UNIVERSITY OF CALIFORNIA, SAN DIEGO

Plasmodium Sexual Development and the Role of Plasmepsin X in *Plasmodium falciparum* transmission to *Anopheles gambiae*

A Dissertation submitted in partial satisfaction of the

Requirements for the Degree of Doctor of Philosophy

in

Biomedical Sciences

by

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2010

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2010

DEDICATION

In recognition of all those who have believed in me and helped me to reach this day:

- my parents, Kingngeun Bounkeua and Dara Strong, who taught me to be compassionate, dedicated and hardworking through their own example
- all of my family, especially my sister, Kay Bounkeua who lent countless hours of early-morning and late-night support, and my step-father, Jeffrey Strong
- my research mentors: Dr. Jeffrey Griffith from the University of New Mexico; Phil Rodacy, Pamela Walker and Susan Bender from Sandia National Laboratories; Dr. Michael Lenardo from the National Institutes of Health, and Dr. Joseph Vinetz, my thesis advisor
- my thesis committee, for their enthusiastic and thoughtful scientific input
- members of the Vinetz lab, for their teachings and for creating an engaging learning environment for the past 4 years, especially Dr. Fengwu Li and Dr. Michael Matthias, Kenny Pettersen, Paula Maguina, Dr. Christian Ganoza and Dr. Shira Abeles
- my graduate colleagues, for their technical help, moral encouragement and friendship, especially Neekesh Dharia, Jennifer Aron and Joshua Theisen
- my dear friends, who loved and supported me during this time: Emily Mathews, Sherry He, Ilya Gertsman, Caleb Stokes, and Tara Herrmann

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

2DGE – two-dimensional electrophoresis

6Fam – 6-carboxyfluorescein

BHQ – black hole quencher

bp – base pairs

cap – central apical pore

cry – crystalloid

CTRP – circumsporozoite and TRAP related protein

DAPI – 4',6-diamidino-2-phenylindole

DHFR – dihydrofolate reductase

DIG – digoxigenin

DMEM – Dulbecco's modified eagle's media

DNA – deoxyribonucleic acid

DOZI – development of zygote inhibited

DTT – dithiothreitol

dv – digestive vacuole

e – erythrocyte

EDTA – ethylenediaminetetraacetic acid

EGTA – ethylene glycol tetraacetic acid

ELISA – enzyme-linked immuno-sorbant assay

EM – electron microscopy

FCS – fetal calf serum

FITC – fluorescein isothiocyanate

FKBP – forkhead binding protein

gc – gametocyte

GEMS – gene-enrichment motif searching

gm – macrogamete

GO – gene ontology

GS – gamete exflagellation/emergence solution

h – hemozoin

HEPES – 4-(2-hydroxyethyl)-1-piperazineethanesulufonic acid

HS – heat inactivated human serum

IACUC – Institutional Animal Care and Use Committee

IFA – immunofluorescence assay

IgG – immunoglobulin subtype G

IgM – immunoglobulin subtype M

IMC – inner membrane complex

IP – immunoprecipitation

IPTG – isopropyl β-D-1-thiogalactopyranoside

kDa – kilodalton

KO – knockout

l – liter

LB-amp – laurel broth with ampicillin

LCCL – *Limulus* factor C, cochlear protein Coch-5b2, and late gestation lung protein Lgl1

Leukostat – modified Wright stain used to visualize *Plasmodium* parasites by light microscopy

m – microneme

M – molar, moles of reagent / liter of solution

mAb – monoclonal antibody

MALDI-TOF – matrix-assisted laser desorption/ionization time-of-flight

MCB – master cell bank

µF – microfarad

µg – microgram

ml – milliliter

µm – micrometer

mM – millimolar

MS – mass spectrometry

mt – mitochondria

MudPIT – multidimensional protein identification technology

n – nucleus

N – normal, gram equivalents of H+ ions / liter of solution

NF – Nijmegen *Falciparum* isolate

nm – nanometer

o – ookinete

OPI – ontology-based pattern identification

PAGE – polyacrylamide gel electrophoresis

PBS – phosphate buffered saline

pCAM-BSD – genetic integration construct containing blasticidin-S-deaminase

PCR – polymerase chain reaction

PFA – paraformaldehyde

Pfs16 – *Plasmodium falciparum* surface protein 16

Pfs25 – *Plasmodium falciparum* surface protein 25

pI – isoelectric point

pm – plasma membrane

PM – plasmepsin

pr – polar ring

pRBC – packed red blood cells

PRISMA – Projectos en informatica medicina salud y agricultura

Puf – Pumilio in *Drosophila melanogaster* and *fem*-3 binding factor in *Caenorhabditis elegans*

Pvg377 – *Plasmodium vivax* gamete protein 377

Pvs230 – *Plasmodium vivax* surface protein 230

Pvs28 – *Plasmodium vivax* surface protein 28

qPCR – quantitative polymerase chain reaction

qRT-PCR – quantitative reverse-transcriptase polymerase chain reaction

r – retort

RBC – red blood cell

RNA – ribonucleic acid

RPMI – Roswell Park Memorial Institute culture media

rPM X-GST – recombinant PM X fusion protein with GST tag

RT – room temperature

RT-PCR – reverse transcriptase polymerase chain reaction

SA – suspended animation solution

SDS – sodium dodecyl sulfate

SOAP – secreted ookinete adhesive protein

SP – signal peptide

spb – spindle pole body

SSC – sodium citrate and sodium chloride

TBS – tris buffered saline

TCA – trichloroacetic acid

TEM – transmission electron microscopy

TM – transmembrane domain

TRAP – thrombospondin-related adhesive protein

UV – ultraviolet

UTR – untranslated region

V – volts

V-gc – mature stage V gametocyte

WARP – von Willebrand Factor A domain-related protein

WBC – white blood cell

WCB – working cell bank

z – zygote, round diploid sexual stage form

LIST OF FIGURES

LIST OF TABLES

ACKNOWLEDGEMENTS

I would like to thank Dr. Joseph Vinetz for four years of support, providing an environment with unique, diverse research experiences, and for his scientific and career mentoring. I would like to acknowledge funding from the University of California San Diego Medical Scientist Training Program, the Biomedical Sciences Graduate Program, US Public Health Service grant T32GM008198 and National Institute of Allergy and Infectious Disease grant R01 AI0459999-06.

Chapter 2 and part of chapter 5 were submitted for publication: Bounkeua, V., Li, F., Prieto, J.H., Yates, J.R. III, Vinetz, J.M. "Biological features and proteomic analysis of *in vitro*-cultivated *Plasmodium falciparum* sexual stage parasites". The dissertation author was the primary author and investigator of this paper.

Chapter 3 will be submitted for publication: Bounkeua, V., Li, F., Abeles, S.R., McClean, C.M., Vinetz, J.M. "*In vitro Plasmodium vivax* ookinete development is not correlated to gametocyte maturity or sex ratio as determined by qRT-PCR". The dissertation author was the primary author and investigator of this paper.

Chapter 4 will be submitted for publication: Bounkeua, V., Pettersen, K.D., Vinetz, J.M. "The role of plasmepsin X in *Plasmodium* transmission to the mosquito vector". The dissertation author was the primary author and investigator of this paper.

Part of chapter 5 will be submitted for publication: Bounkeua, V., Li, F., Prieto, J.H., Yates, J.R. III, Vinetz, J.M. "Proteomic analysis of *Plasmodium vivax* macrogametes." The dissertation author was the primary author and investigator of this paper.

