The Case for Sequencing the Genome of the Blood-Feeding Hemipteran Insect, *Rhodnius prolixus*

***Rhodnius* Research Community- Re: Genome Sequencing :**

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Rationale:

There is strong justification to sequence the genome of this remarkable insect because it impacts human biology on many levels and sequence information will contribute to significant advances in biomedical and biological research. The multifold rationale includes: (1) the disease impact of *Rhodnius prolixus* as a vector for Chagas' disease; (2) the significant potential for development of novel biopharmaceuticals; (3) basic cell biology and physiology particularly amenable to research in *Rhodnius,* such as ion and water transport, evolution of cellular immunity, cell-cell interactions, cytoskeletal biology, reproductive physiology, germ cell biology; (4) Comparative genomics and evolutionary biology as, despite the importance of insects in particular in the biological world, no hemipteran or any other hemimetabolous insect has been sequenced. Sequences available or currently being done include only holometabolous insects of the Diptera, Lepidoptera, Hymenoptera and Coleoptera. Considering the millions of years when evolutionary divergence occurred in these insect groups (eg, Diptera and Lepidoptera some 290 – 350 million years) and the fact that the following array of insects sequenced, in progress or planned (*Drosophila melanogaster and 10 other related species;, Anopheles gambiae, Aedes egypti and A. triseriatus, A. albopictus, and Culex pipiens., Bomby morix, Manduca sexta, Tribolium castaneum and Apis mellifera*) includes no hemipterans there

is a need to address this. *Rhodnius* is an especially excellent sequencing candidate, as it is of major medical importance and also provide many other biomedical and biological opportunities as we hope to make clear in this proposal.

Availability of these other genomes will allow identification of evolutionarily conserved genes, investigation of evolutionary divergence and mechanisms underlying cellular processes associated with cell division, chromosome structure and function and with feeding, digestion, excretion and reproduction in blood-feeding insects (mosquitoes, *Rhodnius*) relative to those with phytophagous diets (*Drosophila, Bombyx*).

Since our initial submission there has been much progress, growth in the research community and initiation of an international triatomine genome sequencing consortium with the aim of integrating and coordination efforts of the community to advance the prospect of having a reduviid hemipteran like *Rhodnius* and related species sequenced. In Nov. 2003 an initial group of 14 scientists from all countries of MERCOSUR met in Montevideo to discuss and decide the first steps of a work plan that should lead in the long term to the sequencing of the genome of different species of Triatominae, important for Chagas disease transmission in Latin-America. This group, which included a number involved in our original proposal and the those of us outside South America have the same overall goals. We have joined forces and are combining into a larger broader international community. Progress to date has been excellent and the concern of the review panel about whether or not the genomic infrastructure (eg. cDNA's, ESTs etc) is in place to exploit *Rhodnius* as a model for biological studies has also been addressed.

The overall objectives of the genome sequencing we all share are as follows: (1) To generate knowledge about the biology of these important Chagas disease vectors and about the vector-parasite interactions. (2) To develop further tools and insights for the design of vector control measures. (3) To exploit the biopharmaceutical potential molecules from *Rhodnius* offer. (4) To study phylogeny and allow for comparative arthropod genomics. (5) To develop tools for genetic manipulation of these vectors. (6) To provide the basic information necessary for proteomic studies. (7) To study tissue and organ specific expression of genes, their functional roles and to provide, through EST sequencing, information about intron/exon boundaries and alternative splicing

Achieving these goals will require work on: (1) full genome sequencing (this proposal). (2) Construction of genomic libraries using DNA from eggs in final stage before eclosion, to avoid contamination with exogenous DNA. This will be DNA source for genome sequencing. (3) Establishment of centers for maintaining triatomine colonies and certification (by means of allozymes and mitochondrial DNA markers and karyotype, chromosome banding and FISH.) This has been done. (4) Characterization of repetitive sequences (content, quantity, dispersion), further detailed analysis of genome size, chromosome structure and content, and identification of markers and definition of linkage groups etc., using ESTs and other markers. Such research will depend on and be facilitated by having the genome sequence data. Some work already underway in various labs. (5) Construction of EST libraries from *R. prolixus* and preliminary sequencing with emphasis of the digestive tract, heart, follicular epithelium, fat body, nervous system, ovaries, embryos, malpighian tubules and haematocytes. Some progress already made. (6) Functional studies: Macroarray and microarray of EST clones for characterization and expression studies are envisaged. A proteomics approachs for expression. Identification of marker genes for various cell processes. RNAi perturbations to illucidate gene function, etc.

We are strongly committed to collaborate internationally to achieve these goals, and to coordinate efforts globally. With the work underway, the tools now available we are at a cross

roads to build on the rich legacy of functional information derived from the efforts of *Rhodnius* researchers worldwide that has accumulated in literature spanning more than 50 years. The community is now particularly well poised to utilize full genome sequence data. A high level of enthusiasm and support for this initiative exists and is increasing, as all of us see a need for sequence information and the significant benefits that would accrue from it. There is already a broad based scientific community including scientists from USA, Argentina, Brazil, Canada, Chile, Columbia, France, Uruguay, and Paraguay with the expectation others will join as this initiative continues to grow. Involved are universities, research institutes and the Washington Univ, Genome Sequencing Center as well as the Pasteur Institute and with supporting interest (CDC and NIAID).

