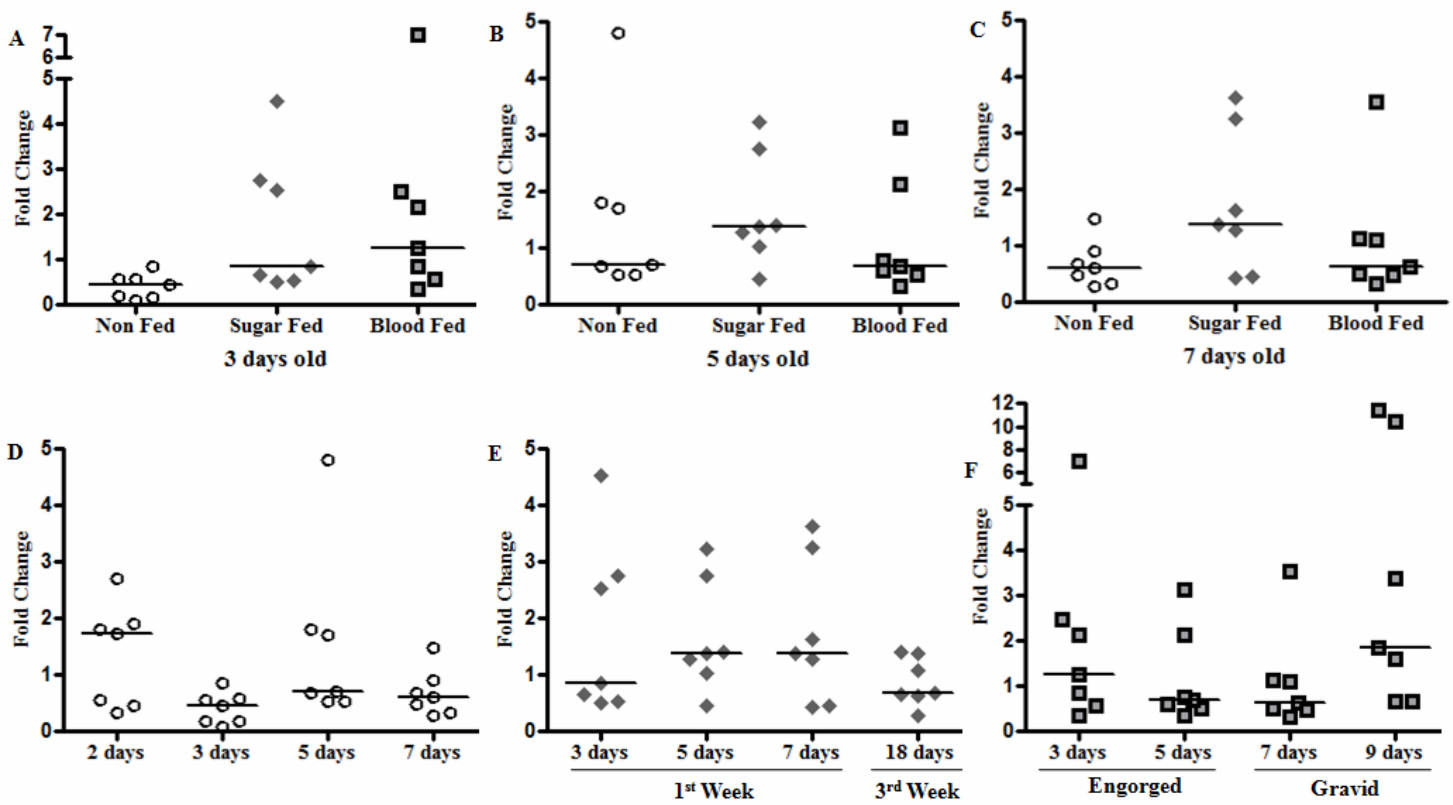

Figure 6.1 Expression profiles were assessed as fold changes (Y axis) over expression of non-fed specimens. Diet influences on *SP12* gene expression are displayed in (A), (B) and (C), representing expression patterns in 3 days, 5 days, and 7 days old *P. papatasi* specimens. Graphs (D), (E) and (F) display ageing influences on expression profiles of *P. papatasi* specimens fed on water (non-fed), sugar (sugar-fed), and blood (blood-fed), respectively. Horizontal bars represent the expression median values between the samples and each sample represents an individual fly. Asterisk (*) indicates statistically significant differences between every two groups analyzed. Diet and senescence influences on gene expression were considered statistically significant at $\alpha = 0.0016$ and $\alpha = 0.0008$, respectively. Circles (non-fed), triangles (sugar-fed), and squares (blood-fed) symbol schemes are shown.

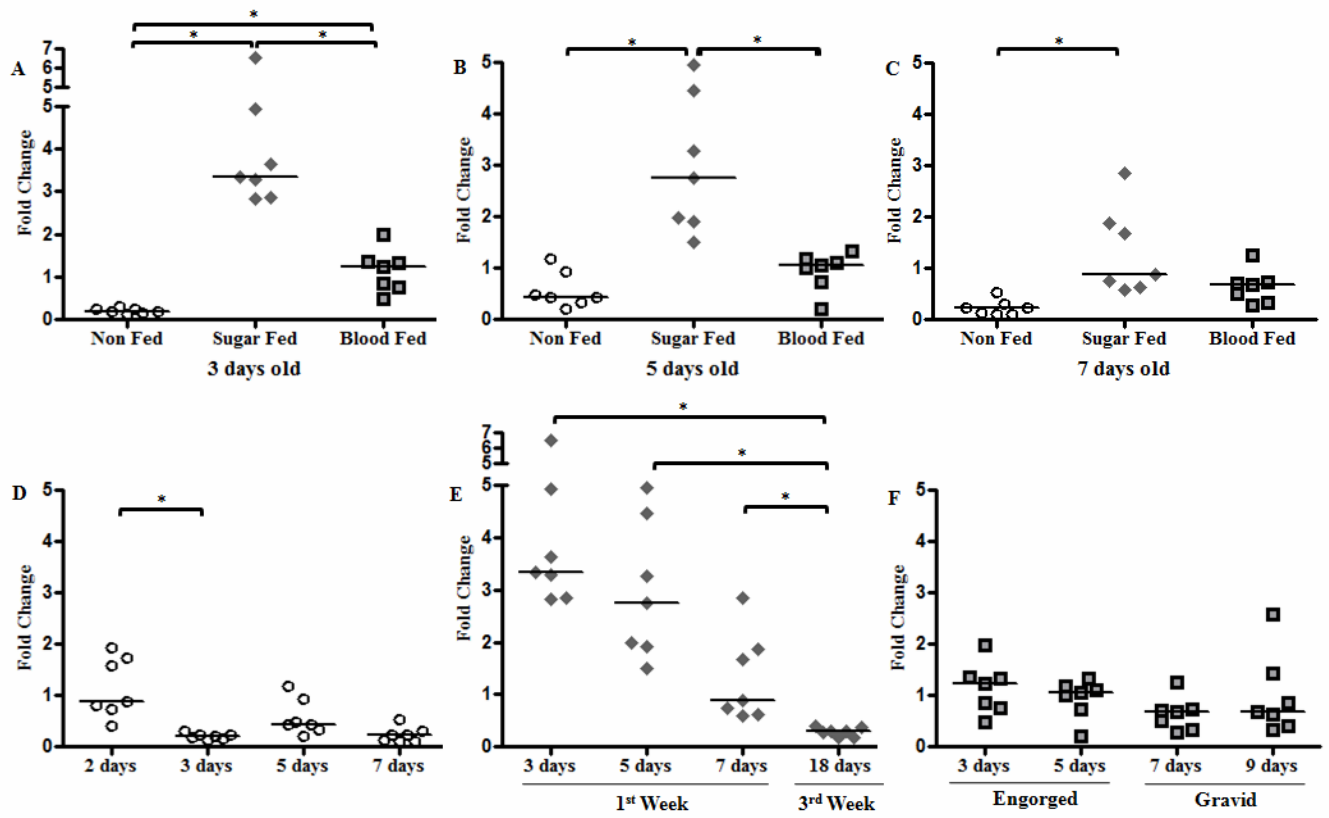

Figure 6.2 *SP44* expression. Expression profiles were assessed as fold changes (Y axis) over expression of non-fed specimens. Diet influences on *SP12* gene expression are displayed in (A), (B) and (C), representing expression patterns in 3 days, 5 days, and 7 days old *P. papatasi* specimens. Graphs (D), (E) and (F) display ageing influences on expression profiles of *P. papatasi* specimens fed on water (non-fed), sugar (sugar-fed), and blood (blood-fed), respectively. Horizontal bars represent the expression median values between the samples and each sample represents an individual fly. Asterisk (*) indicates statistically significant differences between every two groups analyzed. Diet and senescence influences on gene expression were considered statistically significant at $\alpha = 0.0016$ and $\alpha = 0.0008$, respectively. Circles (non-fed), triangles (sugar-fed), and squares (blood-fed) symbol schemes are shown.