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ABSTRACT OF THE DISSERTATION

Plasmodium Sexual Development and the Role of Plasmepsin X in *Plasmodium falciparum* transmission to *Anopheles gambiae*

by

Viengngeun Bounkeua

Doctor of Philosophy in Biomedical Sciences

University of California, San Diego, 2010

Professor Joseph M. Vinetz, Chair

This work explored sexual development of the lethal human malaria parasite, *Plasmodium falciparum.* A method to cultivate *Plasmodium falciparum* sexual stage parasites *in vitro* was optimized and increased ookinete yield > 20-fold over previous reports. This method was adapted for *Plasmodium vivax,* a neglected human malaria parasite, and found to generate *Plasmodium vivax* ookinetes. The method was essential for investigation of the role of a novel aspartic protease, Plasmepsin X, in sexual stage parasite development. Antibodies to Plasmepsin X were found to significantly decrease *Plasmodium* transmission to mosquitoes by 20-40% *in vivo* but did not affect ookinete development *in vitro*. This was the first demonstration that a non-food vacuole Plasmepsin facilitated *Plasmodium falciparum* midgut infection of mosquitoes. Finally, the numbers of parasites generated with this method were

sufficient for proteomic analysis of sexual stage parasites. The *Plasmodium falciparum* zygote and ookinete proteome has never before been available, and this was the first time that any stage of *Plasmodium vivax* has been analyzed using a proteomic approach. The ability to generate *Plasmodium falciparum* sexual stage parasites, especially zygotes and ookinetes, has permitted detailed molecular analyses essential for transmission-blocking vaccine studies as well as global analyses that generated insights into *Plasmodium* sexual stage biology.

CHAPTER 1

INTRODUCTION

Malaria is a devastating disease that affects more than 500 million people and kills an estimated 900,000 people each year (*WHO, 2008*). Malaria is caused by infection with the *Plasmodium* parasite, and the majority of malaria is caused by two human malaria parasites, *Plasmodium falciparum* (*P. falciparum*), which causes severe and occasionally lethal disease, and *Plasmodium vivax* (*P. vivax*). Although *Plasmodium* can be treated with chemotherapeutics, resistance to commonly used anti-*Plasmodial* drugs is an increasing problem. Much of the morbidity associated with malaria infection is due to re-infections with the parasite (*Kita et al., 2009*). It is clear that the ability to control, let alone eradicate, malaria is dependent on new technologies to prevent malaria infection.

Despite intensive research efforts, the immune correlates of protection from malaria are poorly understood, and a vaccine has remained elusive. In the vertebrate host, *Plasmodium* spends the majority of its life cycle as an intracellular erythrocytic parasite, and much of malaria vaccine research is focused on the *Plasmodium* erythrocytic cycle (*Beeson et al., 2008*). However, as an intra-erythrocytic parasite, *Plasmodium* has limited exposure to both humoral and cellular immune responses (*Bannister et al., 2000*). Although there are reports of acquired immunity, whereby previously exposed adults are protected from severe malaria, this 'immunity' is short-lived. Previously 'immune' individuals that leave a malaria-endemic area for an extended period are as likely to develop severe malaria as naive individuals (*Gupta et al.,1999, Heyburn et al., 2005*).

Malaria vaccine research has recently focused on transmission-blocking vaccines, which would prevent *Plasmodium* infection of the mosquito vector (*Saul,*

2007). Transmission-blocking vaccines, like transmission-blocking interventions, are population-based approaches to reducing malaria burden. They would prevent people from transmitting the parasite to its *Anopheles* mosquito vector (*Greenwood et al., 2005, Greenwood, 2008*). Transmission-blocking vaccine strategies would target antigens expressed on or secreted from sexual stage parasites, which develop in the mosquito midgut. In this paradigm, individuals would be vaccinated against sexual stage parasite antigens. The resulting antibodies would then be ingested with infectious gametocytes during a mosquito blood meal. These ingested antibodies would then prevent parasite sexual development and/or ookinete invasion of the mosquito midgut with the goal of blocking parasite transmission. One major advantage of targeting sexual stage proteins over blood stage proteins is that these antibody targets would not be subject to immune pressure since they are not expressed until sexual development in the mosquito midgut (*Carter et al., 1989*). Indeed, sexual stage proteins appear to exhibit minimal antigenic variation globally (*Kaslow et al., 1989, Richards et al., 2006*). Immune selection against blood stage antigens has been a problem that has plagued successful development of a blood stage vaccine.

Plasmodium is a complex parasite with multiple life cycle forms in both the vertebrate host and the mosquito vector. When a *Plasmodium*-infected mosquito takes a blood meal from a vertebrate host, it simultaneously deposits sporozoites, the infectious form to humans, into the skin. Sporozoites then migrate to the liver where they invade and establish infection in hepatocytes. *Plasmodium* parasites then replicate within hepatocytes as hepatic schizonts that, upon maturation, erupt from infected cells as merozoites. Merozoites infect erythrocytes and initiate the intra-erythrocytic

cycle of the *Plasmodium* life cycle. Upon infection of an erythrocyte, they subsequently develop into ring- and trophozoite-stage forms. Trophozoites undergo asexual replication inside the erythrocyte to become erythrocytic schizonts. When schizonts mature, they erupt from erythrocytes as 8-32 daughter merozoites that continue the intra-erythrocytic cycle (*Gaur et al.2004,* for review). Current blood stage vaccines target proteins expressed on merozoites. Unfortunately, a major disadvantage of targeting this form is the efficiency of merozoite invasion of new erythrocytes, which occurs within 10-20 seconds (*Dvorak et al., 1975*). This is a short duration of time that merozoites could potentially be exposed to humoral immunity.

Plasmodium sexual development begins with the formation of sexually differentiated gametocytes. During the intra-erythrocytic cycle, specific environmental cues, many of which have yet to be identified, stimulate a small subset of ring stage parasites to develop into gametocytes. *P. falciparum* gametocytes undergo morphologically distinct development (*Alano, 2007*, *Kooij and Matuschewski, 2007* for review). Gametocytes terminally differentiated, which means that they are no longer capable of undergoing asexual replication. They are sexually dimorphic and are present as either a male microgametocyte or a female macrogametocyte.

Mature, sexually dimorphic gametocytes are taken up with the mosquito blood meal (Figure 1.1). Environmental changes encountered in the midgut, including a drop in temperature and an increase in pH, stimulate gametocyte emergence from infected erythrocytes as micro- and macrogametes in a process known as gametogenesis. Male microgametocytes undergo three rounds of DNA replication to generate up to eight microgametes from one microgametocyte. One macrogametocyte only generates one

macrogamete. Microgametes fertilize female macrogametes to generate zygotes. The parasite then undergoes sexual recombination and meiotic replication followed by transformation into a polarized, motile, constitutively secreting ookinete (*Baton et al., 2005*). The ookinete must first migrate out of the blood bolus towards the midgut barrier (*Vlachou et al., 2006, Siden-Kiamos et al., 2006a and 2006b, Hirai et al., 2006, Ishino et al., 2006, Tremp et al., 2008, Moon et al., 2009*). Ookinete recognition of and initial binding to the midgut barrier is then mediated by interactions with midgut proteoglycans (*Dinglassan et al., 2007a and b*). The midgut barrier is composed of: the peritrophic matrix, a proteinaceous, chitin-containing material secreted by the midgut epithelia as a protective barrier against noxious substances in a blood meal; and the midgut epithelia, a single layer of cells. Multiple enzymes secreted by the ookinete were shown to be essential for parasite transmission to the mosquito and are presumed to digest components of the peritrophic matrix (*Huber et al., 1991, Vinetz et al., 2000, Vinetz et al., 2004* for review). After traversal of the peritrophic matrix, the ookinetes migrate via an intra- or inter-cellular route through the midgut epithelia. Once the ookinete penetrates the midgut barrier, it comes to rest against the basal lamina. Extracellular matrix components of the basal lamina, such as collagen and laminin, stimulate the ookinete to round up and develop into an oocyst. Oocysts harbor developing sporozoites, the form of the parasite responsible for causing human disease.