Evidence of genomic infrastructure and potential to capitalize on sequence information is evident as individual groups have published sequences for individual genes, such as the aquaporin gene in the *Rhodnius* Malpighian tubules and in genes coding for various factors necessary for blood-feeding (anticoagulants, vasodilators, platelet aggregation inhibitors and immune response modulators) and other work mentioned later in this revised proposal..

Under the auspices of either the South American Consortium (coordinated by the Rio de Janiero group) or in the USA at Washington Univ or in Canada at U of Manitoba (yet to be determined) we plan a meeting/workshop in 2005 to integrate the various international research groups and individual labs into a Global *Rhodnius prolixus* Genome Project Consortium with the aim to promote and facilitate international cooperation and collaborations and insure integration of the genome sequencing plan. The foundation for this has already been set by the multi-country South American group. This will foster discovery of new technologies and tools to control this medically important vector and illucidate fundamental biological processes that have far reaching biomedical significance to man and provide new information important for comparative genomics and evolutionary biology.

Rhodnius prolixus offers a unique opportunity for multifacetted benefits to arise from having the genome sequenced. Exciting new work these past two years including generation of EST's, libraries, new proteomics, fundamental cell biology research and so on, undertaken by a dedicated and active vibrant international group of *Rhodnius* researchers provides concrete evidence that the community in general is well poised to utilize and exploit sequencing information. Total genome sequencing funded by NIH would play a crucial role in advancing the many efforts now underway such as those involving karyotyping, tissue gene expression studies, tissue specific gene markers, tissue specific cDNA libraries, and tissue specific EST' work already underway to progress and yield significant advances.

The time is right. There is an opportunity to build on the international efforts already begun by the South American community to make this truly a global effort. We invisage this will involve international coordination with other initiatives that are in planning or early stages of start-up; these include the NHGRI (we hope)– Washington University and University of Manitoba, Winnipeg and other Canadian and USA and South American consortium of research labs; the Pasteur Institute -Genopole, EC; CDC - Ellen Dotson; and the TIGR - Najib El Sayed. The magnitude to the total genome sequencing requires a true international effort.

The extensive experience and capabilities of the Washington U Genome Sequencing Facility is vital. Considering the magnitude of the task and the capacity and expertise at the St. Louis facility, we anticipate the major sequencing activity to be focussed at the Washington U Sequencing Facility, with coordinated activity and effort also involving sequencing activities presently underway at the Pasteur Institute and certain South American research labs.

An annotation system and well designed data storage and aquisition system that is user friendly, and readily available to all researchers has already been established in Rio de Janeiro (elaborated on below). We feel the community is in an excellent position to capitalize on the availablity of the genome sequence data. There is an air of excitement and cooperative enthusiasm in the community as well as interest in the CDC (Ellen Dodson) and certain NIAID labs at the prospect of this genome project comming to fruition.

The sequencing of *Rhodnius* will provide a central model which will be complimented by physical mapping and sequencing of EST's in various other reduviids that are also important vectors for Chagas disease. *Rhodnius* is the ideal choice for sequencing as it has a smaller genome size compared with other reduviids, there is a wealth of molecular and biochemical work already done or in progress , and significant potential to link sequence data to stucture and function in basic cell biology and physiology . As this would be the first hemimetalous insect and first hemiteran sequenced it would fill a major void in comparative genomics as well.

As the genome is sequenced an array of post genomic tools can be brought to bear on to use the technologies of genomics towards devising new approaches towards *R. prolixus* control as it is a major disease vector and also for investigating selected cell biology and physiology problems that are especially accentuated in *Rhodnius*. Tools include transformation (transposons were recently discovered in reduviids); bioinformatics; transcriptone analysis (eg. Ribeiro publications); disruption of gene function with RNAi (Manitoba, Zoology has just recruited an RNAi expert); and use of various expression systems . The implications and applications span - disease transmission; molecular diagnostics and biopharmaceuticals; germ cell biology, development, cell biology, biodiversity and speciation, and evolution .

With the above update and overview of the rationale and the research community the following sections briefly summarize information of the biology of *Rhodnius* and the reduviid group of hemipterans it belongs to, and details indicating why we feel this is an ideal candidate for genome sequencing and on the substantive community worldwide that can capitalize on sequence information now and into the foreseeable future. Some of these were more fully iterated in the initial proposal but are abbreviated here due to space restriction.

General overview of the biology of *Rhodnius prolixus:*

This insect is a member of the Reduviidae hemipterans native to the Americas. They are hemimetabolous insects having 5 nymphal instars and the adult stage. They are highly evolved efficient blood feeders with the blood meal being crucial to molting, progression through the life cycle and reproduction. The fact that their life cycle and basic biology is so highly regulated by a blood meal led the renowned insect physiologist Sir V.B. Wigglesworth to use *Rhodnius* as an experimental lab model. His work yielded many key discoveries about how insect molting, growth and reproduction are regulated.

