Proposal for Sequencing the Genome of the Sand Flies, *Lutzomyia longipalpis* and *Phlebotomus papatasi*

Mary Ann McDowell[1]	Center for Tropical Disease Research and Training, Department of Biological Sciences, University of Notre Dame, USA
Frank Collins	Center for Tropical Disease Research and Training, Department of Biological Sciences, University of Notre Dame, USA
Marcelo Ramalho-Ortigao	Laboratory of Malaria and Vector Research, National Institute of Allergy and Infectious Diseases, National Institutes of Health, USA
Jesus Valenzuela	Laboratory of Malaria and Vector Research, National Institute of Allergy and Infectious Diseases, National Institutes of Health, USA
Shaden Kamhawi	Laboratory of Parasitic Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, USA
Rod Dillon	Liverpool School of Tropical Medicine, UK
Paul Bates	Liverpool School of Tropical Medicine, UK
Michael Lehane	Liverpool School of Tropical Medicine, UK

[1] Contact: Center for Tropical Disease Research and Training, Department of Biological Sciences, University of Notre Dame, Notre Dame, IN 46556; E-mail: mcdowell.11@nd.edu; Phone: 574-631-9771; FAX: 574-631-7413.

I. Overview

This proposal represents a collaborative effort by the international sand fly research community to initiate the first large-scale genome sequencing project of two medically important sand fly species. As important vectors of human disease, phlebotomine sand flies are of global significance to human health, transmitting protozoan, bacterial, and viral pathogens. Completed genome sequences of these medically important vectors will foster development of novel technologies to control these devastating diseases. Furthermore, phlebotomine sand fly research has served as a key model for studies concerning vector/parasite/host interactions by revealing novel mechanisms defining vector competence, propelling the field of vaccine research into promising areas, and identifying novel therapeutics for human use. This genome project will accelerate progress in these areas, as well as complement and enhance ongoing comparative genomics efforts.

Phlebotomine sand flies are members of the family Psychodidae, which includes a diverse group of vectors that vary widely in geographic distribution, ecology and the pathogens they transmit. **Here we propose to sequence the genomes of two different phlebotomine sand flies, *Phlebotomus papatasi* and *Lutzomyia longipalpis*, that exhibit distinct distributions, behavior and pathogen specificity.** A comparative approach will provide substantial added-value, both technical and scientific, and will accelerate the discovery of regulatory and biochemical pathways within this family as potential biopharmaceuticals, vaccine candidates, and targets for insecticide development. Moreover, comparative genome sequence analyses between these and other available genomes will elucidate the pathways that lead to arthropod blood-feeding and immunity, inform arthropod phylogenetic relationships and enhance our comprehension of the evolutionary mechanisms that define genome organization.

II. Rationale for Sand Fly Genome Project

Improving Human Health. Sand flies serve as vectors for several established, emerging and re-emerging infectious diseases. One of these infections, Carrion's Disease, or bartonellosis, is caused by the gram-negative bacterium *Bartonella bacilliformis*. This pathogen infects human red blood cells resulting in two main clinical manifestations, nodular cutaneous eruptions and a frequently fatal anemia, Oroya fever. Although not a global disease (restricted to the Andes region of South America), Oroya fever is extremely dangerous; if untreated this infection causes one of the highest death rates (40-85%) of all infectious diseases. Sand flies also transmit viral infections; the sand fly fever viruses found in Africa, the Mediterranean basin, Middle East and Central Asia, in particular are a significant health problem. Three sand fly serotypes are currently circulating, Sicilian virus (SFSV), Naples virus (SFNV), and Toscana virus (TOSV). All three viruses cause a febrile, influenza-like illness, with TOSV resulting in aseptic meningitis. Moreover, the viral hemorrhagic fever virus, Rift Valley Fever Virus (RVF), also can be transmitted by phlebotomine sand flies (1, 2). All of these viruses are considered important as potential tools for bioterrorism (3).

The most devastating of the sand fly transmitted diseases are the leishmaniasis, causing substantial morbidity and mortality in much of the world. The clinical forms of leishmaniasis in humans range from disfiguring cutaneous lesions to systemic fatal disease, depending primarily on which of several parasite and sand fly species initiates the infection. These diseases are global, occurring in 88 countries on 5 continents, putting 350 million people at risk for contracting leishmaniasis. Currently 12 million people are infected and 2 million new cases occur annually. In 2002 the number of recorded deaths due to *Leishmania* infection was 59,000, considered a significant underestimate of the total mortality due to leishmanial diseases. These diseases also contribute substantially to morbidity, causing a disease burden that ranks above African trypanosomiasis, schistosomiasis, Chagas' disease, and dengue fever; the average metric Disability Adjusted Life Years (DALYs) due to the leishmaniasis is 2,357,000 (4). Political instability and warfare is expanding *Leishmania*-endemic regions and increasing the propensity for epidemics world-wide. These factors are coupled with the increase of visceral disease due to HIV co-infection, spurring the World Health Organization to classify leishmaniasis as one of the world's epidemic-prone diseases.

This extremely important tropical disease has become a particularly difficult health problem for the US military. Thirty-two cases of leishmaniasis were identified in military personnel deployed during the 1990-1991 Desert Storm Campaign, twelve of which were characterized as viscerotropic (a syndrome where parasites spread to visceral organs). **More recently, leishmaniasis in this region has had a profound effect on US troops; approximately 1200 soldiers deployed during Operation Iraqi Freedom have contracted cutaneous leishmaniasis and many additional cases are predicted to occur in the future**. In addition, several soldiers in Afghanistan have contracted leishmaniasis, including 2 cases of visceral disease (5). As 90% of the world's leishmaniasis cases occur in areas where US military forces are currently operating and America's interests persist, this problem will continue to expand.

The current chemotherapies for leishmaniasis are expensive and toxic: high cost, drug toxicity and the emergence of drug resistance demand a vaccine or alternative control measures. A significant effort towards the production of efficacious vaccines for leishmaniasis as well as sand fly fever has been expended over the years, however, no vaccine that shows effective protection in human populations has emerged so far from conventional approaches. Therefore, new control strategies to combat these diseases must be developed, combining attacks on human infections and on the arthropod vectors. As with insecticide resistance in mosquito populations insecticide-resistant sand flies have begun to emerge [for review see (3)]; furthermore, sand flies have been shown to have detoxification mechanisms that could lead to insecticide cross resistance (6). Rising insecticide-resistance in these vector populations underscores the need for the development of novel sand fly control measures.

A number of novel and potentially safe and effective approaches to the control of pathogen transmission could be developed from a better understanding of important sand fly behaviors such as host seeking for both human and animal reservoir hosts. This has been an important control target for vectors of African trypanosomes, where specific vector behavior is used to attract the tsetse fly vectors to insecticide impregnated, artificial targets. The genes responsible for host-seeking behaviors in tsetse flies as well as in the mosquitoes *Anopheles gambiae* and *Aedes aegypti* are now major features of interest that have justified genome projects for these vectors. In *An. gambiae* in particular, the genome project has opened a very active and productive field of research targeted at the molecular mechanisms responsible for host seeking and other important odor-mediated behaviors. With a clear understanding of the molecular physiology of host selection by sand flies, similar decoy control strategies could be envisioned. In addition, with elucidation of the molecular events involved in host finding, antagonists of specific steps in this process could prove to be effective targets for repellants. Analysis of the genome of important sand fly vectors will provide not only the knowledge base for developing safe sand fly attractants, repellants, or insecticides that could be used operationally in the field, but it could also be the source of information on those aspects of sand fly biology that are so important in initiating the vertebrate infection.

Approximately 20 different species of *Leishmania* are transmitted by 40 or so different sand fly species, which are subdivided into two genera, *Phlebotomus* and *Lutzomyia* (7). *Phlebotomus* species are responsible for transmitting leishmaniasis throughout parts of Africa, southwest Asia, the Middle East, and the Mediterranean; *Lutzomyia* species are vectors throughout South and Central America (8). There is a close ecological relationship between a *Leishmania* species and the vector(s) that transmit that species in nature. For example, *P. sergenti* will only transmit *L. tropica* and *P. papatasi* only transmits *L. major*. The restriction of *P. papatasi* for *L. major* is dictated by a midgut lectin that binds to a *L. major*-specific carbohydrate domain on the major *Leishmania* surface molecule lipophosphoglycan (LPG) (9). Although all vectors are specific under natural conditions, some such as *P. argentipes*, the natural *L. donovani* vector, and *Lu. longipalpis*, the natural vector of *L. chagasi* (=*L. infantum)*, can transmit a range of *Leishmania* species under laboratory conditions (10). This has given rise to the concept of “restricted” and “permissive” vectors, for example *P. papatasi* and *Lu. longipalpis*, respectively. The mechanisms that dictate vector competence for these other sand fly/*Leishmania* combinations remain to be completely elucidated.

Hematophagous vectors of disease are not just “flying syringes” for the inoculation of pathogens, they also dispense a plethora of pharmacologically active compounds that prevent host haemostasis. Many of these active compounds are immunogenic and elicit host immune responses that reduce the feeding efficiency (11) and fecundity of the arthropod vectors (12-16). Furthermore, these molecules act as immune effectors that influence the ability of blood-feeding vectors to transmit pathogens (17-19). Repeated exposure to sand fly bites causes a delayed-type hypersensitivity (DTH) response recognized by local inhabitants as a painful skin disease called ‘harara’ (20). Recently, elicitation of this response has been suggested to be an evolutionary advantage for sand flies, by increasing blood-flow at the bite site and, therefore decreasing the amount of time it takes for a sand fly to take a full blood meal (21). Although advantageous for sand flies, the DTH elicited by repeated exposure to sand fly bites (22) or salivary gland homogenate (23) protects against *Leishmania* infection. To identify the saliva immunogens that are being neutralized in this system as potential vaccine candidates, Valenzuela et al. have identified *P. papatasi* and *P. ariasi* salivary proteins that elicit immune responses(24, 25), and are testing these in murine models. Immunization with one of these proteins (SP15, *P. papatasi*) provides the same protection as saliva pre-exposure in mice. Recently, a *Lu. longipalpis* salivary gene was identified that when used as a DNA vaccine protected against visceral disease in a hamster model (J. Valenzuela, unpublished results).