The ookinete is a potential target of transmission blocking interventions because it is a population bottleneck for *Plasmodium*. Of 10^3 gametocytes ingested during a mosquito blood meal, 10^2 successfully develop into ookinetes, and less than

10^1 penetrate the midgut barrier to become oocysts. From this point, however, one ookinete that penetrates the midgut wall to form an oocyst has the potential to generate thousands of sporozoites (*Poudel et al., 2008, Sinden and Billingsley, 2001*). This makes the ookinete an attractive target for blocking malaria transmission.

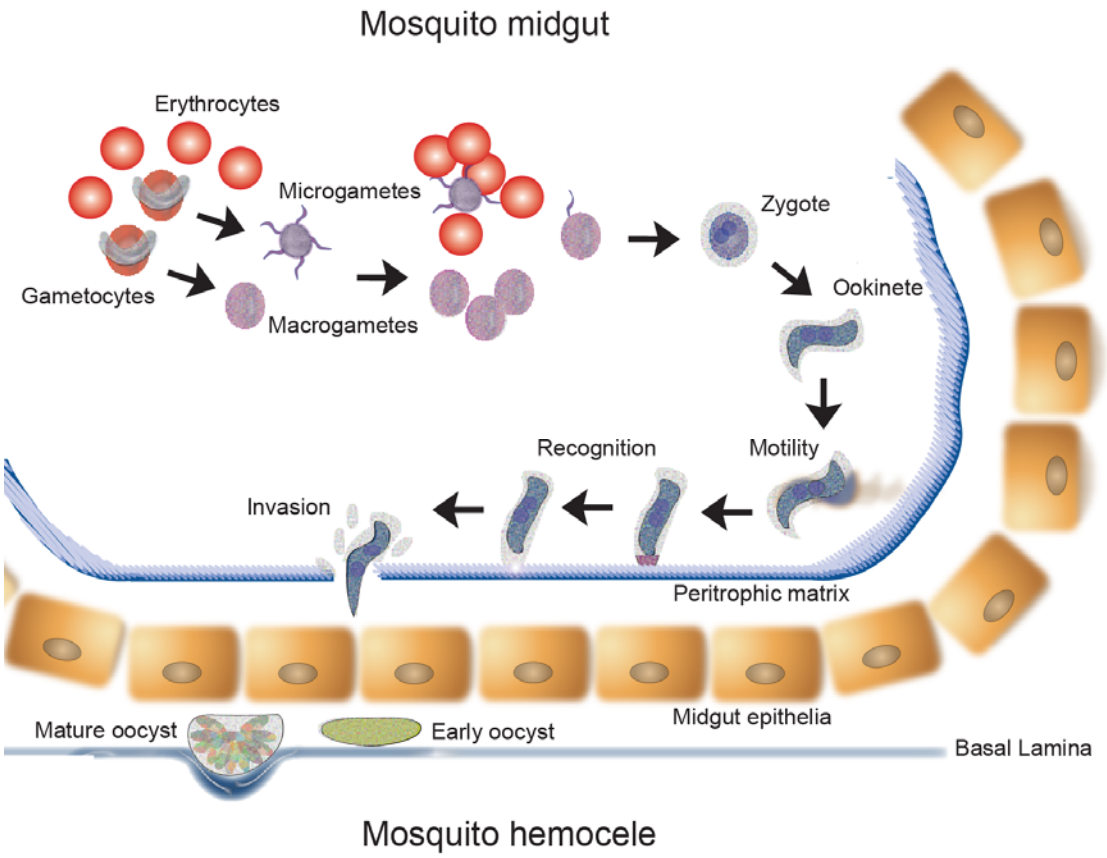

Figure 1.1
***Plasmodium* sexual development in the mosquito midgut.**

Much remains to be learned about the biology of the *Plasmodium* sexual cycle and the mechanisms underlying ookinete invasion of the mosquito midgut wall. For example, ookinete secretion of proteolytic enzymes is important for parasite

penetration of the midgut barrier, but only a handful of proteins have been identified and characterized (*Vinetz, 2005* for review, *Kariu et al., 2006*, *Ecker et al., 2007, Ecker et al., 2008*). A major impediment to the study of these important processes in *P. falciparum* is the inability to generate large quantities of sexual stage parasites either *in vitro* or *ex vivo*. The ability to produce large numbers of *P. falciparum* zygotes and ookinetes would not only enhance our understanding of *P. falciparum* sexual stage biology, it could also greatly accelerate transmission-blocking vaccine research. A sexual stage cultivation method would provide a means to study the mechanisms underlying transmission-blocking interventions, which is not practically feasible for *P. falciparum*. The availability of large numbers of sexual stage parasites would also permit the application of methodologies used in systems biology, such as proteomic analysis, to understand *Plasmodium* sexual stage parasite development.

The application of systems biology, transcriptomics and proteomics, to the study of *Plasmodium*, can lead to the identification of many proteins that are important for *Plasmodium* invasion of its host cells. The advantage to a systems biology approach over the traditional targeted, protein-specific approaches is the efficiency of *in silico* discovery. Although the *in silico* findings must be biologically confirmed, they also permit a more comprehensive understanding of *Plasmodium* processes than was previously achieved. Proteomic analysis of *Plasmodium* intra-erythrocytic parasites and sporozoites generated valuable insights into these parasite life stages (*Florens et al., 2002, Hall et al., 2005, Ecker et al., 2008, Lasonder et al., 2002, Lasonder et al., 2008*). Proteomic analysis of *Plasmodium* sexual stage parasites,

particularly for the lethal human disease-causing agent, *P. falciparum* could greatly accelerate transmission-blocking vaccine research

This study focused on *P. falciparum* sexual stage parasite development. Chapter 2 described development of a method to generate *P. falciparum* sexual stage parasite forms to address the lack available zygotes and ookinete forms for study. This method resulted in the generation of up to 10^6 ookinetes per 10 ml culture, a > 20-fold increase over previous reports. Chapter 3 described the adaptation of this method to *P. vivax* zygotes and ookinetes. In Chapter 4, this *P. falciparum in vitro* sexual stage cultivation method was then used to complement investigations of a potential target of transmission-blocking vaccines, Plasmepsin X (PM X). In PM X studies, this method facilitated separate evaluation of sexual stage development from ookinete invasion of the mosquito midgut. Finally, in Chapter 5, *P. falciparum* sexual stage parasites were used a global, proteomic approach to identify mechanisms important for sexual stage parasite development.

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CHAPTER 2

BIOLOGICAL FEATURES OF *IN VITRO*-CULTIVATED SEXUAL STAGE PARASITES OF *PLASMODIUM FALCIPARUM*

Abstract

Plasmodium transmission to the mosquito vector depends on the ability of the *Plasmodium* ookinete, an invasive form of the parasite, to invade and establish infection on the mosquito midgut. Blocking this process could stop the proprogation of malaria infections. The biology of the *Plasmodium falciparum* ookinete is poorly understood because sufficient quantities of ookinetes have not been producible. A sexual stage cultivation method was developed that produced 5-50 x 10^6 *P. falciparum* ookinetes per 10 ml culture with gametocyte-to-ookinete transformation rates of approximately 25%. This was a > 20-fold improvement from previous reports. Transmission electron micrographs of cultured parasites confirmed ookinete development. The ability to consistently produce and purify ookinetes will allow for the direct study of *Plasmodium falciparum* sexual stage forms at a biochemical level and will facilitate the development of novel approaches to eradicate malaria.