The order Hemiptera, Suborder heteroptera, family Reduviidae, subfamily Triatominae contains 5-6 tribes, with 17 genera, counting more than 130 species. The genera *Rhodniu*s, *Panstrongylu*s and *Triatom*a are the most important as Chagas disease vectors, of which the primary representatives are *R. prolixus* (present in Brazil, Colombia, Venezuela, Guyana, Central America and the southern Mexico), *Triatoma dimidiata* (present in Mexico, Central America, Colombia, Ecuador, Peru), and *Triatoma infestans* (present in Argentina, Paraguay, Brazil and Bolivia). *R. prolixus*, has a shorter lifecycle (3 months versus 6 months for T. infestans), and is easier to rear and there is considerable ongoing biological and biochemical research on *Rhodnius* including cDNA's and ESTs . For example Oliveira has two cDNA libraries partially sequenced,

one from whole midgut and another from ovarian follicular epithelium, with about 1000 reads being worked out for each; Ribeiro has a full inventory of salivary gland ESTs for the pharmacologically rich and diverse proteins of the Rhodnius salivary glands., Panzera has much work on chromosome structure, FISH mapping and genome size and many others have various functional studies underway.

Importance of *Rhodnius prolixus* as a disease vector:

Chagas' disease (American Trypanosomiasis), caused by the pathogen *Trypanosoma cruzi* and transmitted by *R. prolixus* and other reduviids, has broad medical and economic importance. The economic and social costs associated with disability and mortality in the endemic countries is enormous and will continue to be so because a high proportion of the 16 to 18 million persons already infected will develop chronic symptomatic Chagas' Some 50,000 will die annually with another 100 million at risk of contracting Chagas' (Kirchhoff, 03; CDC; WHO and others). The importance of reduviids as vectors is in their adoption of domiciliary habits, with *Rhodnius* being the most effective in adopting this mode (Schofield et al.., 99).

Chagas' disease is one of the triumvirate of parasitic diseases - Leishmaniasis, Chagas' and African Sleeping sickness - all caused by parasites of the Trypansomatidae family. The relationship between insect vectors and the parasites and pathogens they transmit involves complex interactions (Lowenberger's lab). Such host-pathogen interactions form the basis for physiological exchanges between organisms from different trophic levels, including the cellular and molecular interactions between parasites and host tissues. These interactions are obligate for the parasite. It must find a suitable host and within this host must recognize chemical signals, find the right receptors and enter or attach to the correct tissue. Then it must develop to a stage infective to a vertebrate host. Throughout this process it must avoid the consequences of activating the immune response of the insect host. Important considerations arise in considering the innate immune response in insect vectors of disease. We need to understand the physiological and molecular factors that allow insects to act as vectors, given that they possess a potent immune response that can eliminate most pathogens. Is the pathogen recognized as self, or is the pathogen inactivating or circumventing the immune response of the vector?

In order to understand Chagas' disease, one must have insight into the interactions between the trypanosomes and *R. prolixus*, especially, in terms of parasite development, multiplication, and transmission in the presence of the vectors normally potent immune response to parasites and pathogens. An important goal is to characterize the innate immune response of *R. prolixus,* namely to determine the repertoire of immune peptides used by this vector, the factors that regulate their expression and their role in determining vectorial capacity.

Much of our knowledge of insect immune peptides and their regulation is derived from studies done with *Drosophila.* Recent studies have demonstrated strong similarities between the signal transduction pathways of the innate immune response of insects and the acute phase response of vertebrates. Lowenberger's research on mosquito-borne pathogens involves screens of the *Drosophila* genome, and more recently the *Anopheles gambiae* genome, for sequence similarities and indication of gene function. As these insects are both higher Diptera they are best used for comparing sequences from other Diptera. There is no available genome for hemimetabolous insects such as *R. prolixus*. In addition, due to differences in evolution and development *R. prolixus* may have peptides with similar functions but with nucleotide sequence significantly different than existing sequences in the databanks. For instance, Lowenberger recently sequenced a defensin gene from *R. prolixus*. Whereas certain regions of the coding sequence for the mature peptide shared about 40% identity with defensins in the databanks, the

pre-pro sequence did not share significant identity with ANY sequence in Genbank at the nucleotide or amino acid level (Lopez et al.., 03). Thus while the peptides share certain characteristics there was no conservation in the pre-pro region. This is surprising as it occurs in one of the most highly conserved of the insect immune peptide families.

Genome information is required for genome-wide screens of gene expression in host tissues that interact with the parasite. These interactions are crucial for parasite maturation and infectivity. Sequencing the *R. prolixus* genome will hasten progress and permit mining the genomic data. Areas that are conserved in the active regions of the peptide could be used to screen the genome for alleles or multiple genes. For example peptides or other compounds expressed in the midgut, a tissue extremely importantant parasite development, could be identified and exploited to reduce the susceptibility of *R. prolixus* to parasite invasion and establishment. The hind gut is critical for development of infective stages. Adenyl cyclase activation and cAMP signaling triggers parasite metacyclogenesis (Salmon). Integration of the *Rhodnius* genome sequence with DNA-microarray analysis will allow better understanding of the intracellular signaling pathways and their role in the host-parasite interaction.