It is clear that sand fly salivary proteins injected at the time of infection can influence the extent of clinical pathology and resistance to *Leishmania* (23, 26-30), but it is not known if this mechanism influences *Leishmania* tissue tropism (visceral vs. cutaneous). Access to completed genome sequences for the vector, parasite, and human host would greatly accelerate the discovery process to answer this question and others concerning this intimate interaction that leads to human disease. With the human genome and *L. major* sequences completed, two crucial parts of the knowledge base are in place. Further, the sequences for two additional *Leishmania* species, *L. infantum* and *L. braziliensis,* are underway (http://www.sanger.ac.uk/Projects/L_major/). All that is lacking are the sand fly vector sequences.

<u>Informing Human Biology</u>. Sand flies have evolved numerous fail-safe activities to combat vertebrate blood clotting, vasoconstriction and platelet aggregation. Salivary glands of phlebotomine sand flies contain a complex array of biologically active molecules that are both conserved and divergent among sand fly species; many of these molecules have immunosuppressive effects. For example, maxadilan, found only in *Lutzomyia* species*,* is the most potent vasodilatory polypeptide known to date (31) and exhibits a range of immunomodulatory activities (32-34). T-cell proliferation and delayed-type hypersensitivity (DTH) in mice (33), and stimulates host haematopoiesis. *Phlebotomus* species utilize different vasodilatory substances to facilitate blood-feeding: *P. papatasi* (35) and *P. argentipes* (36) secrete adenosine and AMP following a bloodmeal, having both vasodilatory as well as anti-platelet aggregation properties (37, 38). The presence of an anti-platelet aggregation enzyme, apyrase, has been identified in the salivary glands of both *Lutzomyia* and *Phlebotomus* vectors (39). Furthermore, both genera contain significant levels of endogenous protein phosphatase-1/2A-like activity (40). There has been significant effort by the laboratories of Jesus Valenzuela and Jose Ribeiro to characterize the “spitome” of phlebotomine sand flies. They have identified novel activities including: secreted α-amylase activity (41), hyaluronidase (42), 5’-nucleotidase, a phosphodiesterase that has vasodilatory and anti-platelet activities (43), secreted adenosine deaminase, likely involved in decreasing pain (44), and likely anti-clotting activities. Although the activities may be present in all blood-feeding sand flies, the molecules themselves are not necessarily

conserved among species. Elucidation of the complexity and novelty of sand fly-specific pharmacologically active compounds and its relevance to human disease is only in its infancy. A tremendous potential exists to exploit these novel compounds as biopharmaceuticals to benefit human health.

The recent blossoming of whole-genome science now allows us to make comprehensive ("global") comparisons between diverse organisms. Along with the completed *Drosophila* and *An. gambiae* (malaria vector) genomes, the sand fly genome project will complement and enhance other ongoing vector genome projects including those for *Ae. aegypti* (yellow fever mosquito), *C. pipiens quinqefasciatus* (southern house mosquito), *Glossina morsitans* (tsetse fly), *Rhodnius prolixus* (Chagas' disease vector) and *Ixodes scapularis* (Lyme's disease tick). Global comparisons between representatives of these vectors will greatly inform the evolutionary relationships among these species and lead to advances in our understanding of genes involved in important phenomena such as vectorial capacity, blood-feeding, immune system modulation, and insecticide resistance. The Diptera is the most species-rich and ecologically diverse order of insects. Both phlebotomine sand flies (family Psychodidae) and mosquitoes (Culicidae) are specified as members of distinct infra-orders within the suborder Nematocera. Scientifically, the Nematocera grouping is paraphyletic and the relationships between infra-orders remains to be elucidated (45). Furthermore, the internal relationships within the assemblage that includes Psychodidae remains a matter of debate. Completed genome sequences of two sand fly species will resolve these remaining questions and clarify the taxonomic groupings that have largely depended on classical morphologic characters. Moreover, genome data will improve gene prediction capabilities and enable the identification of conserved arthropod-specific genes, divergent orthologs and differentially expanded paralogous gene families and metabolic pathways amongst the Arthropoda.

Comparative genomics underpins the analysis of the human genome and has proven essential for annotation and the identification of functional genes. Comparative analysis between the human and sand fly genome will likely uncover novel human orthologs that have not been identifiable using other systems as has been the case for both the Honey Bee and *An. gambiae* (46, 47). Furthermore, phylogenetic footprinting is one of the most effective methods for identifying conserved regulatory motifs within non-coding regions. Because we propose a comparative approach between two species from closely related genre we are likely to uncover novel, rapidly evolving regulatory sequences.

<u>Expanding Understanding of Basic Biological Processes.</u> Sand flies have a complex sexual ecology involving an intricate interaction of courtship behavior (including acoustic signals), mate preference, aggression, host attraction, and pheromones (48). These flies form a lek-like aggregation on or near hosts where both mating and female blood-feeding occur; the males arrive first followed by females (49). The progression involves semiochemical factors from the flies (pheromones) and host-derived kairomones (50). This elaborate olfactory system distinguishes sand flies an excellent model for unraveling the genetics of complex odorant systems in species with striking olfactory behaviors. Furthermore, studies in sand flies are likely to contribute to our general understanding of the neurobiology that gives rise to complex courtship behaviors.

<u>Providing Additional Surrogate Systems for Human Experimentation</u>. The innate immune response can be elicited by a variety of receptors that recognize specific molecules on the surface of pathogens (51). The field of invertebrate immunology, dominated by *Drosophila* research, has identified various innate immune pathways responsible for one or more of such events. For example, the Toll and Imd innate immunity pathways, first discovered in *Drosophila*, are homologous to the Toll-like receptor (TLR) and TNFR pathways in mammals (51). A genome wide analysis of immune responses in *Drosophila* has led to the identification of 230 genes that are induced and 170 that are repressed during microbial infection (52). It further has been demonstrated that these pathways can discriminate between fungal and bacterial "infections" and gram positive and gram negative bacteria (53); however it has been suggested that these responses may only be a general defense against non-pathogenic micro-organisms (54). As these studies are typically initiated by injection, rather than natural infection, it is not unexpected that the pathways uncovered have broad specificity. Naturally co-evolving systems, like that of *Leishmania* species and their respective sand fly vectors, offer better opportunities for discovering relevant innate immunological mechanisms.

III. Status of Sand Fly Genome Effort

Although sand fly research has been somewhat delayed in exploiting the burgeoning field of genomics, recent ongoing efforts by the applicants of this proposal have been expended to develop the tools necessary to support *Lu. longipalpis* and *P. papatasi* genome projects. There are no current independent efforts by other organizations to perform large-scale genome sequencing of these two species.

IV. Utility of Sand Fly Genome Sequences

The work outlined in this proposal will build on and complement research that is ongoing in many laboratories throughout the world. The primary intended beneficiary communities of the sand fly genomes include laboratories that study genomics, genetics, various aspects of sand fly biology, vector competency, *Leishmania* immunity, arthropod evolutionary biology and the broader vector biology community in general. As the analytical and molecular methodologies that have revolutionized vector biology research have not yet been fully exploited in the field of sand fly biology, we anticipate that many researchers will engage in molecular analysis of the sand fly genomes. As outlined below the existing research expertise within the scientific community will enable investigators to maximally exploit the genome tools

that we will generate and the full sand fly genome sequences. Although cDNA microarrays will be available from the EST sequencing projects, the full genome sequencing will facilitate the development of high density oligonucleotide micro-arrays, possibly combining *Leishmania* and sand fly expressed genomes. Full genome sequence also will facilitate constructing genome tiling arrays to study "intronic anti-sense messages", as well as permit large scale transcription factor binding site promoter studies using "Chip-Chip" methods (55).

Sand fly/pathogen/host relationships. These genome projects offer an unprecedented opportunity for the sand fly biology field to study the vector/pathogen/host interaction on a global scale. Several laboratories are currently investigating the molecular mechanisms governing the development of the *Leishmania* parasites in the gut of the sand fly. The Liverpool group has constructed a set of cDNA microarrays containing 15,000 clones based on ESTs from the Jacobina strain of *Lu. longipalpis*. The arrays will initially be used to investigate the transcriptome during bloodfeeding and ultimately to examine gene expression in *Lu. longipalpis* in response to the development of the agent of visceral leishmaniasis *L. infantum.*

The Dillon-Bates-Lehane group at the Liverpool School also have embarked on a molecular ecology-based study of the prevalence and diversity of bacteria associated with sand flies. The purpose of the study is to: A) survey for novel intracellular symbionts in wild caught populations, B) define the residential bacteria in the midgut of adult female sandflies, and C) detect sand fly-borne bacteria that might be associated with emerging infections in humans and animals. This study will build on a previous investigation that focused on cultivatable gut bacteria (56). Moreover, the presence of a gut bacterial population may naturally regulate *Leishmania* development as the entire vector-borne phase of the *Leishmania* parasite is confined to the gut lumen. Future strategies will assess the feasibility for displaying anti-parasitic molecules, identified by the genome studies, using gut bacteria**.** The Liverpool group also is working with the NIH group to study the development and transmission of *B. bacilliformis* in Phlebotomine sand flies.