Introduction

The high prevalence of asymptomatic infection in malaria-endemic regions is a barrier to malaria eradication as these infected people are reservoirs of disease (*Drakeley et al., 2006*). Recent studies have examined the potential of transmission-blocking vaccines to control, even eradicate, malaria. Although the efficacy for transmission-blocking vaccines has been robustly demonstrated in animal models of malaria, the precise mechanism(s) by which these interventions block mosquito infection remain poorly understood (*Gwadz, 1976, Carter and Chen 1976*). Only a few antigens from *P. falciparum,* the agent of severe human malaria, have been

characterized (*Vermeulen et al., 1985, Lavazec and Bourgouin, 2008*). The validation of transmission-blocking antigens has been limited by the inability to generate large quantities of *P. falciparum* sexual stage parasites *in vitro*. The pursuit of whole-ookinete transmission-blocking vaccines, whereby attenuated ookinetes could be used as a human vaccine to elicit a humoral response to sexual stage antigens (*Carter and Chen, 1976*), has been dismissed as technically infeasible for this same reason.

Our knowledge of *Plasmodium* sexual stages has come primarily from studies of animal models of malaria, particularly the rodent-infected *Plasmodium. berghei* (*P. berghei*) and the bird-infecting *Plasmodium gallinaceum* (*P. gallinaceum*). This was largely because of the ability to generate large numbers of sexual stage *P. berghei* and *P. gallinaceum* parasites *in vitro*. However, fundamental biological differences between the animal- and human-infecting *Plasmodium* species, particularly in parasite interactions with their respective mosquito vectors, make the use of these animal models increasingly unappealing (*Alavi et al., 2003, Aguilar et al., 2005, Dong et al., 2006*). Published methods used to produce *P. falciparum* ookinetes *in vitro* did not reliably generate ookinetes, generated low yields, or generated unknown quantities of parasites that were not definitively identified as ookinetes (*Ono and Nakabayashi, 1989, Carter et al., 1987, Warberg and Schneider, 1993, Hurd et al., 2003, Dinglasan et al., 2007, Zhou et al., 2008*). Here, we report a method for consistently generating large quantities of *P. falciparum* ookinetes *in vitro*, and we indisputably identify these parasites as ookinetes using transmission electron microscopy. This technical advance permits direct study of *P. falciparum* sexual stage parasites and facilitates the development of novel interventions relevant to human malaria.

Methods

Provenance of *P. falciparum* strain NF54 used in this study

The NF54 isolate of *Plasmodium falciparum* used in these experiments was received from Dr. Stephen Hoffmann (Sanaria, Rockville, MD USA). NF54 is one of the 13 initial NF (Nijmegen *Falciparum*) isolates derived from a Dutch patient (*Ponnudurai et al., 1983*). It was characterized and adapted for cell culture then produced as a monoclonal parasite line by limiting dilution from the NF36 isolate (*Ponnudurai et al., 1981*). NF54 produced gametocytes in culture that developed to sporozoites in *Anopheles* mosquitoes that were then able to infect humans through the bite of infected mosquitoes (*Ponnudurai et al., 1982, Chulay et al., 1986*). NF54 has been maintained at the University of Nijmegen since its original selection, and Sanaria's NF54 parasites were obtained directly from the University of Nijmegen in January 2005 (NF54 Ny 09/29/04). From this vial, Sanaria prepared a NF54 MCB (RKV01-092505) and a WCB (RKV02-102905), both of which were also released in June 2006.

P. falciparum gametocyte cultures

P. falciparum strain NF54 was maintained in continuous asexual culture according to standard protocol with the exception that no antibiotics were used in the complete medium (*Read and Hyde, 1993*): RPMI 1640, 25 mM HEPES, 2 mM L-glutamine, 2.4 g/L $NaHCO_3$, 50 mg/L hypoxanthine, 10% heat-inactivated AB^+ human serum (Interstate Blood Bank, Memphis, TN USA). Human blood used for *in vitro* culture was freshly drawn from volunteers following informed consent according

to protocol approved by the University of California, San Diego Institutional Review Board for human subjects.

Gametocytes were cultured as previously described (modified from *Ifediba and Vanderberg, 1981*). Asexual cultures at 5-15% parasitemia were diluted with 0.9 ml freshly washed, packed human red blood cells for an initial gametocyte culture parasitemia of 0.5%. Generally, asexual stage cultures at 8-10% parasitemia generated gametocyte cultures with the highest yields. Gametocyte cultures were changed with 15 ml of 37^{o}C complete medium daily until they reached 5–10 % parasitemia, usually by day 3-4, at which point 25 ml of 37^{o}C complete medium was used for the remainder of the culture period. Gametocyte cultures were maintained in a low oxygen environment, and medium was changed within 24 hours for the entire culture period using complete medium prepared more than 7 days previously (Figure 2.1). Additionally, approximately 10 ml of spent medium was left in the culture flask during each medium change (*Williams, 1999*). Parasite transformation into mature gametocytes was detected as early as 12 days for microgametocytes and 14 days for macrogametocytes. The majority of parasites developed into mature, stage V gametocytes at 16-18 days and as late as 22 days as detected by light microscopy.

For parasite quantification, thin smears were fixed with methanol and stained using Leukostat dyes (15 seconds in Leukostat 1: 0.1% eosin Y, 0.4% Na_2HPO_4, 0.1% formaldehyde; 30 seconds in Leukostat 2: 0.04% methylene blue, 0.04% Azure A, KH_2PO_4, Na_2HPO_4). A minimum of 10 fields at 100 X were examined using an Olympus BX51 microscope (Olympus, Center Valley, PA, USA); pictures were taken with an Olympus DP71 camera (Olympus, Center Valley, PA, USA).

P. falciparum ookinete cultures

Gametocyte cultures at the peak of microgametocyte development, which was defined as ≥ 2% morphologically mature microgametocytes, and cultures at the peak of macrogametocyte development, which was defined as ≥ 5% morphologically mature macrogametocytes, were tested for gametogenesis as follows. Mature microgametocytes were tested for the ability to exflagellate (*Carter et al., 1993*), on day 12-17, and only cultures with a minimum of 4 exflagellation centers per 40 X field were subsequently used. Mature macrogametocytes were tested for the ability to emerge and the ratio of gametes-to-gametocytes was determined by counting Leukostat-stained blood smears one hour post-emergence. Only cultures with a minimum ratio of 4:1 macrogametes-to-gametocyte were subsequently used.

Two gametocyte cultures with mature, exflagellation-competent micro-gametocytes and two cultures with mature, emergence-competent macrogametocytes were combined to make one ookinete culture. For gametogenesis and fertilization, parasites were centrifuged at 800 x g for 3 minutes, no brake, and the resulting pellet was resuspended with 9 pellet-volumes of 19-23ºC heat-inactivated AB^+ human serum for 30 minutes. Gametes, zygotes and remaining gametocytes were centrifuged at 800 x g for 3 minutes, no brake, and the resulting pellet was resuspended with 4-9 volumes of freshly made, filter-sterilized ookinete medium (RPMI 1640, 25 mM HEPES, 2 mM L-glutamine, 2 g/L $NaHCO_3$, 50 mg/L hypoxanthine, 15% heat-inactivated AB^+ human serum or heat-inactivated fetal calf serum (FCS), pH 8.2 - 8.4 with NaOH). Parasites in ookinete medium were transferred to a 25 cm^2 flask and gently rocked at

19-23ºC for 36 - 48 hours, though ookinetes were observed in culture for up to 72 hours in ookinete medium.