In terms of control of the vectorial transmission in Chagas' disease the benefits that could result from this genome project are enormous. Since parasite and vector biology are inextricably linked, knowing the *Rhodnius* genome would also complement the work of Urmenyi in Brazil and investigators at NIAID, CDC and other agencies that are deciphering the *T.cruzi* genome and genetics. This approach would parallel that used for studies of malaria, where determining the *Anopheles gambiae* genome has been viewed as a major contribution to the efforts combating mosquito-borne diseases. See also Toure et al. 04. Current methods of Chagas' disease control rely mainly on elimination of the domestic vector populations. Lanfredi, a parasitologist and participant in this application, has interest in this project since it will provide the genome of an important Chagas' disease vector, strengthen our understanding of Triatomines development and phylogeny, and identify new targets for insecticide development aiding the control of pathogen transmission. Novel methods of control will require detailed understanding of the molecular biology of development, reproduction, physiology and behavior of the vector. Between the CDC in Atlanta, the NIH, the WHO, and other groups there is ample basic, epidemological and human impact information available to justify the importance of knowing the genome of both the parasite and the vector. The availability of the genome will accelerate identification and exploitation of new target genes in this insect vector. This provides unique opportunities to improve on existing vector control tools and to generate new tools within a global partnership.

Importance for the development of novel biopharmaceuticals:

Because these insects have evolved a multitude of exquisitely effective molecules that serve as anticoagulants, vasodilators and modulators of other hemodynamic processes, a tremendous potential exists to exploit such compounds for treatment of human medical conditions. These agents have been characterized through elegant studies of the salivary glands and feeding mechanisms done by Ribeiro and co-workers at the NIH and elsewhere. Ribeiro is supportive of our quest to have the entire genome sequenced. These insects have a sophisticated cocktail of potent pharmacological compounds. Recent advances in transcriptome and proteome research allows unprecidented insight into the complexity of these compounds and that their molecular diversity as well as diversity of their targets is still larger than previously thought. *Rhodnius* is well equiped to disarm the host's hemostatic machinery, triggered to prevent blood loss following tissue injury such as neutralization of nucleotide release by injured cells; neutralization of vasoconstrictors, stimulation of vasodilation and inhibition of pllatlet

aggregation. Ribeiro et al.'s 04 paper on the Sialome of *Rhodnius* provides a complete inventory of the salivary gland proteins including a number of new ones illucidated from EST sequences. See also the review Ribeiro and Francischetti 03.

The importance of *R. prolixus* as a vector of Chagas' disease depends on its lifestyle as a blood feeder. In this regard, knowledge of the interactions that allow the insect to circumvent the hemostatic and immune responses of its vertebrate hosts is critical. Numerous salivary proteins that contribute to these interactions have been described. Saliva from this insect was shown to release the vasoldilator nitric oxide (NO), and in fact this was the first report of NO production in any insect (Ribeiro et al.., 90). Subsequently it was shown that the NO was ligated to heme proteins, and these proteins have several remarkable or even unique properties including fully reversible NO binding which is pH dependent (tight binding at low pH, ready release at high pH), and pronounced resistance to autooxidation (Ribeiro et al.., 93). A series of four NO-binding heme proteins were purified and cloned from salivary glands of fifth-instar nymphs (Champagne et al.., 95) and given the name "nitrophorins". The ability to express large amounts of functional recombinant nitrophorin (Andersen et al.., 97) led to the solution of a crystal structure for three of the four known nitrophorins (Weichsel et al.. 98; Andersen et al.., 98; Andersen and Montfort 00). Moreira et al.., (03) have investigated the changes in nitrophorin profile during the *Rhodnius* life cycle. The pH dependence of NO binding is now understood in terms of a conformational change in the structure of the protein that results in trapping of the NO in association with the heme (Weichsel et al.,00). This provides an elegant storage and delivery system for the NO: it is stored tightly bound to nitrophorin in the acid (pH 5) environment of the salivary gland, and when injected into the skin the pH shifts to between 7 and 7.4, the protein conformation changes, and the NO dissociates. Ultimately the NO produces vasodilation in the blood vessels by activating guanylate cyclase and increasing cGMP levels in the muscle surrounding the vessel.

Other remarkable properties are also associated with the nitrophorins. These molecules have a high affinity for histamine (Ribeiro and Walker, 94) and can out-compete the vertebrate histamine receptor for its ligand. As a result, the itching and pain that results from histamine is blocked for as long as the insect is feeding and injecting saliva, so the host remains unaware of the attack. In addition, nitrophorin 2 inhibits clotting by preventing formation of the complex that converts inactive factor X to the activated Xa, dramatically slowing thrombin formation and ultimately the entire clotting cascade (Ribeiro et al.., 1994; Sun et al.., 1996; Zhang et al.., 1998). The other nitrophorins lack this activity or participate very weakly. Nitrophorins also have thiol oxidase activity that may contribute to antihemostatic functions (Ribeiro, 1995). Another of the multiple functions of NO is as a microbicide as being studied by Silva-Neto.