Recent work by Bates and colleagues has shown that there are still new aspects of the sand fly-*Leishmania* interaction to be discovered, even regarding a fundamental issue such as the transmission mechanism of leishmaniasis (57). The discovery that a gel-like material (promastigote secretory gel, PSG) is injected along with the parasites during sand fly bite has added a new dimension to understanding the transmission process. In the *L. mexicana*/*Lu. longipalpis* system it is clear that the main component of PSG is of parasite origin, a glycoprotein called filamentous proteophosphoglycan (fPPG), but it is not certain if this is the only component and/or whether minor components could be exerting some biological effect. Advances in understanding the transmission mechanism would be greatly facilitated by the availability of sand fly genome resources, as these would enable the rapid identification of sand fly molecules involved in the transmission process and lead to ways in which to investigate their role. Additionally this work has opened a potential new target for vaccine development, where a sand fly component needs to be incorporated into any applied aspects of vaccine development.

Population genetics. Understanding the population structure of geographically separated vector populations is essential for planning intervention strategies. One of the most popular tools that have emerged during the molecular revolution of population genetics is microsatellite DNA markers. These markers have been used extensively to understand the complex population structure of malaria vectors, research that has led to applied theory concerning the spread of insecticide resistance and the spread of genetically modified refractory mosquitoes. There are only two reports of microsatellites isolated from sand flies (58, 59), this deficiency is due to the relative difficulty in isolating these variable number tandem repeats in phlebotomine sand flies by traditional digest and probing methods in phlebotomine sand flies (59). The recent blossoming of sequencing technology has made it much more efficient and cost effective to identify microsatellite regions by sequencing genomic DNA. The availability of genomic sequence of these important vectors will catapult the field of sand fly population genetics into the 21st century by allowing researchers to identify molecular markers that can by used to ask evolutionary questions on a whole genome scale.

Genetic Basis for Disease and Vector Competence. The developmental cycle of *Leishmania* in the digestive tract of phlebotomine sand flies is complex, consisting of several distinct stages that begin with ingested amastigotes and end with transmission of infective metacyclics. Each of these stages involves specific interactions between *Leishmania* and sand fly midgut molecules. Most of our knowledge of such interactions comes from studies of the parasites. Several key molecules of *Leishmania* and their role in sand fly midgut– *Leishmania* interactions have been well characterized. In contrast, the corresponding molecules in the sand fly vector are poorly known, if at all. As an example, compared to the multitude of biochemically characterized lipophosphoglycan molecules from different *Leishmania* species, and on which vector competence studies were based, only a single sand fly midgut receptor was recently characterized from *P. papatasi* (60). The development of transmission blocking vaccines is based on the interruption of the life cycle of *Leishmania* in its sand fly vector; this relies on finding the species-specific sand fly molecules involved in this life cycle. A genome project will provide an excellent source for mining such information.

Genetic variability within a species also can influence vector competence. The *Lu. longipalpis* gene maxadilan differs genetically by as much as 23% from different sibling species of the *Lu. longipalpis* complex. This genetic variability has no effect on vasodilatory activity (61); however, significant differences in the amount of maxadilan mRNA in these species has been reported (62). Moreover, bites from sibling species collected from Central and South America produce different sized erythemas at the bite site and it has been postulated that differences in maxadilan expression in these vectors

contributes to these responses and to the atypical cutaneous disease caused by *L. infantum* in Costa Rica and visceral disease caused by *L. infantum* in Brazil (62). The availability of full genome sequences, particularly regulatory regions, will allow future exploration distinguishing how genetic variability within species complexes influences parasite transmission and ultimate disease pathology.

Comparative Genomics. The order Diptera contains the vectors of many of the most important pathogens of man and his domesticated animals. These vectors include the mosquitoes, tsetse flies, ceratopogonid midges, tabanids, black flies as well as the sand flies. We now have the full genome sequence of *An. gambiae* (63), the genome sequence of a second mosquito, *Ae. aegypti*, is nearing completion and that of a third mosquito *Culex*, has begun. In addition we now have substantial sequence information for the tsetse fly *Glossina morsitans morsitans* (64). We are also very fortunate in that the leading genetics model organism *Drosophila*, with the wealth of information available for it, is also a Diptera and is an invaluable asset for comparative purposes. The addition genome sequence of two sand fly species to the information available will be a tremendous help in unraveling biological processes that are common to blood sucking insects, including genes and pathways associated with blood feeding, host-seeking behavior, and immune responses to pathogens (65). More importantly it will help identify elements which are unique to sand flies. The usefulness of comparative genomics in the insect field has been more broadly discussed by Kaufman and colleagues (66).

Vaccine Research. *Leishmania* parasites are transmitted to their vertebrate hosts by infected phlebotomine sand flies. Arthropod's saliva modifies the physiology of the host at the site of the bite (67), making it more permissive for pathogen invasion (30). However, immune responses to these sand fly bites precludes the establishment of the pathogen in the vertebrate host (22), indicating that salivary molecules could be used as components of an anti-*Leishmania* vaccine. To identify these potential vaccine candidates, Valenzuela et al. have identified *P. papatasi* (25) and *Lu. longipalpis* (68) salivary proteins that provide protection against *L. major* and *L. chagasi* infection in rodent models respectively. Furthermore, they have designed a high-throughput cloning DNA plasmid to be used as a delivery and expression system of these salivary genes in animal skin and cultured human cells to identify vaccine candidates that elicit strong immune responses in target hosts. They have successfully cloned transcripts coding for secreted salivary proteins from *Lu. longipalpis*; *P. ariasi, P. argentipes*, *P. perniciosus, Lu. intermedia*, and *P. papatasi*.

Arthropod Immunity. Innate immunity plays a crucial role in fighting invading pathogens in arthropods. Genome wide analyses of immune responses been conducted in vectors such as *An. gambiae* (69-71) with the identification of genes potentially involved in response to malaria parasites. By contrast only a very limited number of studies have been done with sand flies, and to date only one anti-microbial peptide (AMP), defensin from *P. duboscqi*, has been characterized in sand flies (72). New AMP and other signaling molecules have been identified in sand flies and are currently being investigated as to their putative roles in *Leishmania* development in the sand fly midgut (M Ramalho-Ortigao, unpublished data). A genome-wide characterization of immune signaling cascade components in sand flies will provide additional tools in understanding different mechanisms of innate response in this as well as other vectors.

Gene Expression. EST sequences of *Lu. longipalpis* and *P. papatasi* under various experimental conditions have already been generated. Recently, an EST library has been generated from *Lu. longipalpis* (Jacobina strain, Bahia, Brazil) at the Liverpool School of Tropical Medicine. Two thousand whole adult sand flies under a range of physiological and *Leishmania* infection histories were used to construct a normalized cDNA library (Soares, University of Iowa) and used for sequencing 32,000 ESTs at the Sanger Institute. The ESTs have been assembled into nearly 10,000 contigs, which have been assigned a provisional annotation by BLAST search against *Drosophila*, *Anopheles* and other databases. Contamination with non-sand fly sequences e.g. *Leishmania*, was found to be extremely low even though > 20% of the sand flies were heavily infected with *Leishmania*. The annotated EST data will be databased in GeneDB thereby facilitating viewing and mining of the data. Further information and access to sequences is available via the Sanger Centre webpage: http://www.sanger.ac.uk/Projects/L_longipalpis/.

Other sand fly cDNA and EST libraries include those generated from *Lu. longipalpis* at the Oswald Cruz Institute, Brazil at the laboratory of Dr. Yara Traub-Cseko. On a much reduced sequencing scale, several ESTs have been identified and some may play important roles during blood feeding or *Leishmania* infection (73). They include a midgut specific chitinase, *LlChit1* (74), a MAP kinase, a *Cactus* homologue (Ikβ) and a TGF-β homologue. Additionally, 4010 cDNA sequences (many full length) comprising 1386 clusters or singletons obtained from *P. papatasi* midgut under different physiological conditions of blood feeding and infection with *L. major* are being investigated. Several novel molecules have been identified some of which may play significant roles in the physiology of this sand fly vector such as reorganization of midgut epithelial cells, innate immune defense, peritrophic matrix synthesis and maturation, and regulation of various physiological processes (Ramalho-Ortigao, unpublished observations).

V. Interest of Scientific Community

There is strong international interest in the *P. papatasi* and *Lu. longipalpis* genome projects from the sand fly research community. We have received letters of support from researchers in the United Kingdom, Europe, Brazil, United States, and Israel (see appendix). Because of their biomedical significance, the sand fly community has conducted decades of

biological research on both of these vectors, including behavior, ecology, phylogenetics, physiology, biochemistry, population genetics, functional genomics, pathogen transmission, insecticide control, and vaccine research. As of June 2005, there were 2,424 *Lutzomyia* and 1,651 *Phlebotomus* entries in Pub Med; clearly availability of complete genome sequences for these organisms would enhance the interest of the sand fly community and attract attention from within and outside the general field of medical entomology.

Recently a Sand Fly Genome Sequencing Consortium (SFGSC) made up of sand fly biologists from around the world was formed with the primary goals of generating genomic resources for the sand fly research community and genome drafts of *P. papatasi* and *Lu. longipalpis*. The first meeting was held at the 5th International Symposium on Phlebotomine Sand Flies in Tunis, Tunisia, April 18th, 2005. One major goal of the consortium is to expand and strengthen ties among international scientists involved in research concerning all aspects of sand fly biology and control. Members of SFGSC are listed below have pledged their support for the genome project.