Chapter 7 - Summary

Despite the high morbidity and mortality caused by leishmaniasis, no specific control procedures are available to eliminate or reduce *Leishmania* transmission, but insecticides. Significant efforts, on the other hand, have been devoted to developing vaccines based on sand fly-derived proteins. The results obtained in this dissertation represent a first step towards development of a sand fly Transmission Blocking Vaccine (TBV) as well as an advanced analysis regarding the importance of expression polymorphism on the effectiveness of salivary proteins-based vaccines.

The sand fly peritrophic matrix (PM) is a significant barrier against *Leishmania* infection. Hence, the molecular components that participate and the PM organization (i.e., peritrophins) and degradation (i.e., chitinase) may be targeted in TBV strategies against leishmaniasis.

For PpChit1, a *Phlebotomus papatasi* midgut-specific chitinase, the functional characterization of this protein regarding *Le. major* vector competence via RNAi was performed. Intra-thoracic injection of 80.5 ng or 144 ng dsRNA significantly reduced *PpChit1* expression at mRNA levels by 72 % and protein levels by 95 %. In addition, knocking down *PpChit1* led to significant reduction in *Le. major* load in *P. papatasi* midguts at 48 h (46 %) and 144 h (63 %) post-infection, before and after PM break down, respectively. Together, these results suggest that PM becomes thicker upon *PpChit1* knock down, leading to *Leishmania* entrapment and mortality within the endoperitrophic space.

The molecular characterization of three *P. papatasi* peritrophins (PpPer1, PpPer2, and PpPer3), whose transcripts had been previously identified in cDNA libraries, was accomplished along with the functional analysis of PpPer1 on *P. papatasi* vector competence. PpPer1, PpPer2, and PpPer3 exhibit four, one, and two chitin binding domain (CBDs), respectively. PpPer3 also displays a mucin-like domain as well as a putative CBD (Pp3put) at the N-termini. This putative CBD lacks the hydrophobic amino acids at the sites predicted to bind to chitin, suggesting it may have undergone neofunctionalization. Regarding expression profiles, *PpPer1* expression is adult midgut-specific and blood-induced, peaking between 12 h and 72 h PBM. *PpPer2* is constitutively expressed in the midgut, but also is expressed in hindguts and in the L2, L3, and L4 larval stages. Although *PpPer3* is expressed in both sugar and blood fed midguts, expression modulation was detected between 12 h and 72 h PBM. Moreover, *PpPer3* also is expressed in

hindguts and Malpighian tubules. Modulation of peritrophin expression upon *Le. major* infection was also evaluated. Whereas *PpPer1* expression was up-regulated at 24 h post-infection (PI), *PpPer3* expression was down-regulated at 24 h and 48 h PI. *PpPer2* expression was not modulated upon *Le. major* infection. Since *PpPer1* is only expressed in midgut tissues, we assessed its functional role on *Le. major* development in the *P. papatasi* midgut through RNAi. Intra-thoracic injection of 80.5 ng of dsRNA targeting *PpPer1* expression reduced mRNA levels by 45% and protein levels by 44 %. In contrast to *PpChit1*, knocking down of *PpPer1* led to an increase in *Le. major* load by 39 % at 48 h and 22 % at 96 h post-infection. Thus, reduction in PpPer1 likely results in a looser PM, allowing the parasite escape to the ectoperitrophic space sooner, or it may contribute to a greater influx of digestive enzymes in the opposite direction. In the latter case, nutrients would become available earlier, speeding up parasite multiplication.

Our data provided new insights in to the functional role of PM proteins in *P. papatasi* vector competence. While PpPer1 acts as a molecular barrier, possibly impairing parasite escape from the endoperitrophic space, PpChit1 activity assists *Le. major* to avoid entrapment within the PM. Moreover, the latter protein also represents a TBV candidate, as it is expected that anti-*PpChit1* antibodies ingested along with an infected blood meal can neutralize chitinase activity in the sand fly midgut, leading to parasite entrapment and posterior elimination with the fecal pellets.

In addition, we assessed the expression variability of nine salivary gland secreted genes in natural populations of the sand fly *P. papatasi*. Female sand flies were collected in the field in Aswan and North Sinai (Egypt) and Swaymeh (Jordan) during different periods of the sand fly activity season, such as early (June), middle (August), and late in the season (September). Of significance, the expression profile comparisons among specimens collected in the different geographic sites (and at the same period of the season) demonstrated that four genes (*SP29*, *SP32*, *SP36*, and *SP42*) displayed up to eight fold greater expression in specimens collected in Swaymeh than in Aswan late in the season (specimens were not collected in North Sinai in September). When seasonal differences in *P. papatasi* expression profiles were assessed, five genes (*SP12*, *SP29*, *SP36*, *SP42*, and *SP44*) exhibited up-regulation (up to six fold) in Swaymeh-collected specimens late in the season. Thus, strong differences in *P. papatasi* salivary gland gene expression were revealed not only between specimens geographically apart, but also between specimens in the same habitat collected in different periods of the sand fly season.

Whether such differences in the amount of sand fly salivary protein inoculated in the host skin can modulate vaccine-mediated immune protection against *Leishmania* needs to be determined.

Sand fly saliva has immunomodulatory properties that define the outcome of *Leishmania* infection in the mammalian host; thereby, sand fly salivary proteins have been used in the development of protective vaccines against leishmaniasis. Although protection mediated by sand fly saliva-based vaccine has been shown in different animal models, including dogs, variation in salivary gland gene expression in natural populations of sand flies can jeopardize the vaccination process because vaccine protection is dose dependent. Hence, geographic and seasonal variations in sand fly salivary gland gene expression may represent a hurdle for the successful deployment of sand fly salivary protein-based vaccines.

The factors (genetic and/or ecological) modulating the expression of sand fly salivary gland genes in natural habitats have yet to be thoroughly assessed. Nonetheless, the low levels of overall genetic variability among *P. papatasi* populations suggest that genetic factors might have little influences on the differential expression of such genes among different geographic populations. Thereby, ecological differences might play a significant role in fine-tuning *P. papatasi* salivary gland gene expression in specimens collected in different habitats and along the season.

Among the ecological factors that might be influencing the expression levels of *P. papatasi* salivary gland genes are the greater numbers of gravid, engorged, and older females present late in the season in dry habitats, such as Swaymeh. In order to assess if such biotic factor could be modulating the expression of such genes in natural habitats, the expression profiles of the salivary gland genes between colonized specimens in such physiological conditions and sugar fed young ones were compared. As only *SP44* displayed expression profiles modulated by aging and diet, and sugar fed flies exhibited greater *SP44* expression then gravid, engorged, and older ones, other factor must be responsible for the modulation of such genes in natural habitats.

Interestingly, the up-regulation in the expression of *P. papatasi* salivary gland genes in specimens collected late in the season in Swaymeh coincides with the reduced water availability at this period of the season. Such a drought effect late in the season reduces plant photosynthesis and sugar production. Thus, reduced sugar availability might be a significant factor modulating the expression of such genes, yet it has to be experimented.