Purification of sexual stage parasites

The majority of uninfected erythrocytes were removed from sexual stage parasite cultures using Lympholyte-H density gradient medium (Cedarlane Laboratories, Burlington, NC, USA). 3 ml of Lympholyte-H was used to underlay one 10 ml culture. The gradient was centrifuged at 800 x g for 20 minutes, no brake. Parasites were collected from the gradient interface, washed twice in ookinete medium and quantified by light microscopy. A minimum of 10 fields at 100X were counted, and 300-1000 parasites were quantified as described.

Immunofluorescence assay

For immunofluorescence assay (IFA), cultured parasites were fixed on glass slides with methanol and blocked in 3% BSA/3% Triton X-100/phosphate buffered saline (PBS) for one hour at room temperature. Parasites were then probed with primary antibodies against *P. falciparum* chitinase and surface protein 25 (Pfs25), 1C3 (*Langer et al., 2000*) and 4B7 (MRA-28, deposited by Dr. David C. Kaslow, Malaria Research and Reference Reagent Resource Center, Manassas, VA, USA, *Barr et al, 1991*), respectively or mouse Ig isotype control at 1:100 dilution in blocking buffer. Slides were washed 6 times in PBS for a total of 30 minutes then incubated with FITC-conjugated anti-mouse secondary and 3 μM DAPI (Molecular Probes, Invitrogen, Carlsbad, CA USA) in blocking buffer for one hour. Slides were washed

an additional 6 times in PBS then mounted with coverslips using Dako mounting medium (Dako, Carpinteria, CA 92013 USA). Parasites were then examined using an Olympus BX51 fluorescence microscope (Olympus, Center Valley, PA, USA) and Olympus DP71 camera (Olympus, Center Valley, PA, USA). For visualization of live parasites, sexual stage parasites were incubated with Hoechst 33342 (Molecular Probes, Invitrogen, Carlsbad, CA, USA) and examined on a glass slide as described.

Western immunoblot analysis

P. falciparum parasites were purified, pelleted and resuspended in 250 µl of lysis buffer (4 M urea, 0.4% Triton X-100, 50 mM Tris, 5 mM EDTA, 10mM $MgSO_4$, pH 8.0) supplemented with Complete protease inhibitor cocktail (Roche Applied Sciences, Indianapolis, IN, USA). Parasites were processed by 3 cycles of freeze-thaw lysis followed by sonication on ice for 5 minutes in 30 second bursts using a Misonix Sonicator 3000 with an output setting of 7 (Misonix, Farmingdale, NY, USA). Protein concentrations were determined by BCA assay (Bio-Rad, Hercules, CA, USA). 100 µg of each sample was mixed with Laemmli SDS-loading buffer (160 mM Tris, 10% SDS, 20% glycerol, 5% 2-mercaptoethanol, 0.01% bromophenol blue, pH 8.0) and boiled for 10 minutes. Proteins were separated on Novex 10-20% SDS-PAGE minigels (Invitrogen, Carlsbad, CA 92008 USA) and transferred to nitrocellulose membranes. Membranes were blocked in 5% non-fat milk/0.05% Tween-20/Tris-buffered saline (TBS), pH 8.0 for 1 hr. Blots were probed with primary antibody diluted 1:2000 in blocking buffer for 1 hr. Following six 10-minute washes in blocking buffer, blots were probed with peroxidase-conjugated anti-mouse secondary

antibody diluted 1:1000 in blocking buffer. Blots were washed 6 times in blocking buffer, twice in TBS and then developed with TMB chromogenic membrane substrate (KPL, Gaithersburg, MD 20878 USA).

Transmission electron microscopy

Ookinete samples were prepared according to standard protocol (*Zheng et al., 2006*). Briefly, samples were immersed overnight in modified Karnovsky's fixative (1.5% glutaraldehyde, 3% paraformaldehyde and 5% sucrose in 0.1 M cacodylate buffer, pH 7.4.) and post-fixed in 1% osmium tetraoxide in 0.1 M cacodyalte buffer for one hour. Samples were stained for 1 hour with 1% uranyl acetate and then dehydrated in ethanol and embedded in epoxy resin. Embedded samples were sectioned with a Reichert ultramicrotome at a section width of 60-70 nm. Sections were placed on Formvar and carbon-coated copper grids for support. Grids were stained with 1% uranyl acetate and lead nitrate. Stained sections were examined using a JEOL 1200EX II transmission electron microscope (JEOL, Peabody, MA, USA) and photographed using a Gatan digital camera (Gatan, Pleasanton, CA, USA).

Results and Discussion

High Yield Ookinete Production

This method produced high yields of *P. falciparum* sexual stage parasites, particularly ookinetes, *in vitro* (Figure 2.1). The generation of large quantities of *P. falciparum* ookinetes depended on the presence of mature micro- and macrogametes, which in turn depended on the production of mature micro- and macrogametocytes

(Figure 2.2). Gametocyte cultures were started 2-3 days apart because micro-gametocytes matured more quickly than macrogametocytes. Gametocyte cultures generated were found to contain up to 8.5% gametocytemia, and 50-90% of these gametocytes developed to stage V gametocytes (up to 5% mature gametocytemia in culture) at the end of the culturing period (Table 2.1). Additionally, using these methods, the level of asexual parasite contamination was less than 0.5% in mature gametocyte cultures. This was a 5- to 8-fold improvement in gametocyte production as well as a significant decrease in asexual stage parasite contamination of gametocyte cultures compared to previously published reports (*Ono and Nakabayashi, 1989, Dixon et al., 2008, Berry et al., 2009*).

Table 2.1
***P. falciparum* gametocyte yields for 5 representative cultures.**
Gametocytemia of unpurified *P. falciparum* gametocyte cultures was determined when the majority of gametocytes were mature, as early as 16 and as late as 22 days of culture; 1100-2400 cells were counted in 10 fields. Average yields were 2 - 20 x 10^7 gametocytes per 25 ml culture. Percent asexual- and gametocyte-stage parasites for five representative cultures used in ookinete preparations are shown.

Culture	**1**	**2**	**3**	**4**	**5**
Asexual parasitemia	0.3%	0%	0%	0.4%	0.4%
Immature gametocytemia	3%	0.5%	2.2%	1%	2%
Stage V gametocytemia	5%	6%	6.3%	5%	5%
Gametocyte yield (x 10^7)	20	3.3	7.5	5	10