UK
Rod J. Dillon, Liverpool School of Tropical Medicine
Michael J. Lehane, Liverpool School of Tropical Medicine
Paul A. Bates, Liverpool School of Tropical Medicine
Paul Ready, Natural History Museum
Richard Lane, Natural History Museum
Gordon Hamilton, Keele University
Richard Ward, Keele University
Karen Grant, Keele University
Rhayza Maingon, Keele University
Al Ivens, Sanger Institute

COLUMBIA
James Montoya Lerma, Universidad del Valle

ISRAEL
Yosef Schlein, Hebrew University
Alon Warburg, Hebrew University
Jake Jacobson, Hebrew University

BRAZIL
Sergio Verjovski-Ameida, University of Sao Paulo
Jeffrey Shaw, Instituto Evandro Chagas
Valdir de Queiroz Balbino, Universidade Federal de Pernambuco
Paulo Pimenta, Fundação Oswaldo Cruz – FIOCRUZ
Yara M. Traub-Cseko, Instituto Oswaldo Cruz-Fiocruz
Alexandre Afranio Peixoto, Fundacao Oswaldo Cruz
Elizabeth Ragel, Instituto Oswaldo Cruz

ITALY
Anna Maria Fausto, University of Tuscia
Massimo Mazzini, University of Tuscia
Michele Maroli, Institute Superior of Sanita

CZECH REPUBLIC
Petr Volf, Charles University

USA
Shaden Kamhawi, National Institutes of Health
Jesus Valenzuela, National Institutes of Health
Marcelo Ramalho-Ortigao, National Institutes of Health
Phillip Lawyer, National Institutes of Health
Jose Ribeiro, National Institutes of Health
David Sacks, National Institutes of Health
Mary Ann McDowell, University of Notre Dame
Frank H. Collins, University of Notre Dame
David Severson, University of Notre Dame
Edgar Rowton, Walter Reed Army Institute of Research
Russell Coleman, Walter Reed Army Institute of Research
Richard Titus, Colorado State University
Steve Beverley, Washington University School of Medicine
Serap Aksoy, Yale School of Public Health
Greg Lanzaro, University of California-Davis

Specific efforts of members of the SFGSC are outlined below.

a) Drs. MA McDowell and F Collins are supported by the Defense Advanced Research Projects Agency (DARPA), Department of Defense (DoD) to develop *P. papatasi* BAC libraries for genome sequencing and to initiate physical mapping of BAC clones on polytene chromosomes.
b) Drs. R Dillon, P Bates, and M Lehane are supported by Wellcome Trust in collaboration with the Sanger Institute to sequence and analyze *Lu. longipalpis* ESTs. These ESTs and accompanying microarrays will act as a community resource and genome annotation tool for the *Lu. longipalpis* genome sequence. The same group will seek independent support for the generation of *Lu. longipalpis* BAC libraries and physical mapping for genome assembly.
c) Dr. J Valenzuela is supported by intramural NIH funding to sequence and analyze *P. papatasi* and *Lu. longipalpis* midgut and salivary gland cDNAs and ESTs. These sequences will act as a community resource and genome annotation tool for the genome sequences.
d) Dr. MA McDowell and M Ramalho-Ortigao funded by the University of Notre Dame will generate additional *P. papatasi* ESTs from additional tissues and develop a *P. papatasi* microarray for the sand fly research community using cDNA clones generated from the project. Additional independent funding also will be pursued for this effort.
e) Dr. P Bates will supply the reference *Lu. longipalpis* strain and Dr. MA McDowell will supply the reference *P. papatasi* strain for sequencing. Dr. E Rowton and P Lawyer will oversee the maintenance and distribution of the reference strains to the community.
f) Drs. F Collins and D Severson will provide expertise on annotation and management of the sand fly genomes. These investigators have extensive experience in analysis of the *An. gambiae*, and *Ae. aegypti*, genomes.

g) Dr. F. Collins is the principal investigator of an NIAID contract grant to develop and manage *VectorBase*, a centralized relational database that will be the primary web interface for the research community interested in information dealing with genomes of arthropod vectors of human pathogens.

VI. Management of Sand Fly Genome Project

Management of the sand fly sequencing projects will be carried out by an assembled steering committee consisting of 11 international scientists. Current member of this committee consist of scientists that currently conduct sand fly research or have experience with genome sequencing efforts. Current members of this committee are: Drs. MA McDowell, F Collins, D. Severson (University of Notre Dame, USA); Drs. M. Rahmalo-Ortigao, S. Kamhawi, J. Valenzuela (NIH, USA); Drs. R Dillon, P Bates, M Lehane (Liverpool School of Medicine, UK); P Ready (Natural History Museum, UK); Dr. S Verjovski-Almeida (University of Sao Paulo, Brazil). In addition, the committee will include a representative of the MSC following selection of the MSC by NIAID. Dr. MA McDowell will serve as chair of the committee, coordinating interaction with the MSC and involvement of other community personnel as needed. Drs. Collins, Severson, and Verjovski-Almeida will provide expert advice, as these individuals have extensive experience in the development and coordination of genome projects.

VII. Suitability of *P. papatasi* and *Lu. longipalpis* for Genome Sequencing

Many phlebotomine sand flies are vectors of human and animal disease. All the proven vectors of leishmaniasis are species of either *Lutzomyia* in the New World or *Phlebotomus* in the Old World. **To enhance the value of the comparative approach we propose to sequence one species from each genus, *P. papatasi* and *Lu. longipalpis.*** In addition to their individual biomedical importance this combination of species was chosen for several reasons. First, these species are the best studied of the phlebotomine sand flies and for which EST and cDNA sequences currently are available. Second, these disease vectors have wide, but distinct geographical distributions. *Lu. longipalpis,* found only in the New World, is widely distributed, ranging from Mexico to Argentina (75); *P. papatasi*, restricted to the Old World, also has a large geographical distribution, encompassing most of the Indian subcontinent, the Middle East, and the Mediterranean sub-region (76). Third, despite the presence of a few common homologous genes, the "spitomes" of these two species contain a large percentage of transcripts unique to each genus (77). Forth, each species shares a high homology with that of other species within each respective genus. A comparison of salivary transcripts of *P. papatasi* with that from different *Phlebotomus* species (*P. ariasi, P. argentipes, P. perniciousus,* and *P. duboscqi*) revealed a high degree of similarity among transcripts and *Lu. longipalpis* shares high homology with *Lu. intermedia* (Valenzuela et al, manuscript in preparation). Fifth, these vectors transmit parasites at opposite ends of the pathological spectrum: *P. papatasi* transmits cutaneous leishmaniasis, while *Lu. longipalpis* transmits visceral leishmaniasis in nature. Lastly, *Lu. longipalpis* is a permissive vector being able to experimentally harbor several *Leishmania* species, while *P. papatasi* is restrictive only being able to carry *L. major* parasites. Although both these sand fly species transmit *Leishmania* parasites and modulate vertebrate haemostasis, the molecular diversity of the species-specific compounds that mediate these functions is large. These differences approach the *An gambiae*/*Ae. aegypti* system where genome organization and sequence differ to an extent that one mosquito species is not a highly informative model for the other.

Life History Traits and Experimental Suitability of *P. papatasi* and *Lu. longipalpis*. Several colonies of both of these sand fly species have been established and the relevant strains are maintained in several laboratories throughout the world. These flies are reared with relative ease in the laboratory by the method of Modi and Tesh (78). The total developmental period from blood meal to emergence of adults varies between 25-40 days depending on the species and ambient temperature. Colonies can be expanded to produce large numbers of larvae, pupae and adults for sequencing.

Estimates of Genome Size. The only analysis of genome size for a *Phlebotomus* species was performed for *P. perniciosus* (59). Based on re-association kinetics the haploid genome of this species is estimated to be approximately 1.7×10^8 bp (170 Mb). The haploid chromosome length of this species is only 1.7% for males and 3.4% for females greater than that of *P. papatasi*, suggesting that the genome size of *P. papatasi* will be similar (79). Recently nuclear staining techniques (80) were used to estimate the genome size of *Lu. longipalpis,* estimating a size of 300 Mb.

Sand Fly Cytogenetics and Mapping. Cytogenetic studies have been undertaken for both *Lutzomyia* and *Phlebotomus* species. The mitotic karotypes of *Lu. longipalpis* sibling species range from 2n=6 to 2n=8 (81-83). The karyotype of *Phlebotomus* (2n=10) consists of five pairs of chromosomes and includes a pair of heterosomes. Polytene chromosomes of *Lu. longipalpis* larval salivary glands have been identified (84). Furthermore, a G-banding technique has been developed for sand fly chromosomes that has been used to distinguish between different sand fly sibling species (82).

Sand Fly Genetic Manipulation. Methods for the genetic modification of mosquitoes have been developed, allowing introduction of effector molecules that target ligands on parasites or their receptors in mosquito tissues interfering with key recognition properties necessary for parasite development (85-90). Some of the strategies utilize transposable elements to stably insert the information in the mosquito genome. Another approach, paratransgenesis, consists in the use of genetically modified bacterial vector symbionts to interfere with parasite development (91). Sand fly symbionts include *Wolbachia* (92) and *B. bacilliformis* (93). Additionally, genetic manipulation in sand flies can be accomplished by means of

RNA interference (RNAi), in a reverse genetics approach to study gene function. *In vivo* RNAi has been proven successful in several disease vectors, including mosquitoes (94, 95) and ticks (96-98). A protocol for injection of dsRNA in sand flies has been developed attaining a good survival percentage of adult insects. Preliminary data using in vivo RNAi in *P. papatasi* has been obtained (Ramalho-Ortigao and Kamhawi, unpublished data) for experiments using a selected number of gene targets.