In addition to the nitrophorins, a series of anti-platelet activities are present that give a novel perspective on the behavior of complex and redundant systems in antagonizing vertebrate hemostasis. Normally platelets respond to ADP released at an injury site, resulting in aggregation, activation, and release of additional ADP (as well as other factors) to promote formation of a platelet plug. A salivary apyrase hydrolyses ADP and reduces concentrations of this platelet agonist from mM to nM levels at the site of feeding (Ribeiro and Garcia, 1980). The apyrase becomes inefficient at ADP concentrations below 100 nM, but platelets can still respond to this level of ADP. To reduce ADP to sub-physiological levels, *Rhodnius* saliva has a series of lipocalins (RPAIs - *Rhodnius* platelet aggregation inhibitors) which bind ADP in a stochiometric manner (Francischetti et al.., 00, 02, Ribeiro et al. 04). Cooperative action of the apyrase and the RPAIs is required to deplete ADP to the point where platelet function is

inhibited. Recently lysophosphatidylcholine has also been described as another anti-hemostatic molecule in *Rhodnius* saliva in Atella's lab (Golodne et al.., in press).

A secreted form of inositol polyphosphatase is also abundantly expressed, but its function is unclear (Champagne unpublished data). Saliva also suppresses the proliferation of mitogen-stimulated T-cells, but the agent responsible has not been characterized.

From the above, it is clear that *Rhodnius* saliva is a potentially rich source of novel pharmaceutical agents for use in cardiovascular and immunological medicine. The range of compounds may be even larger, as recent work suggests that each developmental stage of the insect produces novel compounds. Antibodies directed against antigens from one stage may well be ineffective against any other stage. As well, *Rhodnius* can exploit many vertebrate hosts for blood, and many of these function as zoonotic reservoirs of Chagas' disease. It is likely that some diversity of salivary molecules is necessary to deal with the varying physiologies of the different hosts.

Regulatory regions for the salivary molecules have not been characterized, and only a genome sequence offers much hope of an understanding of how the complex process of producing and replenishing salivary components is integrated into the physiology of the whole insect. This in turn could offer insight into aspects of the insect's biology that might be manipulated to help reduce its role as a Chagas' vector.

The ingestion of large volumes of blood in a single meal and hydrolysis of vertebrate hemoglobin in the midgut results in the release of very high amounts of heme, which has been shown to be a powerful generator of free radicals (Ritter and Tyrrel, 00). Oliveira has suggested that during their evolutionary history these animals must have developed efficient mechanisms to counteract these deleterious effects. His lab investigates antioxidant mechanisms and heme detoxification pathways (Oliveira et al.., 99, 00,02) including antioxidant enzymes; catalases, superoxide dismutases, glutathione peroxidases, thioredoxin, thioredoxin reductases; enzymes of the urate synthesis pathway (urate is a free radical scavenger); heme oxygenases and proteins involved in aggregation of heme into the sequestering molecule, an insoluble dark brown pigment hemozoin (a process occurring in the gut lumen). Their interests include studying control of gene expression after an oxidative challenge and after a blood meal. Presently they are sequencing ESTs from a midgut cDNA library of adult *Rhodnius* females, a project that has synergism with the genome project. Additionally, to deal with metabolic consequences of blood feeding, the excretory system is adapted to transport specific waste products of blood metabolism and excrete the nitrogenous waste uric acid and potentially toxic organic anions resulting from catabolism of amino acids after the blood meal (Maddrell and Gardiner, 75; O'Donnell et al.., 83).

Other potential areas for exploitation include various CRF-like diuretic peptides and other neuropeptides and circadian clock proteins as studied in the laboratories of Orchard and Steel. Similarly researchers like O'Donnell, Orchard and Morales working on ion and water transporters and their regulation could reveal novel approaches to deal with various human diseases involving membrane transport impairment. Other "vertebrate" hormones and/or receptors for them are also reported in *Rhodnius,* opening other avenues as well. Sevala et al.., (92) reported insulin-like immunoreactivity and Kim et al.., (99) showed that vertebrate thyroid hormone T3 bound to follicle cell membranes.

Thus, knowing the *Rhodnius* genome would have a major impact on opening new avenues to exploit a host of biopharmaceuticals for human benefit.

Importance of *Rhodnius prolixus* for fundamental biological research:

It is ironic that a creature that causes so much human misery also provides us with an exquisite model to study cell biology because evolution has accentuated certain cell processes as a consequence of blood feeding and optimized reproductive strategies. The details relating to the various fundamental cell biology research problems *Rhodnius* provides a good model for have been reduced in this revised proposal due to space constraints, however more details can be found in the initial proposal.