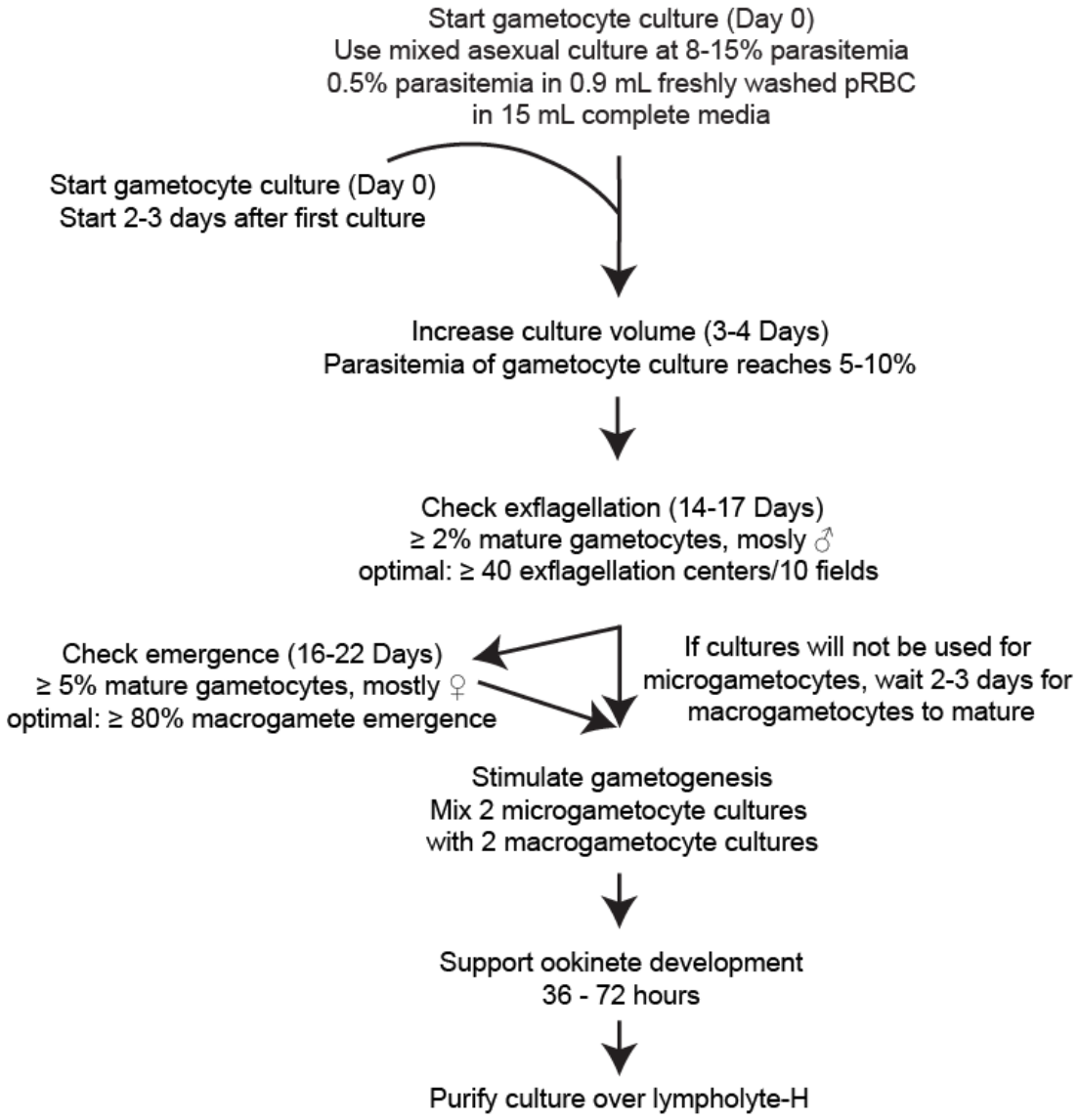

Figure 2.1
Schematic of *P. falciparum in vitro* sexual stage cultivation protocol.
Mature, exflagellation-competent microgametocytes were mixed with mature, emergence-competent macrogametocytes, and stimulated to undergo gametogenesis. The resulting gametes fertilized to become zygotes, and the parasites were incubated at 19°C – 21°C for 36-72 hours in ookinete medium at 10% hematocrit to support ookinete development. Sexual stage parasites were then purified over Lympholyte-H density gradient medium. Average time for gametocyte development and maturity are shown, but individual cultures could take additional time to reach maturity.

Combining two cultures of ≥2% mature microgametocytes with two cultures of ≥5% mature macrogametocytes resulted in optimal production of sexual stage parasites. After gametogenesis, one macrogametocyte will develop into one macrogamete while one microgametocyte will undergo 3 rounds of mitotic replication and become 8 microgametes, all of which are capable of fertilizing one macrogamete (*Sinden et al., 1978*). Therefore, theoretically only one eighth as many microgametocytes are required for the generation of sufficient microgametes to fertilize the resulting macrogametes. Increasing parasite density or adding uninfected red blood cells, both shown to improve *P. falciparum* transmission to mosquitos (*Carter et al., 1993*), did not affect ookinete transformation rates (Figure 2.2).

Intuitively, the production of ookinetes was dependent on the fertilization of macrogametes by microgametes to generate zygotes. Gamete production was dependent on the ability of gametocytes to undergo gametogenesis, which was ultimately dependent on gametocyte maturity. Previously published *P. falciparum* ookinete protocols failed to explicitly examine gametogenesis or only considered microgametogenesis when determining gametocyte maturity (*Warburg and Schneider, 1993, Carter et al., 1987, Ono and Nakabayashi, 1989, Dinglasan et al., 2007, Zhou et al., 2008*). Both macrogametogenesis as well as microgametogenesis were important indicators of gametocyte maturity and were independently predictive of high ookinete yield (Figure 2.2). These cultures had an average transformation rate of 25% ookinetes with a yield of 5-50 x 10^6 ookinetes per 10 ml culture (Table 2.2).