VIII. Goals and Stategy for *P. papatasi and Lu. longipalpis* Genome Project

The long-term goals of the sand fly genome effort are: (i) to generate an extensive set of cDNA sequences that can be used to build cDNA microarrays and assist in annotation of the genomes, (ii) to produce additional information including physically mapped, end-sequenced BACs and a carefully analyzed set of sequenced BACs that will assist will assist in assembly and annotation of the genomes, and (iii) to generate drafts of the *Lu. longipalpis* and *P. papatasi* genomes that are sufficiently complete as to permit genome analysis of both coding and regulatory regions. (Not all components of this work will be carried out by the MSC.) **Due to the relative deficiency in available genome data, we propose an initial project in Phase I based on extensive sequencing of normalized sand fly cDNAs combined with complete sequencing of selected large genomic DNA containing BAC clones, BAC-end sequencing (BES), and physical mapping of the BACs to sand fly polytene chromosomes for Phase I.** The information generated by these studies will provide sequence data that the community can begin to use and will allow a preliminary analysis of the genome to guide Phase II**.** While BAC end sequencing for assembly use need not be done in Phase I, it will be important to have some end-sequenced BACs for physical mapping, which needs to be started immediately. **In Phase II, we propose whole genome random shotgun sequencing (WGS) to 8X coverage of each genome.** The 8X coverage is recommended to enable the use of comparative genomics to identify both regulatory and protein coding regions of the genomes. Particularly important will be the mapping of sequence-tagged BAC clones to the polytene chromosomes, as these data will permit the assembled scaffolds to be directly assigned to locations with specific orientations within the sand fly genome. A draft genome is a desired end point when sequencing a new species as the physical organization and structure of the genome and genetic elements are revealed making it easier to study the genome and to carry out comparative genome studies. The direct products from this project include an index of expressed genes (and thus a first stage microarray), a BAC based physical map of the sand fly genome, and a draft genome sequence of both *Lu. longipalpis* and *P. papatasi.*

Phase I – Initial sequencing

Gene Discovery. The first genome activity we propose is the production and extensive sequencing of a normalized *P. papatasi* cDNA library. An EST database has already been generated for *Lu. longipalpis* (see above). We will target one normalized library prepared from as diverse a background of tissue material as possible to provide a wide range for gene discovery. This tissue will include embryos staged at a variety of intervals, each of the larval instars, pupae, newly emerged male and female adults, older (3-14 days) adults, heads dissected from uninfected male and female sand flies, heads from females at various stages before, during, and after blood meals, from males and females that are in the process of swarming (collected at night), and from sand flies that have fed on an *L. major* infected blood meal. These infected individuals will include sandflies isolated immediately after, 6hr., 24hrs, 2 days and 6 days following the infective blood meal. A comparable library (Normalized A*nopheles* Pool or NAP) was produced for the mosquito *An. gambiae*, and of four different normalized libraries, it proved both the richest from the point of view of gene coverage (almost 11,000 contigs and singletons assembled at 96% identity from 25,000 ESTs) and it contained 85% of all the ESTs found in the other three libraries. An *A. gambiae* head library was the next richest resource, thus our emphasis on head tissue in the *P. papatasi* library (F. Collins, personal communication). A cDNA library made from blood fed only *P. papatasi* females has already been generated and extensively sequenced (99).

Tissue samples will be generated at the University of Notre Dame and the normalized cDNA library will be produced at Express Genomics (Fredrick, Maryland). We recommend sequencing this normalized library in increments of 5,000 sequences until the yield of new ESTs falls off markedly. In the NAP library above, yield of new sequences fell off at a constant rate out to 25,000, where the yield was about 0.4 new ESTs for every EST sequenced. If the of novel ESTs from *P. papatasi* follow this pattern, we would recommend sequencing approximately 40,000 clones from the normalized library. Drs. McDowell and Ramalho-Ortigao will develop a *P. papatasi* EST microarray for global gene-expression analyses in consultation with Drs. Collins and Severson who have experience with *An. gambiae* and *Ae. aegypti* microarray development and analysis.

Justification. Besides their utility in full length gene cloning, genome annotation and the development of microarrays, the ESTs generated from these studies will underpin ongoing proteomics studies, inform on the repertoire of expressed genes and gene families in sandflies and permit evolutionary comparisons between invertebrate genes. ESTs will provide the sand fly research community with information on the types of genes and gene families that are expressed during sand fly development, within tissues, and will also enable the identification of genes involved in blood feeding, host finding, and pathogen transmission. EST sequencing of 40,000 clones will be done as part of the MSC project. Some additional EST sequencing and microarray production will be done independently.

Genome size determination. The DNA content of both *P. papatasi* and *Lu. longipalpis* will be estimated by Cot analysis as previously described (100). The genome size will be estimated by comparing the reassociation rate of denatured *P. papatasi* DNA to that of an *Escherichia coli* DNA standard. Only the unique, or late, component of the *P. papatasi* reassociation curve will be compared. Briefly, unlabeled, or "driver" DNA will be fractionated by sonication and sized by agarose gel electorphoresis by comparing the mobility of sheared DNA to lambda DNA digested with *Hind*III. "Tracer" DNA will be radiolabeled with ^{3}H-dTTP by nick-translation, fractionated, and sized on a 3% alkaline agarose gel. Driver and tracer will be suspended in a ration of 200:1 and denatured by boiling and allowed to reassociate at 60°C. Single-stranded DNA will be isolated on hydroxyapatite columns following the specific reassociation times and measured by beta counting. Cot measurements will be made form 10^{-3} to 10^4. Five Cot measurements (10^{-2} – 10^2) will be made for *E. coli* as a standard. This work, which has already been initiated, will be done independently of the MSC project.

BAC genomic DNA library generation and sequencing. BAC libraries will be generated for both *Lu. longipalpis* and *P. papatasi.* We will generate 10X coverage genomic DNA BAC libraries (average insert size of approximately 120kb) from sand fly larvae. Genomic DNA from *Lu. longipalpis* will be provided by Dr. R Dillon and *P. papatasi* larvae will be provided by Dr. MA McDowell and Dr. S Kamhawi. *Lu. longipalpis* library will be generated with external funding independent of the MSC. The *P. papatasi* library will be at the Clemson University Genomics Center in collaboration with Dr. J. Tomkins, and will be done independently of the MSC. The Collins laboratory, which has produced BAC libraries for *An. gambiae*, will assist in this aspect of the work. Complete sequencing of 20 randomly chosen and 20 selected BAC clones from each of the two libraries will be performed. The latter will be selected in consultation with the sand fly research community. This will give a preview of the architecture of each of these genomes prior to full shotgun sequencing, and the assembly and annotation of these BACs will be subject to manual overview and some RT-PCR gene analysis. These annotated BACs will be an important resource for estimating the quality of genome assemblies done in phase II.

We propose BAC end sequencing of ~18,000 clones (~36,000 reads) for the *P. papatasi* library (10X coverage of 120 kb insert clones with about 15% failure). The preliminary genome size estimate for *Lu. longipalpis* is 300 Mb, which would require end sequencing of about 32,000 BAC clones to achieve the same level of coverage as the 170 Mb *P. papatasi* genome. [We suspect that this 300 Mb estimate of genome size may be an overestimate.] The BAC end sequencing and sequencing of 40 BAC clones for each species would be done by the MSC.

Justification. Complete BAC sequences will provide preliminary but valuable information on genome organization, including repetitive sequences, gene structure and density. BACs also can be used to test the feasibility of random shotgun sequencing and genome assembly. Complete BACs and BAC-end sequences will provide sequence data for the community to begin analyzing the sand fly genome. The end-sequenced BACs will be physically mapped to provide data for use in linking the assembled genome sequence to the sand fly chromosomes.

Phase II – Draft of *Lu. longipalpis* and *P. papatasi* Genomes

Whole genome random shotgun sequencing via plasmid libraries is proposed for Phase II of the project. The goal will be an 8X coverage without completely finishing each genome; gap closure will be performed by independent research groups interested in the areas surrounding the gaps. To ensure that the features of interest (exons, regulatory regions, etc.) are uninterrupted and are covered by high quality sequence with a low error rate, a large-scale (scale of tens of kilobases) contiguity is proposed. To account for statistical fluctuations and cloning biases an 8x coverage is requested. Assembly of contigs into scaffolds will be facilitated by the BAC-end sequences. The MSC will generate the genomic libraries and proceed with sequencing and automated annotation. The exact cloning and sequence strategy will be determined by the MSC based in part on the results of phase I. Costs associated with the MSC will be governed by the by the MSC contract guidelines negotiated with the NIAID. The *VectorBase* Bioinformatics Resource Center will assume responsibility for display and future curation of the genomes on completion by the MSC.

Public Release of Genome Data. All sequence data will be approved by the steering committee and released to the public domain. We propose that un-annotated genome data made available to the scientific community through a dedicated MSC website such as the TIGR *Ae. aegypti* website. Annotated sequence will be released to the appropriate database at NCBI and to *VectorBase.*

IX. Colony Choice and Availability of DNA

Consultation with the sand fly research community has resulted in identifying suitable strains as the logical targets for this genome effort. The Jacobina strain, Bahia, Brazil of *Lu. longipalpis* and the Israeli strain of *P. papatasi* will be used for this sequencing effort. The ESTs currently available have been derived from these strains. The *Lu. longipalpis* Jacobina strain colony at the Liverpool School of Tropical Medicine was originally established by Richard Ward in 1988 by flies caught in Jacobina, Bahia State, Brazil and has been maintained continuously since establishment. At several times during this time, the colony has fluctuated in population size and has been expanded from a small number of flies. The Israeli strain of *P. papatasi* was originally was given to Walter Reed Army Institute of Research (WRAIR) in 1983 from the Hebrew University, Jerusalem. The population size of this colony also has fluctuated over time. Currently the colony is being expanded from a small number of flies at WRAIR and will undergo one more expansion once transferred to the University of Notre Dame before sequencing will begin.