In all, this dissertation brings significant contributions for the development of sand fly-based vaccines, as PpChit1 was shown to be a feasible target for TBVs. Moreover, the expression polymorphisms of *P. papatasi* salivary gland genes revealed in this study can have a significant impact in the deployment of such vaccines in endemic settings.

Appendix A - Transmission Blocking Vaccines to Control Vector-borne Diseases

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Abstract

Insect-borne diseases are responsible for severe mortality and morbidity worldwide. As control of insect vector populations relies primarily on the use of insecticides, the emergence of insecticide resistance as well to unintended consequences of insecticide use pose significant challenges to their continued application. Novel approaches to reduce pathogen transmission by disease vectors are been attempted, including transmission-blocking vaccines (TBVs) thought to be a feasible strategy to reduce pathogen burden in endemic areas. TBVs aim at preventing the transmission of pathogens from infected to uninfected vertebrate host by targeting molecule(s) expressed on the surface of pathogens during their developmental phase within the insect vector, or by targeting molecules expressed by the vectors. For pathogen-based molecules, the majority of the TBV candidates selected as well as most of the data available regarding the effectiveness of this approach come from studies using malaria parasites. However, TBV candidates also have been identified from midgut tissues of mosquitoes and sand flies. In spite of the successes achieved in the potential application of TBVs against insect-borne diseases, many significant barriers remain. In this review, many of the TBV strategies against insect-borne pathogens and their respective ramification with regards to the immune response of the vertebrate host are discussed.

Key Words: Transmission-blocking Vaccines; TBVs; Vector-borne disease control.

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Introduction

Insect-borne diseases cause about 1.5 million human deaths every year (Hill et al., 2005). Besides mortality, morbidity resulted from infection with such diseases responds for major economic losses mainly in developing countries. The burden of insect borne diseases such as malaria and leishmaniasis in disability adjusted life years (DALY) reach almost 50 million, an indication that current strategies are not effective and new tools are necessary to combat the spread of such diseases (Hill et al., 2005). Among the many different strategies targeting disease vectors or the pathogens they transmit (Valenzuela, 2004, Thomas & Read, 2007, Billingsley et al., 2008, Coutinho-Abreu et al., 2009, Mcmeniman et al., 2009, Oliveira et al., 2009), transmission-blocking vaccines (TBVs) are thought to represent a significant step in that direction, and have now moved from the bench top to clinical trials (Saul et al., 2007, Wu et al., 2008).

TBVs aim at interfering and/or blocking pathogen development within the vector, halting transmission to non-infected vertebrate host (Figure A.1). TBVs usually rely on immunization of vertebrate hosts (either infected or uninfected) with molecules derived from the pathogen or the vector in order to reduce pathogen transmission from infected to uninfected hosts. Such molecules (i.e., antigens) may be inoculated into the vertebrate host as purified proteins inducing the host immune system to produce specific antibodies (Singh & O'hagan, 1999). Alternatively, antibodies can be raised by inoculating the host with recombinant DNA plasmids containing the gene encoding such molecules (Lobo et al., 1999, Coban et al., 2004, Kongkasuriyachai et al., 2004, Leblanc et al., 2008). The expression and secretion of the specific protein into host tissues induce the immune system to produce antibodies against such proteins (Abdulhaqq & Weiner, 2008). To boost the immune response of the vertebrate, antigens are usually inoculated in conjunction with adjuvants. Nevertheless, the mechanisms by which adjuvants improve the immune response are still poorly understood (Singh & O'hagan, 1999, Aguilar & Rodriguez, 2007). The specific antibodies produced against pathogen and/or vector antigens will interfere with the development of the pathogen within the vector following a blood meal on a vaccinated and infected individual.

For a molecule to be an effective TBV candidate certain basic principles must be followed. First, it has to induce high antibody titers in order to block completely pathogen development within the insect (Kubler-Kielb et al., 2007). Additionally, in case the TBV

candidate is presented in an antigen/adjuvant combination, this combination has to be safe enough to the vertebrate host in order to prevent significant side effects following immunization (Saul et al., 2007, Wu et al., 2008). Ideally, a TBV candidate antigen will display low levels of polymorphisms (in field isolates) so that a unique antigen may be used to produce a TBV capable of recognizing all the field variants of that specific antigen (Kocken et al., 1995, Drakeley et al., 1996, Duffy & Kaslow, 1997, Sattabongkot et al., 2003). Alternatively, an effective TBV may need to combine different antigens because the combined action of the antibodies against such antigens may produce a more efficient transmission-blocking result (Duffy & Kaslow, 1997, Gozar et al., 1998, Kongkasuriyachai et al., 2004).

In light of the abundance of data regarding TBVs targeting human parasites, we felt necessary to restrict the discussion on TBVs associated within this subject. Salivary antigen-based vaccines (Valenzuela, 2004, Oliveira et al., 2009) that in spite of the conferred protection do not prevent transmission were not included in this review. Moreover, insecticidal vaccines (Foy et al., 2003) are only briefly mentioned as per their potential to reduce vectorial capacity (Billingsley et al., 2006). These subjects were the recent focus of many review articles (Willadsen, 2004, Billingsley et al., 2006, Titus et al., 2006, Billingsley et al., 2008, Dinglasan & Jacobs-Lorena, 2008, Oliveira et al., 2009). In this review, we limited our discussion to insect-based TBVs, and to those results based on clearly identified target molecule(s).

Transmission-blocking vaccines (TBVs)

Parasite antigen-based TBVs

Most of the studies on TBVs to date were conducted using antibodies targeting antigens expressed on the surface of sexual stage of malaria parasites (Figure A.1; Table A.1). *P. falciparum* proteins Pfs25, Pfs28, Pfs48/45, and Pfs230, and their orthologs in *Plasmodium vivax*, were tested in transmission-blocking assays (Quakyi et al., 1987, Kaslow et al., 1988, Duffy & Kaslow, 1997, Hisaeda et al., 2000, Sattabongkot et al., 2003, Malkin et al., 2005, Outchkourov et al., 2008). Following is a discussion of each one of these *Plasmodium*-derived TBV molecules, different expression systems utilized to produce them and combination with different adjuvants.

***P. falciparum*-derived TBV candidate – Pfs25**

Pfs25 is a 25 kDa protein expressed on the surface of zygote and ookinete stages of *P. falciparum* and consists of four tandem epidermal growth factor (EGF) domains (Kaslow et al., 1988). The TBV potential of Pfs25 was demonstrated using the Vaccinia virus as delivery systems of this antigen to mammalian hosts (Kaslow et al., 1991), or using recombinant Pfs25 expressed in yeast (Barr et al., 1991, Kaslow et al., 1991). With the Vaccinia virus system, infectivity of *Anopheles freeborni* with *P. falciparum* following an infection on artificial blood meal was reduced to 40 % when 25 μg/ml of Pfs25 monoclonal antibodies was added to the blood meal. Infectivity was almost eliminated when the concentration of monoclonal antibodies was increased to 200 μg/ml, and transmission blocking activity was complete when polyclonal antibodies were used (Kaslow et al., 1991). A recombinant Pfs25 expressed in yeast (Pfs25-B) also exhibited blocking activity. Sera from mice injected with Pfs-25-B in the presence of adjuvants, such as Freund's or muramyl tripeptide (MTP-MF59), reduced *P. falciparum* infection of *An. freeborni* to less than one percent of the mosquitoes tested. Likewise, mosquitoes fed on blood of *Aotus trivigatus* monkeys infected with *P. falciparum* and immunized with Pfs25-B in combination with MTP-MF59 adjuvant exhibited infectivity of less than 10 % (Barr et al., 1991).

As a monomer, Pfs25 was shown to be poorly immunogenic (Kubler-Kielb et al., 2007), and different forms of this protein, including various Pfs25 dimers (obtained from single or two-step linkage conjugations) or Pfs25 linked to immunogenic domains (such as OVA and the *Pseudomonas aeruginosa* recombinant exoprotein A), were tested for higher induction of antibody titers. Two of the Pfs25 dimers obtained via two-step conjugation of amide linkages displayed the greatest immunogenicity, which was further increased when injected with alum (Kubler-Kielb et al., 2007).