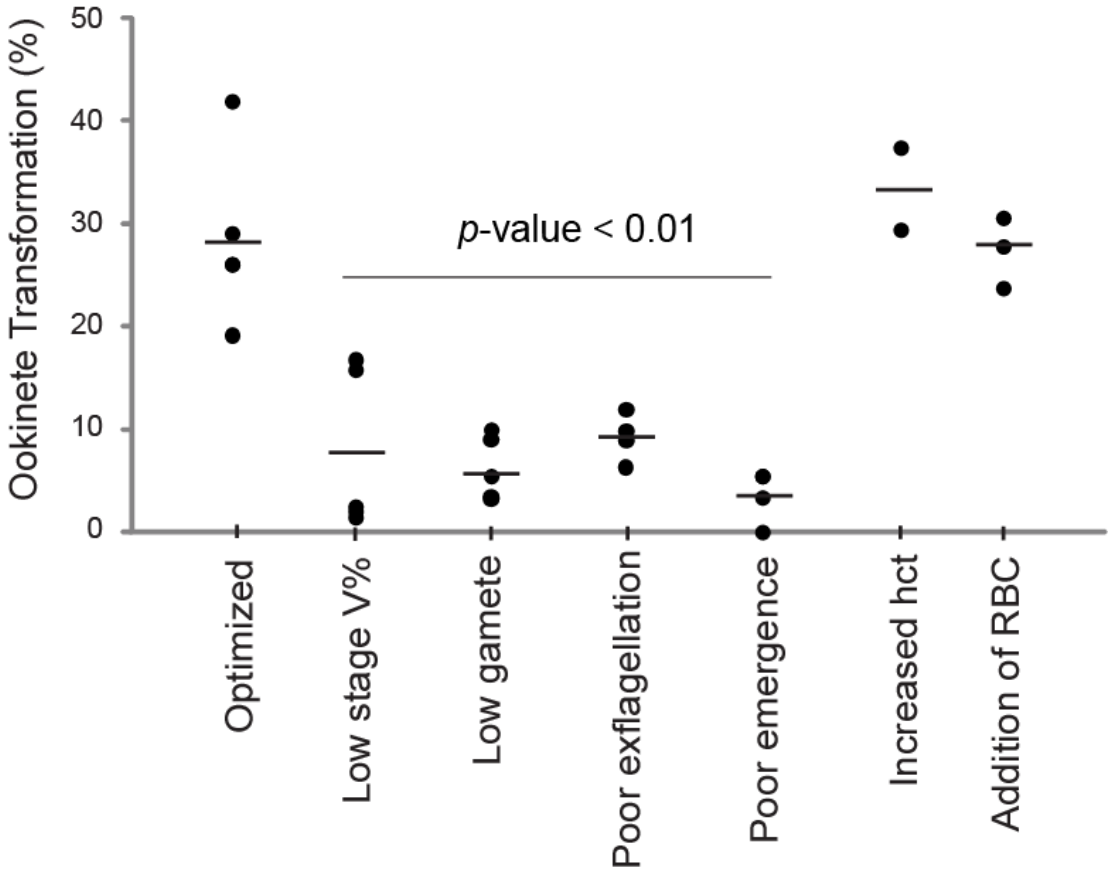

Figure 2.2
***In vitro P. falciparum* ookinete transformation was dependent on gametocyte density and gamete maturity.**
Sexual stage parasite cultures contained 19-42% ookinetes and were reproducibly generated by mixing ≥ 5% macrogametocytemic cultures with ≥ 2% microgametocytemic cultures at a final hematocrit of 10-20% (Optimized culture). Gametocyte cultures with ≤ 2% mature gametocytemia resulted in sexual stage cultures with 1-17% ookinetes (Low stage V%). Low rates of both gamete emergence (< 80%) and exflagellation (<4 exflagellating centers per 40x field) resulted in sexual stage cultures with 3-10% ookinetes (Low gamete*). Low rates of either exflagellation (Low exflagellation*) or emergence (Low emergence*) resulted in 6-12% and 3-5% ookinetes per culture, respectively. Increased hematocrit of sexual stage cultures from by two-fold (Increased hct) did not significantly affect ookinete development, with ookinete yields at 24% – 31%. The addition of fresh erythrocytes (Addition of RBC) to ookinete cultures did not significantly affect ookinete transformation; yields were 29% – 37%. Statistical significance was determined by ANOVA.

Table 2.2
P. falciparum* ookinete densities *in vitro.
Sexual stage parasite densities for five representative cultures were determined; each 10 ml culture yielded 5 – 60 x 10^6 ookinetes.

Culture	**1**	**2**	**3**	**4**	**5**
% Gametocytes	39%	45%	37%	32%	13%
% Gametes	24%	16%	12%	31%	67%
% Zygotes/Retorts	11%	9%	9%	11%	8%
% Ookinetes	26%	31%	42%	26%	12%
Ookinete yield (x 10^6)	10	57	31	10	24

Identification of novel parasite forms by light microscopic examination of *in vitro*-cultivated *P. falciparum* sexual stage parasites

Sexual stage parasite cultures were examined by light microscopy and were found to contain a mixture of round macrogametes and zygotes, as well as banana-shaped gametocytes and ookinetes (Figure 2.3). In light micrographs, zygotes were distinguished from macrogames by the presence of a prominent nucleus. Ookinetes were distinguished from gametocytes by the presence of one to two prominent nuclei and the lack of a surrounding red cell membrane (Figure 2.3).

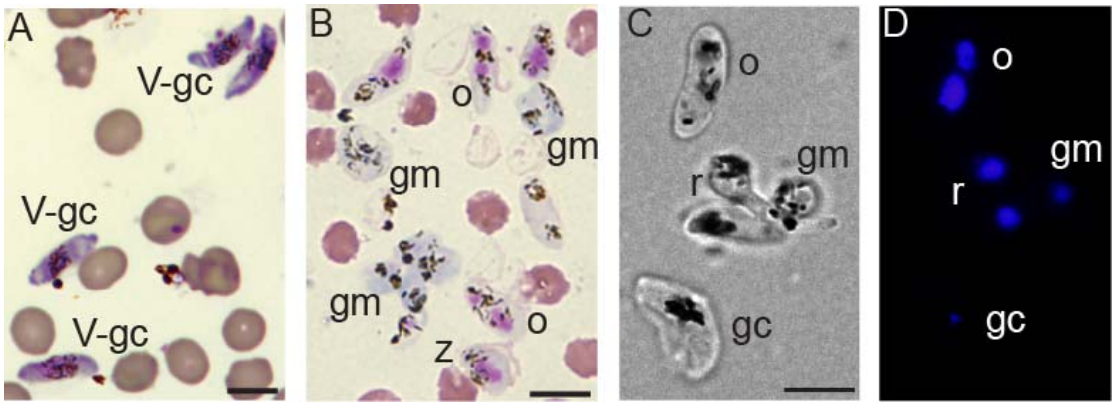

Figure 2.3
Leukostat-stained light micrographs of *in vitro*-cultivated *P. falciparum* sexual stage parasites.
A, Leukostat-stained thin-smears of unpurified gametocyte cultures contain mature stage V gametocytes (V-gc). Scale bar: 5 μm.
B. Leukostat-stained thin-smears of *in vitro* cultured *P. falciparum* sexual stage parasites. Ookinetes (o) were distinguishable from gametocytes (gc) by the presence of one to two large eosinophilic nuclei and lack of a surrounding erythrocyte membrane. Macrogametes (gm) were slightly basophilic while zygotes (z) and retort ookinetes (r) had a large eosinophilic center. Hemozoin appeared as dark brown pigment crystals. Scale bar: 5 μm.
C. Bright field images of live sexual stage parasites stained with D. Hoechst 33342 showed an ookinete (o) with two prominent nuclei while a gametocyte (gc), seen within the erythrocyte, had only one small nucleus. Retort ookinetes (r) and a gamete (gm) each had one nucleus. Scale bars: 5 μm.

At this junction, it was not ascertained whether the presence of two nuclei in ookinetes indicated a failure of macro- and microgamete nuclear fusion or nuclear fusion and replication followed by a single round of nuclear division prior to oocyst development. Studies from *P. berghei* indicated that gamete fertilization and zygote formation is followed by a meiotic replication to diploid or tetraploid levels (*Sinden and Hartley, 1985, Janse et al., 1986*), but the observation of two nuclei in ookinetes has not previously been reported. To clarify this issue, one would cross two gametocyte producing sub-strains with distinct genomic signatures, such as deletion of specific sub-telomeric *var* genes or integration of blastocidin-S-deaminase and

dihydrofolate reductase (DHFR) resistance cassettes, in an *in vitro* ookinete culture. Then, using fluorescence *in situ* hybridization could be done using distinctly labeled probes specific for each strain. It could then be determined whether the two-nuclei ookinetes had a nuclear fusion defect (each nucleus only recognized by one probe) or whether they underwent a single round of nuclear division prior to oocyst development (each of the two nuclei recognized by both probes). If each nucleus were only recognized by one probe, this may indicate that these ookinetes had a nuclear fusion defect that was possibly a result of the *in vitro* ookinete culture method. If each of the two nuclei were to be recognized by both probes, it would definitively demonstrate for the first time that *Plasmodium* ookinete nuclear division occurred prior to the stimulation of oocyst development. In either scenario, the findings would then need to be verified using gametocytes that developed into ookinetes within the mosquito midgut.

Pfs25 and chitinase are found in gametocytes and ookinetes

To further identify elongated parasites as gametocytes or ookinetes, gametocytes and *in vitro*-generated ookinete cultures were examined by western immunoblot analysis and IFA using previously characterized antibodies to chitinase (*Langer et al., 2004*) and Pfs25 (*Barr et al, 1991*) or mouse Ig isotype control antibody. Ig isotype control antibody recognized neither gametocytes nor ookinetes on either western immunoblot analysis or IFA.

Chitinase, an enzyme that cleaves chitin, has been shown to be secreted by ookinetes to facilitate penetration of the chitin-containing peritrophic matrix that

surrounds the parasite-containing mosquito blood meal. These data showed that antibodies to chitinase recognized a 28-32 kDa protein in mature gametocytes, macrogametes and zygote and ookinete cultures. This band was consistent with the predicted size of *P. falciparum* chitinase (Figure 2.4). On IFA, antibodies to chitinase exhibited a diffuse intracellular staining pattern on both gametocytes and ookinetes (Figure 2.4). Although chitinase was previously thought to be an ookinete-specific enzyme, this finding was consistent with previous observations that suggested that chitinase was expressed in mature gametocytes (*Li and Patra, unpublished*). These findings confirmed that chitinase was not strictly ookinete-specific and that chitinase expression could not be used to confirm the generation of *P. falciparum* ookinetes.