X. Availability of Other Funding Sources

Other funding that directly or indirectly supports the goals of this project is being contributed by several sources. Drs. Dillon, Bates, and Lehane have funding from the Wellcome Trust to support cDNA library construction and sequencing of *Lu. longipalpis* ESTs at the Sanger Institute. Dr. Collins is the lead PI on a NIAID contract to develop and maintain *VectorBase*, the database and bioinformatics resource tool for invertebrate vectors of human pathogens. Drs. McDowell and Collins have been awarded funding from DARPA (DoD) to develop *P. papatasi* BAC libraries and to initiate physical mapping of BAC clones on chromosomes. University of Notre Dame Graduate School and the Liverpool School of Tropical Medicine are contributing further funding. Consortium scientists also plan to solicit additional support for the genome effort. Possible sources of direct or indirect funding that currently are being investigated include NIAID, DoD DOE, WHO and the Wellcome Trust.

XI. References

1. Turell, M. J., and P. V. Perkins. 1990. Transmission of Rift Valley fever virus by the sand fly, Phlebotomus duboscqi (Diptera: Psychodidae). *Am J Trop Med Hyg 42:185*.
2. Dohm, D. J., E. D. Rowton, P. G. Lawyer, M. O'Guinn, and M. J. Turell. 2000. Laboratory transmission of Rift Valley fever virus by Phlebotomus duboscqi, Phlebotomus papatasi, Phlebotomus sergenti, and Sergentomyia schwetzi (Diptera: Psychodidae). *J Med Entomol 37:435*.
3. Sidwell, R. W., and D. F. Smee. 2003. Viruses of the Bunya- and Togaviridae families: potential as bioterrorism agents and means of control. *Antiviral Res 57:101*.
4. WHO. 2002. The World Health report 2002, reducing risks, promoting health life.
5. 2004. Two cases of visceral leishmaniasis in U.S. military personnel--Afghanistan, 2002-2004. *MMWR Morb Mortal Wkly Rep 53:265*.
6. El-Sayed, S., J. Hemingway, and R. P. Lane. 1989. Susceptibility baselines for DDT metabolism and related enzyme systems in the sandfly *Phlebotomus papatasi* (Scopoli) (Diptera: Psychodidae). *Bulltin of Entomological REsearch 79:679*.
7. Cunningham, A. C. 2002. Parasitic adaptive mechanisms in infection by leishmania. *Exp Mol Pathol 72:132*.
8. Sacks, D., and S. Kamhawi. 2001. Molecular aspects of parasite-vector and vector-host interactions in leishmaniasis. *Annu Rev Microbiol 55:453*.
9. Kamhawi, S., G. B. Modi, P. F. Pimenta, E. Rowton, and D. L. Sacks. 2000. The vectorial competence of Phlebotomus sergenti is specific for Leishmania tropica and is controlled by species-specific, lipophosphoglycan-mediated midgut attachment. *Parasitology 121:25*.
10. Lainson, R., R. D. Ward, and J. J. Shaw. 1977. Experimental transmission of Leishmania chagasi, causative agent of neotropical visceral leishmaniasis, by the sandfly Lutzomyia longipalpis. *Nature 266:628*.
11. Wikel, S. K. 1982. Immune responses to arthropods and their products. *Annu Rev Entomol 27:21*.
12. Alger, N. E., and E. J. Cabrera. 1972. An increase in death rate of Anopheles stephensi fed on rabbits immunized with mosquito antigen. *J Econ Entomol 65:165*.
13. Nogge, G., and M. Giannetti. 1980. Specific antibodies: a potential insecticide. *Science 209:1028*.
14. Sutherland, G. B., and A. B. Ewen. 1974. Fecundity decrease in mosquitoes ingesting blood from specifically sensitized mammals. *J Insect Physiol 20:655*.
15. Kemp, D. H., R. I. Agbede, L. A. Johnston, and J. M. Gough. 1986. Immunization of cattle against Boophilus microplus using extracts derived from adult female ticks: feeding and survival of the parasite on vaccinated cattle. *Int J Parasitol 16:115*.
16. Chinzei, Y., and H. Minoura. 1988. Reduced oviposition in Ornithodoros moubata (Acari: Argasidae) fed on tick-sensitized and vitellin-immunized rabbits. *J Med Entomol 25:26*.
17. Ramasamy, M. S., M. Sands, B. H. Kay, I. D. Fanning, G. W. Lawrence, and R. Ramasamy. 1990. Anti-mosquito antibodies reduce the susceptibility of Aedes aegypti to arbovirus infection. *Med Vet Entomol 4:49*.
18. Ramasamy, M. S., and R. Ramasamy. 1990. Effect of anti-mosquito antibodies on the infectivity of the rodent malaria parasite Plasmodium berghei to Anopheles farauti. *Med Vet Entomol 4:161*.
19. Lal, A. A., M. E. Schriefer, J. B. Sacci, I. F. Goldman, V. Louis-Wileman, W. E. Collins, and A. F. Azad. 1994. Inhibition of malaria parasite development in mosquitoes by anti-mosquito-midgut antibodies. *Infect Immun 62:316*.
20. Adler, S., and O. Theodor. 1957. Transmission of disease agents by phlebotomine sandflies. *Annual Review of Entomology 2:203*.
21. Belkaid, Y., J. G. Valenzuela, S. Kamhawi, E. Rowton, D. L. Sacks, and J. M. Ribeiro. 2000. Delayed-type hypersensitivity to Phlebotomus papatasi sand fly bite: An adaptive response induced by the fly? *Proc Natl Acad Sci U S A 97:6704*.
22. Kamhawi, S., Y. Belkaid, G. Modi, E. Rowton, and D. Sacks. 2000. Protection against cutaneous leishmaniasis resulting from bites of uninfected sand flies. *Science 290:1351*.
23. Belkaid, Y., S. Kamhawi, G. Modi, J. Valenzuela, N. Noben-Trauth, E. Rowton, J. Ribeiro, and D. L. Sacks. 1998. Development of a natural model of cutaneous leishmaniasis: powerful effects of vector saliva and saliva preexposure on the long-term outcome of Leishmania major infection in the mouse ear dermis. *J Exp Med 188:1941*.
24. Oliveira, F., S. Kamhawi, A. E. Seitz, V. My Pham, P. M. Guigal, L. Fischer, J. Ward, and J. Valenzuela. In Press. From transcriptome to immunome: Identification of DTH inducing proteins from a *Phlebotomus ariasi* saalivary gland cDNA library. *Vaccine*.
25. Valenzuela, J. G., Y. Belkaid, M. K. Garfield, S. Mendez, S. Kamhawi, E. D. Rowton, D. L. Sacks, and J. M. Ribeiro. 2001. Toward a defined anti-Leishmania vaccine targeting vector antigens: characterization of a protective salivary protein. *J Exp Med 194:331*.
26. Theodos, C. M., J. M. Ribeiro, and R. G. Titus. 1991. Analysis of enhancing effect of sand fly saliva on Leishmania infection in mice. *Infect Immun 59:1592*.
27. Norsworthy, N. B., J. Sun, D. Elnaiem, G. Lanzaro, and L. Soong. 2004. Sand fly saliva enhances Leishmania amazonensis infection by modulating interleukin-10 production. *Infect Immun 72:1240*.