Nasal immunization with Pfs25 in the murine model also was tested (Arakawa et al., 2005). Pfs25 expressed in yeast (*Pichia pastoris*) was administered intranasally with a cholera toxin adjuvant in mice. This route of immunization induced both mucosal and systemic antibodies that were capable of eliminating *P. falciparum* oocyst emergence in *Anopheles dirus* (Table A.1) (Arakawa et al., 2005) .

A phase I (human safety assessment) trial of *Pichia pastoris*-expressed Pfs25 antigen using Montanide ISA 51 adjuvant was carried out (Wu et al., 2008). Although anti-Pfs25 human

serum inhibited *P. falciparum* oocyst intensity in *An. stephensi* by more than 90 %, reactogenicity (local and systemic) in human volunteers prevented Montanide ISA 51 to be used as an adjuvant with Pfs25 (Wu et al., 2008).

TBV potential of DNA-based Pfs25 vaccines also have been assessed in murine (Lobo et al., 1999) and non-human primate models (Coban et al., 2004). For both animal models, vaccination was carried out using plasmids encoding Pfs25 or Pfg27, or a combination of plasmids expressing either one of these proteins, or expressing a chimeric Pfs25-Pfg27 protein. Pfg27 antigen is mainly expressed on the surface of *P. falciparum* gametocytes (Lobo et al., 1999, Coban et al., 2004). In the mouse model, DNA immunization with the plasmids encoding Pfs25 alone or in combination with Pfg27-encoding plasmids induced high antibody titers after only two inoculations without adjuvants (Lobo et al., 1999). Injection of plasmids encoding Pfs25 alone displayed the greatest TBV activity (oocyst infectivity reduction: 96.2 % to 96.6 %), followed by co-immunization with plasmids encoding Pfs25 or Pfg27 (oocyst infectivity reduction: 94.8 % to 96.4 %). Immunization with plasmids expressing Pfg27 alone did not induce effective TBV potential (Lobo et al., 1999). In contrast, immunization of Rhesus monkeys (*Macaca mulatta*) with plasmids expressing Pfs25 or a chimeric Pfs25-Pfg27 protein did not stimulate the production of high antibody titers, even after four inoculations, and was incapable of inhibiting *P. falciparum* development in *An. stephensi* (Coban et al., 2004). However, subsequent single immunization with the yeast-expressed Pfs25 protein, along with Montanide ISA 720 adjuvant, significantly reduced *P. falciparum* oocyst numbers by up to 95 % in *An. stephensi* (Coban et al., 2004). Heterologous boosting with recombinant Pfs25 did not induce TBV activity in the immunization with plasmids expressing the chimeric Pfs25-Pfg27 (Coban et al., 2004). Therefore, heterologous boosting of Pfs25 DNA-based TBV vaccine with a recombinant protein plus adjuvant seems to be a reliable option for a phase 1 trial as this vaccination strategy induced strong reduction of *P. falciparum* development in mosquitoes as well as was unable to affect the health of non-human primates (Coban et al., 2004).

An attempt to boost the immunogenicity of a Pfs25-based DNA vaccine was performed via *in vivo* electroporation (Leblanc et al., 2008). This method elicited similar anti-Pfs25 antibody titers in mice as immunization carried out with 100 fold more plasmid without electroporation. Additionally, membrane feeding assays using either serum, derived from Pfs25-DNA plasmid immunization after electroporation or from Pfs25-DNA plasmid (100 fold

concentrated) immunization without electroporation, induced similar *P. falciparum* oocyst intensity reduction in *An. gambiae* (approximately 85%). *In vivo* electroporation of Pfs-25 encoding plasmids could be tested to boost the immune response of TBVs in non-human primates and eventually in phase 1 trials (Leblanc et al., 2008).

P. falciparum-derived TBV candidate – Pfs28

Pfs28 is a 28 kDa *P. falciparum* conserved protein expressed on the surface of retorts, a transitional stage between zygote and ookinete. This antigen also was tested in transmission blocking activity assays. Antibodies produced by the injection of yeast-expressed Pfs28 (yPfs28), in the presence of alum, significantly reduced the infectivity of *An. freeborni* mosquitoes with *P. falciparum*. Lower infectivity was exhibited when vaccination was carried out with yPfs28 and yPfs25 antigens injected together (Duffy & Kaslow, 1997).

Transmission blocking activity against *P. falciparum* was further improved when Pfs25 and Pfs28 were expressed as a unique chimeric protein in yeast, the 25-28c recombinant protein. Vaccination with the 25-28c recombinant protein led to complete arrest of oocyst development earlier, using a lower dose and for a greater amount of time, than vaccination with either Pfs25 or Pfs28 alone or a combination of both (Gozar et al., 1998).

P. falciparum-derived TBV candidate – Pfs48/45

Another TBV candidate to control spread of *P. falciparum* is Pfs48/45. The *Pfs48/45* gene encodes a unique protein that migrates as a double band under non-reducing conditions (Milek et al., 2000). This protein is expressed on *P. falciparum* gametocyte and gamete surfaces and has a central role in male gamete fertility (Van Dijk et al., 2001). Attempts to express this protein in its native form using different systems, such as bacteria (Milek et al., 1998b), Vaccinia virus (Milek et al., 1998a), and yeast (Milek et al., 2000) have failed due to improper folding. However, expression of a truncated form of Pfs48/45 (Pfs48/45-10C) in *E. coli* followed by steps of chemical-induced refolding produced 10-20 % of correctly folded Pfs48/45-10C (Outchkourov et al., 2007). Mice polyclonal antibodies produced against Pfs48/45-10C recombinant protein (in the presence of Freund's adjuvant) reduced the intensity of *P. falciparum* oocysts in *An. stephensi* by more than 15 fold (Outchkourov et al., 2007). Additionally, antibody titers were sufficient to induce transmission blocking effects (Outchkourov et al., 2007). Much improved refolding of the truncated Pfs48/45-10C was obtained through co-expression of *E. coli*

chaperones (i.e., 90 % of the recombinant protein was properly folded and stable) (Outchkourov et al., 2008). Immunization of mice with this recombinant protein led to production of antibody titers that were capable of reducing *P. falciparum* oocyst intensity in *An. stephensi* by at least 88 % in 11 out of 12 assays (Outchkourov et al., 2008).

Furthermore, with regards to the application of Pfs48/45 as a potential TBV against malaria, the variability of Pfs48/45 from culture and field isolates from many countries was analyzed (Kocken et al., 1995, Drakeley et al., 1996). The results obtained indicated low levels of polymorphism in the overall gene among either *in vitro* cultures or field isolates (Kocken et al., 1995, Drakeley et al., 1996).

P. falciparum-derived TBV candidate – Pfs230

Another *P. falciparum* protein tested in TBV assays was Pfs230, a 230 kDa protein expressed on the surface of gametocytes. Although antibodies against Pfs230 blocked the development of *P. falciparum* in the midguts of *An. freeborni*, the transmission blocking activity of anti-Pfs230 monoclonal antibodies was completely lost when complement was inactivated. Thus, the blocking activity of anti-Pfs-230 antibodies was detected only when complement proteins were present (Quakyi et al., 1987).

P. vivax-derived TBV candidates – Pvs25 and Pvs28

P. vivax sexual stage surface proteins, orthologs of *P. falciparum* TBV candidates, also have been isolated and tested in transmission blocking experiments. Pvs25, a Pfs25 ortholog, is expressed on the surfaces of the insect-stages, zygotes and mature ookinetes, whereas Pvs28, a Pfs28 ortholog, is mainly expressed on retorts and mature ookinetes (Hisaeda et al., 2000).

Transmission blocking experiments using antibodies against either Pvs25 or Pvs28 were tested (Hisaeda et al., 2000). Four species of mosquitoes were artificially fed on a mixture of *P. vivax*-infected chimpanzee blood in the presence of antibodies (raised in mice co-injected with alum). *P. vivax* ookinete development was completely blocked by the anti-serum against Pvs25 (Hisaeda et al., 2000). Vaccination against Pvs25 and Pvs28 also presented efficient transmission blocking activity against *P. vivax* isolated from human patients, despite polymorphism in these proteins (Sattabongkot et al., 2003).