Rhodnius has long served as an important physiological laboratory model. Since Wigglesworth's pioneering work on moulting and reproduction, a large body of functional literature has accumulated worldwide on a myriad of aspects including: studies of oogenesis and spermatogenesis (Huebner), the role of haemolymph proteins and lipids in oogenesis (Oliveira, Silva-Neto, Muchado, Atella, Gondim, and their co-workers) etc.

Recent exciting work on comparative triatomine genomics, chromosome structure, genome size and chromosome events in mitosis and meiosis in Panzera's lab highlight the impact genome sequence data would have and also strengthen the rationale for choosing *Rhodnius*. They are researching repetitive sequences, interspersed repeats or mobile elements or transposable elements, cytogenetics, the special holocentric chromosomal structure characteristic of hemipterans and the implications of this for mitosis and meiotic divisions (see Panzera et al. 98, Marcilla et al. 02, Perez et al. 97,00,02). They have cloned and characterized retrotranscriptasa sequences from several triatomines of *Triatoma* (Pérez et al.. 04) and identified a sequence which is very similar to the Het-A retrotransposon of *D. melanogaster*. This retrotransposon locates in the telomere in this species and can be a good candidate to analyse chromosomal ends in triatomines. Panzera proposes a similar approach to analyse retrotransposon in *Rhodnius*.

Other cell biology and cell physiology research areas that a number of *Rhodnius* researchers have focused on are briefly summarized below. Much of the basic structure and functional work is at a stage where the availability of genome sequence data and the array of molecular tools would greatly further the identification of tissue specific gene markers, gene function in cell differentiation regulation, identity and role of membrane transporters, germ cell markers, germ cell determination and hormonal regulation and receptor function to name a few.

These animals have evolved an impressive set of cellular ion and water transport mechanisms to insure homeostasis so they can cope with ingestion of a blood meal, which may equal 10 times an insect's unfed weight. Complex membrane transport and hormonal interactions involving the crop, Malpigian tubules and hindgut are instrumental. (details can be found in Reynolds, 75 Farmer et al.. 81, Maddrell and O'Donnell,92, Maddrell et al.., 1993, Haley et al.., 97; Haley and O'Donnell, 97, Quinlan et al.., 97, Te Brugge et al.., 02, Ianowski et al.., 02). Sequencing of the *Rhodnius* genome will permit precise molecular identification of the serotonin and CRF-related peptide receptors, adenylyl cyclase, and ion transporters such as the H+-ATPase and Na+:K+:2Cl- cotransporters. An important consequence of such studies is that the genes for specific ion transporters or receptors can be expressed in *Xenopus* oocytes or cell lines. Such expression studies will aid pharmacological characterization of the transporters and receptors and identification of novel agents which act as insecticides or alter the midgut environment essential for survival of *T. cruzi*. The various papers from the Morales lab have focussed on aquaporin (Falkenstein et al.., 02) and sodium potasium ATPase in Malpighian tubules this complements their research on kidneys (eg. Schweibert et al.. 98). Whittembury's lab has recently also identified a new water channel (RP-mip) (Echevarria et al.., 01). Research on regulation of molting and circadian rhythms is a focus in the Steel lab (Vafopoulou and Steel,

01)

Phytochemicals with hormonal, antihormonal or toxic effects show promise as potential compounds for control of *Rhodnius* and other triatomines. Sequencing the *Rhodnius* genome will permit precise identification of the mode of action of such compounds which is important for developing pesticides with increased efficacy and decreased effect on non-target organisms. Plants which produce juvenile hormone (JH) analogues or precocenes (which destroy the JH-producing cells), interfere with developmental or reproductive processes in *Rhodnius* (Azambuja and Garcia, 1991). Azadirachtins, isolated from the neem tree, inhibit moulting by blocking release or production of prothoracicotrophic hormone, which stimulates production of the moulting hormone ecdysone by the prothoracic glands (Gonzalez et al.., 99). Other phytochemicals are the lignans such as burchellin, which interefere with excretion both by direct effects on the Malpighian tubules and through effects on diuretic or antidiuretic hormone release (Garcia et al.., 00).

Rhodnius provides an excellent model for investigating the cell biology and regulation of reproduction as it has highly regulated reproduction and a unique ovariole cytoarchitecture. Labs of Huebner, Machado, Oliveira, Silva-Neto, Atella, Gondim and Masuda investigate many aspects of oogenesis and embryogenesis extensively. These include the storage and ulilization of various yolk proteins such as the vitellogenins and lipophorins as well as heme-binding proteins (Oliveira, Machado, Silva-Neto, Masuda) , the basic biology of oogenesis and embryogenesis (Matsuda, Huebner),the mobilization of yolk during embryogenesis (Machado), cell interaction and cytoskeletal dynamic during oogeneseis (Huebner), characterization and role of bioelectrical currents in cell polarity and differentiation (Huebner), germ cell determination and germ cell markers like Vasa (Huebner et al.., 02, CSH Germ Cell Conf.).