28. Samuelson, J., E. Lerner, R. Tesh, and R. Titus. 1991. A mouse model of Leishmania braziliensis braziliensis infection produced by coinjection with sand fly saliva. *J Exp Med 173:49*.
29. Bezerra, H. S., and M. J. Teixeira. 2001. Effect of Lutzomyia whitmani (Diptera: Psychodidae) salivary gland lysates on Leishmania (Viannia) braziliensis infection in BALB/c mice. *Mem Inst Oswaldo Cruz 96:349*.
30. Titus, R. G., and J. M. Ribeiro. 1988. Salivary gland lysates from the sand fly Lutzomyia longipalpis enhance Leishmania infectivity. *Science 239:1306*.
31. Ribeiro, J. M., A. Vachereau, G. B. Modi, and R. B. Tesh. 1989. A novel vasodilatory peptide from the salivary glands of the sand fly Lutzomyia longipalpis. *Science 243:212*.
32. Bozza, M., M. B. Soares, P. T. Bozza, A. R. Satoskar, T. G. Diacovo, F. Brombacher, R. G. Titus, C. B. Shoemaker, and J. R. David. 1998. The PACAP-type I receptor agonist maxadilan from sand fly saliva protects mice against lethal endotoxemia by a mechanism partially dependent on IL-10. *Eur J Immunol 28:3120*.
33. Qureshi, A. A., A. Asahina, M. Ohnuma, M. Tajima, R. D. Granstein, and E. A. Lerner. 1996. Immunomodulatory properties of maxadilan, the vasodilator peptide from sand fly salivary gland extracts. *Am J Trop Med Hyg 54:665*.
34. Soares, M. B., R. G. Titus, C. B. Shoemaker, J. R. David, and M. Bozza. 1998. The vasoactive peptide maxadilan from sand fly saliva inhibits TNF-alpha and induces IL-6 by mouse macrophages through interaction with the pituitary adenylate cyclase-activating polypeptide (PACAP) receptor. *J Immunol 160:1811*.
35. Ribeiro, J. M., O. Katz, L. K. Pannell, J. Waitumbi, and A. Warburg. 1999. Salivary glands of the sand fly Phlebotomus papatasi contain pharmacologically active amounts of adenosine and 5'-AMP. *J Exp Biol 202:1551*.
36. Ribeiro, J. M., and G. Modi. 2001. The salivary adenosine/AMP content of Phlebotomus argentipes Annandale and Brunetti, the main vector of human kala-azar. *J Parasitol 87:915*.
37. Dionisotti, S., C. Zocchi, K. Varani, P. A. Borea, and E. Ongini. 1992. Effects of adenosine derivatives on human and rabbit platelet aggregation. Correlation of adenosine receptor affinities and antiaggregatory activity. *Naunyn Schmiedebergs Arch Pharmacol 346:673*.
38. Collis, M. G. 1989. The vasodilator role of adenosine. *Pharmacol Ther 41:143*.
39. Valenzuela, J. G., Y. Belkaid, E. Rowton, and J. M. Ribeiro. 2001. The salivary apyrase of the blood-sucking sand fly Phlebotomus papatasi belongs to the novel Cimex family of apyrases. *J Exp Biol 204:229*.
40. Katz, O., J. N. Waitumbi, R. Zer, and A. Warburg. 2000. Adenosine, AMP, and protein phosphatase activity in sandfly saliva. *Am J Trop Med Hyg 62:145*.
41. Ribeiro, J. M., E. D. Rowton, and R. Charlab. 2000. Salivary amylase activity of the phlebotomine sand fly, Lutzomyia longipalpis. *Insect Biochem Mol Biol 30:271*.
42. Ribeiro, J. M., R. Charlab, E. D. Rowton, and E. W. Cupp. 2000. Simulium vittatum (Diptera: Simuliidae) and Lutzomyia longipalpis (Diptera: Psychodidae) salivary gland hyaluronidase activity. *J Med Entomol 37:743*.
43. Ribeiro, J. M., E. D. Rowton, and R. Charlab. 2000. The salivary 5'-nucleotidase/phosphodiesterase of the hematophagus sand fly, lutzomyia longipalpis. *Insect Biochem Mol Biol 30:279*.
44. Charlab, R., E. D. Rowton, and J. M. Ribeiro. 2000. The salivary adenosine deaminase from the sand fly Lutzomyia longipalpis. *Exp Parasitol 95:45*.
45. Yeates, D. K., and B. M. Wiegmann. 1999. Congruence and controversy: toward a higher-level phylogeny of Diptera. *Annu Rev Entomol 44:397*.
46. Zdobnov, E. M., C. von Mering, I. Letunic, D. Torrents, M. Suyama, R. R. Copley, G. K. Christophides, D. Thomasova, R. A. Holt, G. M. Subramanian, H. M. Mueller, G. Dimopoulos, J. H. Law, M. A. Wells, E. Birney, R. Charlab, A. L. Halpern, E. Kokoza, C. L. Kraft, Z. Lai, S. Lewis, C. Louis, C. Barillas-Mury, D. Nusskern, G. M. Rubin, S. L. Salzberg, G. G. Sutton, P. Topalis, R. Wides, P. Wincker, M. Yandell, F. H. Collins, J. Ribeiro, W. M. Gelbart, F. C. Kafatos, and P. Bork. 2002. Comparative genome and proteome analysis of Anopheles gambiae and Drosophila melanogaster. *Science 298:149*.
47. Whitfield, C. W., M. R. Band, M. F. Bonaldo, C. G. Kumar, L. Liu, J. R. Pardinas, H. M. Robertson, M. B. Soares, and G. E. Robinson. 2002. Annotated expressed sequence tags and cDNA microarrays for studies of brain and behavior in the honey bee. *Genome Res 12:555*.
48. Soares, R. P., and S. J. Turco. 2003. Lutzomyia longipalpis (Diptera: Psychodidae: Phlebotominae): a review. *An Acad Bras Cienc 75:301*.
49. Quinnell, R. J., and C. Dye. 1994. Correlates of the peridomestic abundance of Lutzomyia longipalpis (Diptera: Psychodidae) in Amazonian Brazil. *Med Vet Entomol 8:219*.
50. Kelly, D. W., Z. Mustafa, and C. Dye. 1997. Differential application of lambda-cyhalothrin to control the sandfly Lutzomyia longipalpis. *Med Vet Entomol 11:13*.
51. De Gregorio, E., P. T. Spellman, P. Tzou, G. M. Rubin, and B. Lemaitre. 2002. The Toll and Imd pathways are the major regulators of the immune response in Drosophila. *Embo J 21:2568*.
52. De Gregorio, E., P. T. Spellman, G. M. Rubin, and B. Lemaitre. 2001. Genome-wide analysis of the Drosophila immune response by using oligonucleotide microarrays. *Proc Natl Acad Sci U S A 98:12590*.
53. Michel, T., J. M. Reichhart, J. A. Hoffmann, and J. Royet. 2001. Drosophila Toll is activated by Gram-positive bacteria through a circulating peptidoglycan recognition protein. *Nature 414:756*.
54. Hultmark, D. 2003. Drosophila immunity: paths and patterns. *Curr Opin Immunol 15:12*.
55. Sikder, D., and T. Kodadek. 2005. Genomic studies of transcription factor-DNA interactions. *Curr Opin Chem Biol 9:38*.

56. Dillon, R. J., E. el Kordy, M. Shehata, and R. P. Lane. 1996. The prevalence of a microbiota in the digestive tract of Phlebotomus papatasi. *Ann Trop Med Parasitol 90:669*.
57. Rogers, M. E., T. Ilg, A. V. Nikolaev, M. A. Ferguson, and P. A. Bates. 2004. Transmission of cutaneous leishmaniasis by sand flies is enhanced by regurgitation of fPPG. *Nature 430:463*.
58. Maingon, R. D., R. D. Ward, J. G. Hamilton, H. A. Noyes, N. Souza, S. J. Kemp, and P. C. Watts. 2003. Genetic identification of two sibling species of Lutzomyia longipalpis (Diptera: Psychodidae) that produce distinct male sex pheromones in Sobral, Ceara State, Brazil. *Mol Ecol 12:1879*.
59. Day, J. C., and P. D. Ready. 1999. Relative abundance, isolation and structure of phlebotomine microsatellites. *Insect Mol Biol 8:575*.
60. Kamhawi, S., M. Ramalho-Ortigao, V. M. Pham, S. Kumar, P. G. Lawyer, S. J. Turco, C. Barillas-Mury, D. L. Sacks, and J. G. Valenzuela. 2004. A role for insect galectins in parasite survival. *Cell 119:329*.
61. Lanzaro, G. C., A. H. Lopes, J. M. Ribeiro, C. B. Shoemaker, A. Warburg, M. Soares, and R. G. Titus. 1999. Variation in the salivary peptide, maxadilan, from species in the Lutzomyia longipalpis complex. *Insect Mol Biol 8:267*.
62. Yin, H., D. E. Norris, and G. C. Lanzaro. 2000. Sibling species in the Llutzomyia longipalpis complex differ in levels of mRNA expression for the salivary peptide, maxadilan. *Insect Mol Biol 9:309*.
63. Holt, R. A., G. M. Subramanian, A. Halpern, G. G. Sutton, R. Charlab, D. R. Nusskern, P. Wincker, A. G. Clark, J. M. Ribeiro, R. Wides, S. L. Salzberg, B. Loftus, M. Yandell, W. H. Majoros, D. B. Rusch, Z. Lai, C. L. Kraft, J. F. Abril, V. Anthouard, P. Arensburger, P. W. Atkinson, H. Baden, V. de Berardinis, D. Baldwin, V. Benes, J. Biedler, C. Blass, R. Bolanos, D. Boscus, M. Barnstead, S. Cai, A. Center, K. Chaturverdi, G. K. Christophides, M. A. Chrystal, M. Clamp, A. Cravchik, V. Curwen, A. Dana, A. Delcher, I. Dew, C. A. Evans, M. Flanigan, A. Grundschober-Freimoser, L. Friedli, Z. Gu, P. Guan, R. Guigo, M. E. Hillenmeyer, S. L. Hladun, J. R. Hogan, Y. S. Hong, J. Hoover, O. Jaillon, Z. Ke, C. Kodira, E. Kokoza, A. Koutsos, I. Letunic, A. Levitsky, Y. Liang, J. J. Lin, N. F. Lobo, J. R. Lopez, J. A. Malek, T. C. McIntosh, S. Meister, J. Miller, C. Mobarry, E. Mongin, S. D. Murphy, D. A. O'Brochta, C. Pfannkoch, R. Qi, M. A. Regier, K. Remington, H. Shao, M. V. Sharakhova, C. D. Sitter, J. Shetty, T. J. Smith, R. Strong, J. Sun, D. Thomasova, L. Q. Ton, P. Topalis, Z. Tu, M. F. Unger, B. Walenz, A. Wang, J. Wang, M. Wang, X. Wang, K. J. Woodford, J. R. Wortman, M. Wu, A. Yao, E. M. Zdobnov, H. Zhang, Q. Zhao, et al. 2002. The genome sequence of the malaria mosquito Anopheles gambiae. *Science 298:129*.
64. Lehane, M. J., S. Aksoy, W. Gibson, A. Kerhornou, M. Berriman, J. Hamilton, M. B. Soares, M. F. Bonaldo, S. Lehane, and N. Hall. 2003. Adult midgut expressed sequence tags from the tsetse fly Glossina morsitans morsitans and expression analysis of putative immune response genes. *Genome Biol 4:R63*.
65. Osta, M. A., G. K. Christophides, D. Vlachou, and F. C. Kafatos. 2004. Innate immunity in the malaria vector Anopheles gambiae: comparative and functional genomics. *J Exp Biol 207:2551*.
66. Kaufman, T. C., D. W. Severson, and G. E. Robinson. 2002. The Anopheles genome and comparative insect genomics. *Science 298:97*.
67. Ribeiro, J. M. 1987. Role of saliva in blood-feeding by arthropods. *Annu Rev Entomol 32:463*.
68. Valenzuela, J. G., M. Garfield, E. D. Rowton, and V. M. Pham. 2004. Identification of the most abundant secreted proteins from the salivary glands of the sand fly Lutzomyia longipalpis, vector of Leishmania chagasi. *J Exp Biol 207:3717*.
69. Christophides, G. K., E. Zdobnov, C. Barillas-Mury, E. Birney, S. Blandin, C. Blass, P. T. Brey, F. H. Collins, A. Danielli, G. Dimopoulos, C. Hetru, N. T. Hoa, J. A. Hoffmann, S. M. Kanzok, I. Letunic, E. A. Levashina, T. G. Loukeris, G. Lycett, S. Meister, K. Michel, L. F. Moita, H. M. Muller, M. A. Osta, S. M. Paskewitz, J. M. Reichhart, A. Rzhetsky, L. Troxler, K. D. Vernick, D. Vlachou, J. Volz, C. von Mering, J. Xu, L. Zheng, P. Bork, and F. C. Kafatos. 2002. Immunity-related genes and gene families in Anopheles gambiae. *Science 298:159*.
70. Dimopoulos, G., G. K. Christophides, S. Meister, J. Schultz, K. P. White, C. Barillas-Mury, and F. C. Kafatos. 2002. Genome expression analysis of Anopheles gambiae: responses to injury, bacterial challenge, and malaria infection. *Proc Natl Acad Sci U S A 99:8814*.
71. Dimopoulos, G. 2003. Insect immunity and its implication in mosquito-malaria interactions. *Cell Microbiol 5:3*.
72. Boulanger, N., C. Lowenberger, P. Volf, R. Ursic, L. Sigutova, L. Sabatier, M. Svobodova, S. M. Beverley, G. Spath, R. Brun, B. Pesson, and P. Bulet. 2004. Characterization of a defensin from the sand fly Phlebotomus duboscqi induced by challenge with bacteria or the protozoan parasite Leishmania major. *Infect Immun 72:7140*.
73. Ramalho-Ortigao, J. M., P. Temporal, S. M. de Oliveira, A. F. Barbosa, M. L. Vilela, E. F. Rangel, R. P. Brazil, and Y. M. Traub-Cseko. 2001. Characterization of constitutive and putative differentially expressed mRNAs by means of expressed sequence tags, differential display reverse transcriptase-PCR and randomly amplified polymorphic DNA-PCR from the sand fly vector Lutzomyia longipalpis. *Mem Inst Oswaldo Cruz 96:105*.
74. Ramalho-Ortigao, J. M., and Y. M. Traub-Cseko. 2003. Molecular characterization of Llchit1, a midgut chitinase cDNA from the leishmaniasis vector Lutzomyia longipalpis. *Insect Biochem Mol Biol 33:279*.
75. Grimaldi, G., Jr., R. B. Tesh, and D. McMahon-Pratt. 1989. A review of the geographic distribution and epidemiology of leishmaniasis in the New World. *Am J Trop Med Hyg 41:687*.
76. Lewis, D. J. 1974. The biology of Phlebotomidae in relation to leishmaniasis. *Annu Rev Entomol 19:363*.
77. Valenzuela, J. G. 2002. High-throughput approaches to study salivary proteins and genes from vectors of disease. *Insect Biochem Mol Biol 32:1199*.