Sera of *Aotus lemurinus griseimembra* monkeys immunized with Pvs25 and Montanide ISA 720 adjuvant also was used in a TBV assay (Arevalo-Herrera et al., 2005). These sera were

capable of blocking human-derived *P. vivax* oocyst intensity in *A. albimanus* by more than 98 %, reaching complete oocyst inhibition in most of the replicates (Arevalo-Herrera et al., 2005).

Similar to the transmission blocking activity of the *Aotus* anti-Pvs25 sera observed previously (Arevalo-Herrera et al., 2005), TBV activity of anti-Pvs25 sera raised in rhesus monkeys (*Macaca mulatta*) also was assessed (Collins et al., 2006). The boosting effect of two adjuvants (alum and Montanide ISA 720) was compared. Immunization with a combination Pvs25 and Montanide ISA 720 induced a stronger antibody response than Pvs25 and alum in rhesus monkeys, reaching 100 % reduction of oocyst intensity in *An. freeborni*, 204 days after immunization (Collins et al., 2006).

Transmission blocking activity of Pvs25 has been evaluated in phase 1 human trials. Antibodies to Pvs25 were raised in healthy human volunteers using Alhydrogel® (aluminium hydroxide gel) as adjuvant, and the sera was mixed with the *P. vivax*-infected blood taken from infected patients from an endemic region. This mixture of human infected blood and human anti-Pvs25 serum was used to artificially feed *An. dirus*. The results from the study revealed significant interference in *P. vivax* development within mosquito midgut caused by the human anti-Pvs25 sera. Additionally, long lasting antibody titers were elevated and no reactogenicity (side effects) was observed (Malkin et al., 2005). Nevertheless, higher antibody titers are necessary for successful control of *P. falciparum* transmission by mosquitoes in endemic areas (Malkin et al., 2005). A second phase 1 trial, also using Pvs25 as a potential TBV was carried out using Montanide ISA 51 as an adjuvant. Due to induced local and systemic reactions in the vacinees, use of this antigen-adjuvant combination in humans was halted (Wu et al., 2008).

Immunogenicity of Pvs25 and Pvs28 DNA-based TBVs was also assessed in a murine model (Kongkasuriyachai et al., 2004). Many immunization schemes, including Pvs25 or Pvs28 plasmids alone, in combination, boosted with the recombinant Pvs25 or Pvs28 proteins plus aluminium hydroxide adjuvant, or a combination of plasmids encoding Pvs25 and Pfs25, were carried out. Overall, all these schemes efficiently elicited comparable levels of antibodies and inhibited *P. vivax* development in *An. freeborni* and *An. gambiae* (at least 74 % oocyst intensity reduction) (Kongkasuriyachai et al., 2004).

Other pathogen molecule-based TBV candidates

In regard to proteins expressed on the surface of parasites (other than *Plasmodium*) transmitted to humans by insect vectors (Table A.1), only a limited number has been tested as potential TBVs (Tonui et al., 2001a, Saraiva et al., 2006).

In *Leishmania major*, the two most abundant surface antigens, LPG and gp63, were tested as transmission blocking vaccines. *Phlebotomus dubosqci* sand flies were partially fed on mice immunized with purified native LPG, recombinant gp63 (rgp63) expressed in bacteria, crude *L. major* lysate (WPA), or a cocktail of LPG and rgp63. The sand flies were subsequently fed on *L. major*-infected mice. The results indicated that serum against WPA and the two protein-cocktail exhibited greater *L. major* blocking activity than sera against either LPG or gp63 (Tonui et al., 2001a). However, blocking of *L. major* development was due to damage of the midgut epithelial layer, probably caused by immune-active substances present in the blood of the pre-vaccinated mice (Tonui et al., 2001b).

Interestingly, a commercially available treatment for canine visceral leishmaniasis (Leishmune®) was recently shown to function as a TBV in sand flies (Saraiva et al., 2006). Leishmune® (FML-vaccine) is a protective vaccine made of *L. donovani* fucose-mannose ligand and the adjuvant saponin, which was successfully tested in a phase III vaccine trial (Da Silva et al., 2000). Although the surface molecule (FML) was isolated from *L. donovani*, Leishmune® exhibited transmission blocking activity in the New World sand fly *Lutzomyia longipalpis* when infected with *Leishmania infantum chagasi* (Saraiva et al., 2006). Antibodies produced in dogs following Leishmune® injection reduced *Lu. longipalpis* infectivity by 79.3 % and parasite load by 74.3 % even after 12 months of immunization (Saraiva et al., 2006).

Insect-based TBVs

Proteins expressed within insect vector tissues and that may interact with pathogens also have been tested as TBV candidates (Table A.2). Insect-based TBV candidates include (structural) proteins that are expressed by the insect midgut (Lal et al., 2001), midgut enzymes that play a role in blood digestion (Lavazec et al., 2007), and parasite receptors expressed by the epithelial cells lining the midgut (Kamhawi et al., 2004, Dinglasan et al., 2007)

In mosquitoes, polyclonal antibodies against *An. gambiae* midgut proteins nearly completely reduced the intensity of *P. falciparum* oocysts (98 %) and sporozoites (96 %) within

An. stephensi tissues. Also, *An. gambiae*-derived anti-midgut monoclonal antibodies inhibited development of *P. falciparum* and *P. vivax* in different *Anopheles* species (Lal et al., 2001). Additionally, these antibodies also can be used to reduce insect vector densities (vector-blocking vaccines) because they reduce vector survivorship and fecundity (Lal et al., 2001). Antibodies against carboxypeptidase cpbAg1 from *An. gambiae* reduced *P. falciparum* infectivity by more than 92% seven days after an infectious artificial blood feeding (Lavazec et al., 2007). In addition to the effect on the number of oocysts per infected mosquito, anti-cpbAg1 strongly reduced mosquito progeny (Lavazec et al., 2007). Antibodies to a midgut aminopeptidase (AgAPN1), which is one of the *P. falciparum* receptors in the *An. gambiae* midgut, were used to reduce *P. falciparum* oocyst intensity in *An. gambiae* and *An. stephensi* by 73 % and 67 %, respectively (Dinglasan et al., 2007).

Sand fly-based TBV have also been suggested as a potential strategy against *Leishmania* transmission, especially in those situations where the disease displays an anthroponotic transmission profile, or where domestic dogs play a critical role as reservoirs of the parasite. The utilization of sand fly molecules as TBVs was contemplated following the characterization of PpGalec, a galectin (galactose-binding) -like protein. PpGalec is a expressed on the surface of the midgut cells in *Phlebotomus papatasi* and serve as a receptor for *L. major* lipophosphoglycan (LPG) attachment (Kamhawi et al., 2004). Interestingly, *P. papatasi* fed artificially on mouse blood containing *L. major* amastigotes and mixed with sera from PpGalec-immunized mice displayed a reduction of 86 % on the levels of *L. major* infection within the sand fly midgut. Moreover, no infectious metacyclic forms were detected from the flies fed on anti-PpGalec sera (Kamhawi et al., 2004).

Although this study clearly demonstrated that using antisera it is possible to block a parasite receptor within the midgut of its vector, the issue here is that PpGalec is present within a vector that is associated with zoonotic disease. To succeed against the transmission of a human parasite, a TBV molecule has to function more broadly, in vector-parasite pairs that are meaningful to human disease.

In a similar approach, anti-sera raised against another sand fly molecule, PpChit1, also characterized from *P. papatasi* (subgenus *Phlebotomus*) inhibited the chitinolytic activity of the midgut of other sand fly species *in vitro*, including *P. duboscqi* (also subgenus Phlebotomus), but even from a sand fly (*P. argentipes*) of a distant subgenus (*Euphlebotomus*) (Ramalho-Ortigao et

al., 2005). These data suggested a significant cross-species activity, which is what is at stake here. Thus, using a model disease vector such as *P. papatasi* for investigations related to the identification of candidate TBVs will provide significant clues to molecules that display a broad spectrum of activity, have counterparts (orthologs or paralogs) in other sand fly vectors, and are potentially directly associated with the development of *Leishmania*. Thus, when such molecules are targeted, via antibodies or other strategies, it will lead to the disruption of the development of the parasite and its transmission to humans and domestic animals.