Hopefully the above, provides sufficient examples to show that sequencing the genome would impact a broad spectrum of important biological topics and greatly increase the utility of *R prolixus* as a model system. The wealth of functional literature already available would help make sense of genomic data and how specific biological functions are regulated and have evolved.

Importance to evolutionary biology and arthropod genome information:

Arthropods are the most morphologically diverse animal group on the planet. They produce positive and negative impacts upon all facets of human life. Insects are the dominant group of arthropods with an ancient history. Comparative insect genomics will be an important tool for understanding evolutionary biology. As noted earlier there is a pressing need to have the genome of a, hemimetabolous insect as genome comparisons show insect groups diverged considerably faster than vertebrates. Attempts to use *Drosophila* probes to isolate *Rhodnius* genes has been of limited success, as the divergence between them is significant. This, coupled with the considerable biomedical importance of *Rhodnius,* and its merits as an excellent model system underscores the need, value and impact sequencing the *R. prolixus* genome would have. As noted earlier, Lowenberger's results on the defensin gene demonstrate that functional similarities of certain peptides between species may differ considerably in nucleotide sequence (Lopez et al.., 03). As this occurs in one of the most highly conserved of the insect immune peptide families it highlights the need to sequence a hemimetabolous insect that clearly is far removed evolutionarily from *Drosophila*.

***Rhodnius* an ideal candidate for sequencing, choice of culture, and species certification:**

There are various *Rhodniu*s colonies worldwide. Recently it was discovered that some long standing colonoies thought to be *Rhodnius* were in fact not. So it is critical a stable, verified

homogeneous *Rhodnius* colony be used in all genome sequencing efforts. The Rio de Janeiro colony obtained from FIOCRUZ 25 years ago were classified as *Rhodnius prolixus* by classical entomologists, based on external morphology. However when insects were submitted to a PCR-based molecular analysis (as described by Monteiro et al. (03, Molecular Ecology 12, 997-1006) the results indicated that they were in fact *Rhodnius robustus.* *Prolixus* and *robustus* are cryptic species and genetic differences are relatively low. Some authors even describe then as a complex of species, similar to what happens with *Anopheles gambiae*. Nevertheless, the species (or sub-species) that is more relevant for Chagas disease transmission is the *prolixus*. Other colonies that were previously classified as *prolixus* turned out to be *robustus* by this PCR analysis. The Winnipeg *Rhodnius* colony originates from the original Wigglesworth Cambridge colony but has not been tested as to authenticate the species identification. Therefore, we intend to use a colony more recently collected in the field, what was obtained from Dr Célia Cordon-Rosales from Guatemala, a part of the Latin America where there *robustus* does not exist. This colony is now in place in Rio de Janeiro. Verifiable populations of *R. prolixus* showed a very low nucleotide diversity using 26 populations from seven Latin American countries (Monteiro et al.., 03). Some preliminary sequencing has already been done using genomic DNA from the Huebner lab colony and also the Rio de Janeiro colony. To avoid potential problems the Rio colony will be used for the full genome sequencing effort. Also to avoid contamination with bacterial symbiont or other exogenous DNA, the DNA source will be from late stage pre-eclosion embryos. Various centers have been set up to maintain certified triatomine colonies. For *Rhodnius* these are: Federal Univ, of Rio de Janeiro – Dept. of Biochemistry (Pedro de Oliveira) and Fiocruz Department for Biochemistry and Molecular Biology (Patricia Azambuja); Universidad de Chile – Facultad de Medicina – Programa de Biologia Celular e Molecular (Aldo Solari; Universidad de la Republica – Facultad de Ciencias – Department for Biology (Francisco Panzera). Once the Winnipeg colony has been certified it could provide an additional center. Thus there is ample back-up should a disaster hit any colony. An incubation malfunction recently greatly depleted the Winnipeg colony for example. Certification of *Rhodnius* identifications will be carried out by Fernando Monteiro (Fiocruz): allozymes and mitochondrial DNA markers and Francisco Panzera : karyotype, chromosome banding and FISH.

Thus of the most relevant as vectors for Chagas disease, *Rhodnius* is the best choice for genome sequencing due to its homogeneity and smaller genome size.

Genome size:

Insect genome sizes vary considerably for example; *Drosophila* about 180MBp; *Anopheles gambiae* 260 MBp; *Bombyx mori* 530 MBp; *Manduca sexta* 530 MBp, *R. prolixus* 670 MBp; *Triatoma infestan* between 1.3 and 1.8 GBp and *Triatoma dimidiata* 955MBp

There has been scarce and contradictory information about genomic size in triatomines however recent work of Panzera et al. (04) has clarified this. The haploid genome in Triatominae varied from 0.686 pg (*R. prolixus*) to 2.666 pg (*Triatoma delpontei*). Members of *Rhodnius* genus have the lowest values of all the species analysed (Panzera et al.. 2004b). The estimate in the initial White Paper of 800 MBp turned out to only be a modest overestimate as the new work now shows the *R. prolixus* genome to be 670 MBp (larger than other insects sequenced thus far).