78. Modi, G. B., and R. B. Tesh. 1983. A simple technique for mass rearing Lutzomyia longipalpis and Phlebotomus papatasi (Diptera: Psychodidae) in the laboratory. *J Med Entomol 20:568*.
79. Kreutzer, R. D., G. B. Modi, R. B. Tesh, and D. G. Young. 1987. Brain cell karyotypes of six species of New and Old World sand flies (Diptera: Psychodidae). *J Med Entomol 24:609*.
80. Nardon, C., M. Weiss, C. Vieira, and C. Biemont. 2003. Variation of the genome size estimate with environmental conditions in Drosophila melanogaster. *Cytometry A 55:43*.
81. Kreutzer, R. D., G. B. Modi, R. B. Tesh, and D. G. Young. 1987. Brain cell karyotypes of six species of New and Old World sand flies (Diptera: Psychodidae). *J Med Entomol 24:609*.
82. Yin, H., J. P. Mutebi, S. Marriott, and G. C. Lanzaro. 1999. Metaphase karyotypes and G-banding in sandflies of the Lutzomyia longipalpis complex. *Med Vet Entomol 13:72*.
83. Jimenez, M. E., F. J. Bello, C. Ferro, and E. Cardenas. 2001. Brain cell karyotype of the phlebotomine sand fly Lutzomyia shannoni (Dyar) (Diptera: Psychodidae). *Mem Inst Oswaldo Cruz 96:379*.
84. White, G. B., and R. Killick-Kendrick. 1975. Proceedings: Demonstration of giant chromosomes in the sandfly Lutzomyia longipalpis (Lutz and Neiva, 1912). *Trans R Soc Trop Med Hyg 69:427*.
85. Ito, J., A. Ghosh, L. A. Moreira, E. A. Wimmer, and M. Jacobs-Lorena. 2002. Transgenic anopheline mosquitoes impaired in transmission of a malaria parasite. *Nature 417:452*.
86. Atkinson, P. W., and A. A. James. 2002. Germline transformants spreading out to many insect species. *Adv Genet 47:49*.
87. Yoshida, S., H. Matsuoka, E. Luo, K. Iwai, M. Arai, R. E. Sinden, and A. Ishii. 1999. A single-chain antibody fragment specific for the Plasmodium berghei ookinete protein Pbs21 confers transmission blockade in the mosquito midgut. *Mol Biochem Parasitol 104:195*.
88. Stowers, A. W., D. B. Keister, O. Muratova, and D. C. Kaslow. 2000. A region of Plasmodium falciparum antigen Pfs25 that is the target of highly potent transmission-blocking antibodies. *Infect Immun 68:5530*.
89. Barreau, C., M. Touray, P. F. Pimenta, L. H. Miller, and K. D. Vernick. 1995. Plasmodium gallinaceum: sporozoite invasion of Aedes aegypti salivary glands is inhibited by anti-gland antibodies and by lectins. *Exp Parasitol 81:332*.
90. Lal, A. A., P. S. Patterson, J. B. Sacci, J. A. Vaughan, C. Paul, W. E. Collins, R. A. Wirtz, and A. F. Azad. 2001. Anti-mosquito midgut antibodies block development of Plasmodium falciparum and Plasmodium vivax in multiple species of Anopheles mosquitoes and reduce vector fecundity and survivorship. *Proc Natl Acad Sci U S A 98:5228*.
91. Durvasula, R. V., A. Gumbs, A. Panackal, O. Kruglov, S. Aksoy, R. B. Merrifield, F. F. Richards, and C. B. Beard. 1997. Prevention of insect-borne disease: an approach using transgenic symbiotic bacteria. *Proc Natl Acad Sci U S A 94:3274*.
92. Ono, M., H. R. Braig, L. E. Munstermann, C. Ferro, and S. L. O'Neill. 2001. Wolbachia infections of phlebotomine sand flies (Diptera: Psychodidae). *J Med Entomol 38:237*.
93. Ihler, G. M. 1996. Bartonella bacilliformis: dangerous pathogen slowly emerging from deep background. *FEMS Microbiol Lett 144:1*.
94. Blandin, S., L. F. Moita, T. Kocher, M. Wilm, F. C. Kafatos, and E. A. Levashina. 2002. Reverse genetics in the mosquito Anopheles gambiae: targeted disruption of the Defensin gene. *EMBO Rep 3:852*.
95. Blandin, S., S. H. Shiao, L. F. Moita, C. J. Janse, A. P. Waters, F. C. Kafatos, and E. A. Levashina. 2004. Complement-like protein TEP1 is a determinant of vectorial capacity in the malaria vector Anopheles gambiae. *Cell 116:661*.
96. Aljamali, M. N., A. D. Bior, J. R. Sauer, and R. C. Essenberg. 2003. RNA interference in ticks: a study using histamine binding protein dsRNA in the female tick Amblyomma americanum. *Insect Mol Biol 12:299*.
97. Narasimhan, S., R. R. Montgomery, K. DePonte, C. Tschudi, N. Marcantonio, J. F. Anderson, J. R. Sauer, M. Cappello, F. S. Kantor, and E. Fikrig. 2004. Disruption of Ixodes scapularis anticoagulation by using RNA interference. *Proc Natl Acad Sci U S A 101:1141*.
98. Pal, U., X. Li, T. Wang, R. R. Montgomery, N. Ramamoorthi, A. M. Desilva, F. Bao, X. Yang, M. Pypaert, D. Pradhan, F. S. Kantor, S. Telford, J. F. Anderson, and E. Fikrig. 2004. TROSPA, an Ixodes scapularis receptor for Borrelia burgdorferi. *Cell 119:457*.
99. Ramalho-Ortigao, J. M., S. Kamhawi, E. D. Rowton, J. M. Ribeiro, and J. G. Valenzuela. 2003. Cloning and characterization of trypsin- and chymotrypsin-like proteases from the midgut of the sand fly vector Phlebotomus papatasi. *Insect Biochem Mol Biol 33:163*.
100. Besansky, N. J., and J. R. Powell. 1992. Reassociation kinetics of Anopheles gambiae (Diptera: Culicidae) DNA. *J Med Entomol 29:125*.