Challenges and perspectives on TBVs

In addition to identifying TBV candidates that are effective and may span different insect vector species, challenges to TBV development also encompass antigenic variability present in field isolates (Kocken et al., 1995, Drakeley et al., 1996, Duffy & Kaslow, 1997, Sattabongkot et al., 2003), immunogenicity of such antigens (Kubler-Kielb et al., 2007), reactogenicity caused by adjuvants (Saul et al., 2007, Wu et al., 2008), non-specific responses (Quakyi et al., 1987, Tonui et al., 2001a), and improper folding of antigens (Kaslow et al., 1994, Milek et al., 1998a, Milek et al., 1998b, Milek et al., 2000). Natural antigenic boosting is another important issue that must be dealt with (Arevalo-Herrera et al., 2005).

Antigens expressed on the surface of insect-stage parasites have been postulated as TBV candidates because they seem not to be under the selective pressure mediated by the vertebrate immune system. Consequently, these proteins could exhibit low levels of polymorphisms, favoring the efficacy of vaccines derived from a unique antigenic variant (Sattabongkot et al., 2003). Even though Pvs25, Pvs28, and Pfs48/45 proteins from different *Plasmodium* field isolates are polymorphic antigens, the amount of variation of these antigens does not preclude their use as TBVs (Drakeley et al., 1996, Duffy & Kaslow, 1997, Sattabongkot et al., 2003). On the other hand, antigenic variability of vector TBV candidates has not yet been assessed.

Another challenge to the development of TBVs is related to the strength of the immune response mediated by the TBV candidate. For example, Pfs25 does not stimulate a strong immune response to generate antibody titers that could reduce parasite development efficiently in mosquito field populations (Kubler-Kielb et al., 2007), nor does Pvs25 administered with Alhydrogel® (Malkin et al., 2005). In order to booster the immunogenicity of Pvs25, a different adjuvant (Montanide ISA 51) has been tested (Wu et al., 2008). Similarly, immunogenicity of

Pfs25 has been improved by modifications of the 3D-structure of this protein (Kubler-Kielb et al., 2007) as well as by the use of different adjuvants (Barr et al., 1991, Kaslow et al., 1994).

An alternate strategy to boost the immune response of TBV antigens was assessed through DNA-based TBV immunization (Lobo et al., 1999, Coban et al., 2004, Kongkasuriyachai et al., 2004, Leblanc et al., 2008). Besides the lower cost and stability of these vaccines (Leblanc et al., 2008), DNA-based TBVs alone or boosted with recombinant proteins elicit high antibody titers (Lobo et al., 1999, Coban et al., 2004, Kongkasuriyachai et al., 2004). These vaccines were used to block transmission of malaria parasites, and have demonstrated safety when used in non-human primates (Lobo et al., 1999, Coban et al., 2004, Kongkasuriyachai et al., 2004). Electroporation of DNA-based TBVs enhanced antibody production in mice and also are considered an alternative to boosting antibody response to DNA vaccines in larger animals (Leblanc et al., 2008).

The use of adjuvants to boost TBV antigen response is a significant issue to be resolved, as certain types of adjuvants induce reactogenicity in humans (Wu et al., 2008) and animal models (Saul et al., 2007). In humans, a combination of Pvs25 and Alhydrogel® was shown to be safe in a phase 1 trail (Malkin et al., 2005). In contrast, Pvs25 and Montanide ISA 51 caused local and systemic adverse effects (Wu et al., 2008). In Rhesus monkeys, while Pvs25-H Montanide ISA 720 immunization induced higher levels (10 fold) of antibodies than the Pvs25-Alhydrogel® (aluminium hydroxide gel) combination, it also led to local reactogenicity (Saul et al., 2007). In a subsequent experiment, the immunization of monkeys with similar or lower doses of Pfs25/Montanide ISA 720 did not induce local reactogenicity or stimulated similar antibody levels following a second round of injection with a dose as high as that in the first experiment (Saul et al., 2007). A qualitative difference in immune response between the first and second boosting may be responsible for the reactogenic reactions caused by Pfs25/Montanide ISA 720. As suggested by Saul et al (Saul et al., 2007), this may be prevented by an immunization scheme using a small priming dose followed by a second dose several months later (Saul et al., 2007). Therefore, combinations of TBV candidate antigens and adjuvants need to be thoroughly tested in order to define the specific dose to be administered, and using the safest antigen-adjuvant combination.

Another interesting aspect of TBVs is the possibility of natural boosting of the immune response of animals infected with a pathogen (i.e., pre-immunized) (Milek et al., 1998a, Arevalo-

Herrera et al., 2005). Hence, candidate TBV proteins expressed on the surface of both insect-stage and blood-stage pathogens may induce activation of the immune response in infected hosts vaccinated with the same antigens (Arevalo-Herrera et al., 2005). However, this approach may not be suitable to every TBV, such as Pvs25 which displays low expression in blood-stage *P. vivax* (Arevalo-Herrera et al., 2005), and has yet to be demonstrated for the *Plasmodium* TBV-antigen candidates that are expressed during gametocytogenesis, for example, Pfs230 (Quakyi et al., 1987) and Pfs48/45 (Milek et al., 1998a).

Proper folding of the TBV candidate protein following expression via recombinant techniques also may affect the efficacy of the vaccinating antigen. As indicated above, expression of Pfs25 in prokaryotic systems resulted in improper folded proteins (Kaslow et al., 1994), which was solved by expression in yeast (Kaslow et al., 1994) or in Vaccinia virus-infected mammalian cells (Kaslow et al., 1991). Conversely, Pfs48/45 expressed in bacteria (Milek et al., 1998b), Vaccinia virus-infected mammalian cells (Milek et al., 1998a), or yeast (Milek et al., 2000) resulted in the production of denatured antigens, improper for host immunization. Properly folded Pfs48/45 was recently obtained by chemically induced re-folding of an *E. coli*-expressed truncated Pfs48/45 (Outchkourov et al., 2007) and through co-expression of this antigen with *E. coli* chaperons (Outchkourov et al., 2008). Thus, the system of choice for recombinant expression can significantly affect the outcome of the TBV candidate.

Future directions on TBV research

The low antibody-responses induced by immunization with *Plasmodium*-surface proteins as well as the reactogenicity caused by some adjuvants seem to be the main constraints on the development of anti-malaria TBVs (Kubler-Kielb et al., 2007, Saul et al., 2007, Wu et al., 2008). Although antigen engineering has been carried out in order to improve antibody-response (Kubler-Kielb et al., 2007), and some immunization routines appear to reduce side effects (Saul et al., 2007), human-safe adjuvants that induce strong antibody responses still need to be developed for TBV antigens to induce sufficient antibody titers to reduce transmission in endemic areas. With regards to other pathogens besides malaria parasites, assessment of potential TBV candidate antigens has yet to be undertaken.

In regards to insect-based TBV candidate molecules, the number of TBV antigens available is still reduced and needs to be extended to target other vector species. In addition to

assessing a TBV candidate molecule that prevents pathogen development within insect vector tissues, an effect on the vector survivorship is also one of the main objectives.

Reduction of vector survival is thought to interfere exponentially with vectorial capacity (Black Iv & Moore, 2004, Billingsley et al., 2006, Billingsley et al., 2008), as the time available for pathogen development within the vector is significantly shortened. Despite several studies showing that insect feeding on blood of animals immunized with insect tissue homogenates exhibit reduced survivorship, most of these studies suffered from high experimental variability (Billingsley et al., 2006). However, one study has shown that immunization with a unique insect molecule (mucin) can induce an immune response capable of killing insect vectors via a cell-mediated response (Foy et al., 2003). Thus, an ideal TBV antigen should reduce parasite development, reducing vector competence (a linear parameter in the vectorial capacity equation), as well as vector survivorship (the exponential parameter). These two effects associated can lead to thorough reduction of vectorial capacity and disease burden in endemic areas.