Annotation, Data Base, Access and dispersion of data and other resources:

A web site will be constructed to provide access to information about triatomine genomics and biology (http://www.triatomine.org), as well as an initial sequence database will be established (http://www.triatomineDB.org). A list server will be established

(triatomine_amsud@fiocruz.br) to promote discussion, interaction and planning.

The annotation of *Rhodnius* will done through a platform named 'Bionotes' (www.bionotes.com.br) already in place in Rio de Janeiro. BioNotes is an annotation system which is under continual development at the Virtual Bioinformatics Institute of Rio de Janeiro, and is being used also in the genome project of *Gluconacetobacter diazotrophicus*. Researchers can access annotations stored in public data sources; execute analysis programs, manually generate new annotations; and analyze current annotations via a user-friendly interface. Researchers can navigate through hyperlinks to annotations stored in external public data sources. For example, the researcher can see a result from BLAST and click on the name of one of the similar sequences to look at the annotations of that similar sequence which are stored in the NCBI database. The researchers can also locate annotations by 'drilling down' the genome. While a genome project is in progress, sequencing of the genome will generate new reads. As a consequence, BioNotes re-executes the analysis programs to generate new versions of the data and offers tools to transfer manual annotations from older to newer versions of the data, as otherwise it would be very discouraging to annotate data from on-going sequencing projects. Besides that, the public data sources are also being constantly updated, so, to remain up-to-date, BioNotes refreshes its data warehouse from time to time by re-accessing the data sources to retrieve new data. Since BioNotes has a Web interface, data distribution is possible. This fact is important because it makes the data access, control, sharing and interpretation more efficient. If other prospects need to be considered this will be done in consultations with the Washington Univ. Sequencing facility.

Individuals of the research community have their individual research grants that provide a broad foundation of peripheral support in resources, additionally a grant has been approved in Argentina for Shanchez for sequencing ESTs from *Triatoma*. The South American group has submited a grant proposal for an EST project to sequence and annotate 15 tissue-specific cDNA libraries to the Brazilian Funding agency (CNPq). Potential assistance in funding from Genome Canada is also being explored by E. Huebner. There is also a small grant as seed money from the Pasteur Institute for AMSUD to certain South American Labs. Clearly, to accomplish a complete genome sequencing requires a much larger sequencing effort. So funding the Washington U facility is essential. At this time interest and initial start-up or planning involves this proposal to the NHGRI with Washington University Sequencing Facility and more limited activity possibly including the Pasteur Institute-Genopole, EC , the CDC - Ellen Dotson, the TIGR - Najib El Sayed.

Sequencing Strategy and Cost:

The Washington University Genome Sequencing Center has extensive experience mapping and sequencing genomes in the range of 5 to 3000 Mb. For the *R. prolixus* genome, with an estimated size of 670 Mb, we would propose a BAC-based physical mapping effort, paired with a whole genome sequencing (WGS) phase composed of reads generated from fosmid and plasmid clones.

BAC and fosmid libraries would be prepared by the CHORI lab (Pieter de Jong). For fosmid libraries, we are now using the copy control vector pCC1FOS. This allows us to maintain clones as single copy to minimize deletion and rearrangement events, as well as to induce high-copy growth for template preparation. With this system, we are able to purify and sequence fosmid clones at the same high rate and low cost as plasmid clones. 80,000 BAC clones would be fingerprinted using standard methods to produce 10X physical coverage of the *R. prolixus* genome. We would further propose that end sequences be generated for

approximately 10,000 of these BACs to provide links between the physical map and WGS scaffolds. Based on our recent experience with the chicken genome, we would expect that these resources would result in a physical map with excellent coverage and contiguity.

The WGS phase of the project would consist of approximately 10 million plasmid and one million fosmid reads. This would generate roughly 8X coverage of the genome sequence. The physical coverage in fosmid clones would be approximately 25-fold, thus providing the resource for any desired targeted or systematic finishing effort for this genome.

At this point, the genome sequence would be of sufficient contiguity and quality for preliminary analyses and gene discovery by comparative methods. To improve upon the utility of the sequence for these and other types of analyses, we would propose one or two rounds of automated primer-directed sequencing ("prefinishing") to extend sequence contigs and close a large fraction of gaps present in the initial assembly. BAC and fosmid clones would serve as the templates for the necessary sequencing reactions. The methods, computational tools and laboratory pipelines for automated finishing are already in place at the Washington University Genome Sequencing Center.

At this point, manual finishing could be employed for targeted regions (or the whole genome, if deemed necessary) to further improve contiguity and sequence accuracy. Our experience with finishing the mouse genome from a combined approach and initial finishing of the *C. briggsae* genome from a whole genome shotgun approach suggests that most of this work would involve using PCR to sequence and/or size regions that were missing, ambiguous or repetitive in content.

The cost for the project as we have outlined it here would be in the $16-20M range. Physical mapping would cost approximately $0.6M, including library construction and BAC end sequencing. The WGS phase would cost approximately $14.5M. A prefinishing phase could be added at a cost of approximately $1M.

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