TBV could also be able to reduce survivorship of different species of insect vectors, via immunization with conserved antigens, as proposed by Canales *et al* (Canales et al., 2009), providing protection to pathogens transmitted by different vectors. However, significant cross-species effects have yet to be demonstrated.

Conclusion

Significant funding and research efforts are currently been invested to develop novel mechanisms to control insect-borne diseases. Among the various strategies investigated, TBVs may soon be applied towards the control of vector borne diseases, especially malaria and leishmaniasis. This assumption is supported by at least two recent developments, the first being the approval of the *P. vivax* Pvs25 antigen as TBV during a phase I human trial (Malkin et al., 2005, Saraiva et al., 2006). Another is the use of the fucose-mannose ligand from *L. donovani* as a vaccine against canine visceral leishmaniasis following a phase III trial for animal application (Da Silva et al., 2000, Saraiva et al., 2006). It turns out, unexpectedly, Leishmune^{TM} function as a TBV preventing the development of *L. infantum chagasi* in its natural vector the sand fly *L. longipalpis* (Malkin et al., 2005, Saraiva et al., 2006).

Currently, many laboratories are investigating several leads that potentially can be applied as TBV to prevent insect-transmitted pathogens. However the outcome of the research

with current TBV candidates, it is clear to investigators that novel strategies are needed in order to reduce the current burden of vector-borne diseases in general.

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Table A.1 Transmission blocking vaccines based on pathogens molecules

TBV antigen (antigen origin)	Antigen production	Antigen name	Vaccinated animal (adjuvant)	Sera dilution (concentration)	Targeted pathogen	Insect vector	Mean oocyst number	Infectivity %	Reference
Pfs25 (*P. falciparum*)	Virus (Vaccinia virus)	rPfs25^	Mouse (Ribi)	1:2 (200 µg/ml) 1:4 (100 µg/ml) 1:8 (50 µg/ml) 1:16 (25 µg/ml)	*P. falciparum*	*An. freeborni*	0.1 1 1.4 3.5	1 % 11 % 16 % 39 %	(Kaslow et al., 1991)
	Yeast (*S. cerevisiae*)	Pfs25-B	Mouse (FCA[1]) (MTP*****)	1:2 1:2	*P. falciparum*	*An. freeborni*	0 0	<1 % 0 %	(Barr et al., 1991)
			Monkey[@] (MTP*)	1:2			0-2.6[#]	0-10 %	
	Yeast (*S. cerevisiae*)	Pfs25-B	Mouse (Alum)	Neat	*P. falciparum*	*An. freeborni*	0	0 %	(Kaslow et al., 1994)
			Monkey[@] (Alum)	Neat			0-3.8[&] 0-12.8[$] 19.9-28.2[$$]	- - -	
	DNA vaccination	VR1020/25	Mouse	1:5 1:10	*P. falciparum*	*An. stephensi*	0.17-0.26 0.19-0.39	3.4-3.8 % 2.6-4.3 %	(Lobo et al., 1999)
	DNA vaccination/ Yeast (*S. cerevisiae*)	VR1020/25 and Pfs25	Monkey[<] (Montanide ISA 720)	1:2 1:4 1:8	*P. falciparum*	*An. stephensi* *An. gambiae*[?]	0.9-3.1 2.4-6.4 3.2-10.4	5-17 % 14-38 % 19-62 %	(Coban et al., 2004)
	Yeast (*P. pastoris*)	Pfs25	Mouse (cholera toxin)	1:2 1:8 1:32	*P. falciparum*	*An. dirus*	0 0 10	0 % 0 % 21.3 %	(Arakawa et al., 2005)
	Yeast (*P. pastoris*)	Pfs25	Human (Montanide ISA 51)	-	*P. falciparum*	*An. stephensi*	-	<10 %	(Wu et al., 2008)
	DNA vaccination (*in vivo electroporation*)	Pfs25	Mouse	1:2[>] 1:4[>] 1:8[>] 1:16[>]	*P. falciparum*	*An. gambiae*	1.0 3.4 9.5 51.9	2.5 % 9 % 24 % 100 %	(Leblanc et al., 2008)

TBV antigen (antigen origin)	Antigen production	Antigen name	Vaccinated animal (adjuvant)	Sera dilution (concentration)	Targeted pathogen	Insect vector	Mean oocyst number	Infectivity %	Reference
Pfs28 (*P. falciparum*)	Yeast (*S. cerevisiae*)	yPfs28	- (FCA+Ribi) (Alum)	Neat[!!]	*P. falciparum*	*An. freeborni*	0-0.33[&] 0.21[&]	0-8 % 3 %	(Duffy & Kaslow, 1997)
		yPfs25+yPfs 28[+]	- (FCA+Ribi)	1:40			0.047-0.16	7.6-9 %	
Pfs25-Pfs28 (*P. falciparum*)	Yeast (*S. cerevisiae*)	25-28C	Mouse (Alum)	Neat[!!]	*P. falciparum*	*An. freeborni*	0	0 %	(Gozar et al., 1998)
Pfs45/48 (*P. falciparum*)	Bacteria (refolded *in vitro*)	Pfs45/48-10C	Mouse (FCA[!])	1:2 (10 µg/ml)	*P. falciparum*	*An. stephensi*	0.45	0.06 %	(Outchkourov et al., 2007)
	Bacteria (w/chaperonins)	Pfs45/48-10C	Mouse (FCA[!])	1:2 (10 µg/ml)	*P. falciparum*	*An. stephensi*	0-5.1	0-12 %	(Outchkourov et al., 2008)
Pfs230 (*P. falciparum*)	Purification	Pfs230^	Rabbit (FCA[!])	- (100 µg/ml)	*P. falciparum*	*An. freeborni*	0.2-4	0.3-5.8 %	(Quakyi et al., 1987)
Pvs25 (*P. vivax*)	Yeast (*S. cerevisiae*)	Pvs25	Mouse (Alum)	1:2	*P. vivax*	*An. stephensi*	0	0 %	(Hisaeda et al., 2000)
	Yeast (-)	Pvs25	Mouse (Alum) Rabbit (Alum)	1:2 1:8 1:2 1:8	*P. vivax*	*An. dirus*	0.18 1.26 4.25 4.06	- - - -	(Sattabongkot et al., 2003)
	DNA vaccination	DV25	Mouse	1:8 1:10	*P. vivax*	*An. freeborni*	3.17 0.4	86 % 87 %	(Kongkasuriyachai et al., 2004)
		DV28		1:10 1:10		*An. freeborni* *An. gambiae*	0.4 1.5	87 % 86 %	
		DV25+DV28		1:8 1:10 1:10		*An. freeborni* *An. gambiae*	0.8 0.04 0.8	93 % 99 % 93 %	
	Yeast (*S. cerevisiae*)	Pvs25	Monkey[1] (Montanide ISA 720)	1:4	*P. vivax*	*An. albimanus*	0.0-0.04	-	(Arevalo-Herrera et al., 2005)
	Yeast (*S. cerevisiae*)	Pvs25H	Human (Alhydrogel®)	Neat[!!] 1:1	*P. vivax*	*An dirus*	- -	- -	(Malkin et al., 2005)

TBV antigen (antigen origin)	Antigen production	Antigen name	Vaccinated animal	Sera dilution (concentration)	Targeted pathogen	Insect vector	Mean oocyst number	Infectivity %	Reference
Pvs25 (*P. vivax*)	Yeast (*S. cerevisiae*)	Pvs25	Human (Montanide ISA 51)	-	-	-	-	-	(Wu et al., 2008)
	Yeast (*S. cerevisiae*)	Pvs25	Monkey[<] (Montanide ISA 720)	1:2	*P. vivax*	*An. freeborni*	0^{++}	0 %	(Collins et al., 2006)
				1:8			1.41	1.6 %	
				1:32			1.3	32.8 %	
			(Alum)	1:2			1.71	2.9 %	
				1:8			1.3	10.6 %	
				1:32			2.05	34.2 %	
Pvs28 (*P. vivax*)	Yeast (*S. cerevisiae*)	Pvs28	Mouse (Alum)	1:2	*P. vivax*	*An. freeborni*	0.91	0.7 %	(Hisaeda et al., 2000)
	Yeast (*S. cerevisiae*)	Pvs28	Mouse (Alum)	1:2	*P. vivax*	*An. dirus*	0.11	-	(Sattabongkot et al., 2003)
				1:8			1.31	-	
			Rabbit (Alum)	1:2			10.73	-	
				1:8			4.79	-	
WPA (*L. major*)	Purified	Whole cell lysate	Mouse	-	*L. major*	*P. duboscqi*	-	25 %	(Tonui et al., 2001a)
rgp63 (*L. major*)	Bacteria	gp63	Mouse	-	*L. major*	*P. duboscqi*	-	40 %	(Tonui et al., 2001a)
LPG (*L. major*)	Purified	LPG	Mouse	-	*L. major*	*P. duboscqi*	-	43.3 %	(Tonui et al., 2001a)
LPG+rgp63 (*L. major*)	Purified + Bacteria	LPG+rgp63	Mouse	-	*L. major*	*P. duboscqi*	-	37.5 %	(Tonui et al., 2001a)
Leishmune® (*L. donovani*)	Purification	FML	Dog (Saponin)	1:1	*L. major*	*L. longipalpis*	-	30.6 %	(Saraiva et al., 2006)

(^) Monoclonal antibodies were used in transmission blocking assays.

(!) Freund's complete adjuvant.

(*) Muramyl tripeptide.

(@) *Aotus trivirgatus*.

(#) Oocysts present in midguts of mosquitoes that fed on sera from monkeys immunized 22 weeks before challenge. No oocysts were present in mosquitoes that fed on sera from animals immunized 12 weeks before challenge.

(-) Undetermined.

(&) Seven days after 3rd immunization.

($) Sixty one days after 3rd immunization.

($$) Eighty nine days after 3rd immunization.

(<) *Macaca mulatta.*

(?) Similar results were observed using *An. Stephensi.*

(>) These results correspond to immunization with 20 μg of plasmid.

(+) Sera diluted 1:40.

(++) TBV assayed 204 days after immunization.

(!) *Aotus lemurinus griseimembra.*

(!!) Sera were not previously diluted prior to mixing with equal or greater amount of blood for insect feeding.

Table A.2 Transmission blocking vaccines based on insect-vector antigens

TBV antigen (antigen origin)	Antigen production	Antigen name	Vaccinated animal (adjuvant)	Sera dilution (concentration)	Targeted pathogen	Insect vector	Mean oocyst number	Infectivity %	Reference
Midgut (*An. gambiae*)	Tissue Extraction	Midgut antigens MG25E[1]	Mice $(FCA)_1$	-	*P. falciparum*	*An. stephensi*	-	2 %	(Lal et al., 2001)
				-		*An. farauti*	-	2.1 %	
						An. stephensi	-	5.2 %	
						An. albimanus	-	6.5 %	
CPBAg1 (*An. gambiae*)	-	CPBAg1	Rabbit (-)	1:1	*P. falciparum*	*An. gambiae*	-	8 %	(Lavazec et al., 2007)
AgAPN1 (*An. gambiae*)	Bacteria	AgAPN1	Rabbit (-)	(200 µg/ml)	*P. falciparum*	*An. stephensi*	9[#]	33 %	(Dinglasan et al., 2007)
						An. gambiae	11[#]	27 %	
Galectin (*P. papatasi*)	Cell free system	PpGalec	Mouse (-)	Neat[!!]	*L. major*	*P. papatasi*	N/A	11 %*****	(Kamhawi et al., 2004)

(**[1]**) MG25E is a monoclonal antibody derived from antigens present in *An. gambiae* midgut lysates.

(-) Undetermined.

(N/A) not applicable.

(**[#]**) Median values, corresponding to 67 % inhibition in *An. gambiae* and 73 % inhibition in *An. stephensi*, respectively.

(*****) Controls presented 55 % *L. major* infectivity.

(**[!!]**) Sera were not previously diluted prior to mixing with equal or greater amount of blood for insect feeding.

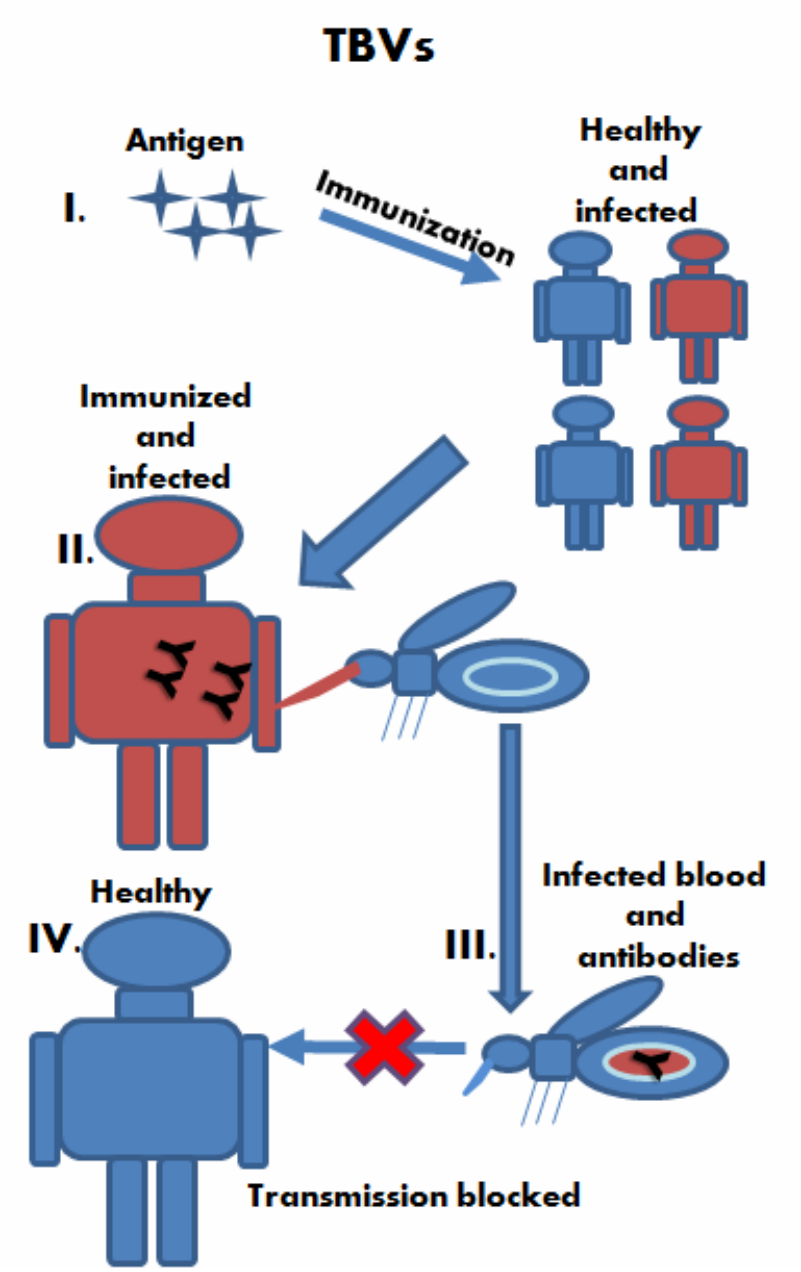

Figure A.1 Transmission blocking vaccines (TBVs). TBV is a strategy designed to prevent transmission of a pathogen by the bite of its infected, natural vector. Frequently, TBVs rely on generating antibodies against vector molecules that are involved in pathogen development. (I) Healthy (blue) and infected (red) individuals are immunized with a TBV antigen; (II) Insect-vectors take an infected blood meal containing TBV antigen-specific antibodies; (III) Specific antibodies produced against the antigen inhibit pathogens development within the insect vector, (IV) preventing transmission to uninfected host(s).