Pfs25 is an ookinete surface protein also thought to be specifically expressed in ookinetes. In western immunoblot analysis, antibody directed against Pfs25 only recognized zygote and ookinete cultures (Figure 2.4). However, in IFA, antibody directed against Pfs25 recognized both gametocytes and ookinetes (Figure 2.4). IFA is a more sensitive technique than western immunoblot analysis, and these findings were consistent with previously published results (*Scholz et al., 2008*). These data indicated that Pfs25 expression cannot be used to confirm generation of *P. falciparum* ookinetes by IFA, as previous groups have suggested (*Dinglasan et al., 2007*). As expression of these two presumed ookinete-specific proteins were detected in mature gametocytes by IFA, the production of ookinetes was definitively confirmed by transmission electron microscopy (TEM) of sexual stage parasite cultures.

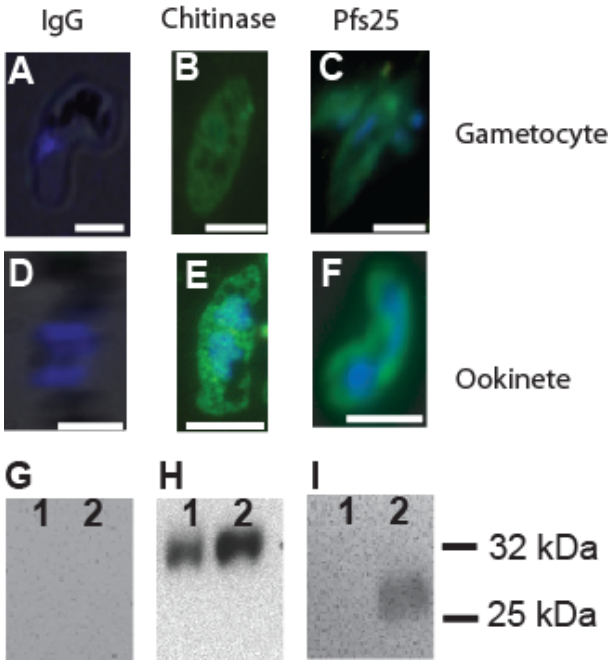

Figure 2.4
Chitinase and Pfs25 expression in *P. falciparum* sexual stage parasites.
Gametocytes and ookinetes were probed with antibodies (green). DAPI (blue) was used to visualize DNA. Scale bars: 5 µm.
Gametocytes (A, B, C) and ookinetes (D, E, F) probed with Ig isotype control (A, D) antibody showed minimal fluorescence compared to anti-chitinase antibody (B, E) and anti-Pfs25 antibody (C, F), which showed diffuse cytoplasmic fluorescence.
Western immunoblot analysis of gametocytes (lane 1) and zygotes plus ookinetes (lane 2) probed with:
G. Ig isotype control antibody showed no reaction.
H. anti-chitinase antibody demonstrated recognition of an ~32 kDa band.
I. anti-Pfs25 antibody recognized a ~26 kDa band in zygote plus ookinetes.

<u>*P. falciparum* ookinete production demonstrated by transmission electron microscopy</u>

Analysis of cultivated parasites by transmission electron microscopy demonstrated the presence of round parasite sections, which could have been cross-sections of macrogametes, zygotes or cross-sections of gametocytes or ookinetes, as well as elongated parasite sections, which could have been gametocytes and ookinetes. Cross-sections of gametocytes could be identified by the presence of a surrounding

erythrocyte while cross-sections of ookinetes could be identified by the presence of subpellicular microtubules and the lack of a surrounding erythrocyte (*Sinden et al., 1976, Sinden et al., 1978, Sinden et al., 1985, Sinden et al, 1987*). Ookinetes and immature gametocytes (stages I-IV) both maintained a rigid, elongated shape with numerous subpellicular microtubules (Figure 2.5). Transmission electron microscopy definitively distinguished elongated, banana-shaped parasites as either gametocytes or ookinetes (Figure 2.5).

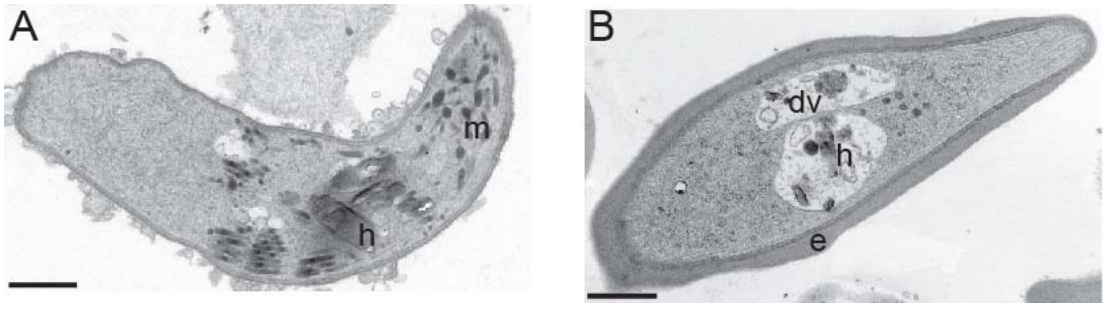

Figure 2.5
Electron micrographs of *P. falciparum* ookinetes and gametocytes.
(A) Cross-section of an ookinete showed micronemes (m), seen as electron-dense round or cigar-shaped organelles, clustered towards the apical end. Scale bar: 1 μm. (B) Cross-section of a gametocyte, which resided within an infected erythrocyte (e) and contained a discrete digestive vacuole (dv) with hemozoin (h). Scale bar: 1 μm.

Ultrastructural analysis of *P. falciparum* ookinete apical end

Examination of erythrocyte-free elongated parasites demonstrated that they exhibited characteristic ookinete ultrastructure, including the apical complex and a pellicle. The apical complex consisted of an apical polar ring, to which the subpellicular microtubules were anchored, and micronemes, the ookinete secretory organelles (*Raibaud et al., 2001*). Elements of the ookinete pellicle were clearly

demonstrated in these electron micrographs, including the parasite plasma membrane, the inner membrane complex and subpellicular microtubules (Figure 2.6). The ookinete plasma membrane was surrounded by a fuzzy, electron-dense layer suggestive of a thick coat of surface proteins. The inner membrane complex could be seen beneath the plasma membrane and became a thick, electron-dense collar as it neared the polar rings and central apical pore (*Canning and Sinden, 1973*). The central apical pore has been associated with secretion of invasion-related proteins (*Langer et al., 2000*). Cross sections of the polar rings, which supported the subpellicular microtubules, were observed just deep to the central apical pore (Figure 2.6).

Micronemes were seen as cigar-shaped or bulbous membrane-bound organelles that have been shown to contain proteins secreted through ducts near the apical end. Numerous micronemes were visible at the ookinete apical end (Figure 2.6) and seen adjacent to subpellicular microtubules; this suggested that they could be trafficked toward the apical end along microtubules as has been demonstrated in other stages of the parasite life cycle (*Bannister et al., 2003, Schrevel et al., 2008*). Additionally, some micronemes could be seen with their long ducts oriented towards the apical end, consistent with their role as organelles containing secreted proteins.

Another common feature found in ookinetes was an organized electron-dense structure previously identified as crystalloid, which appeared to be specific to ookinetes and was not seen in developing oocysts (*Garnham et al., 1962, Aikawa et al., 1984, Sinden et al., 1985*). Based on the observation that crystalloid bodies were often seen surrounded by membrane-bound micronemes in *P. falciparum* ookinetes, it was tempting to speculate that microneme proteins are initially produced as crystalloid

and then packaged into developing micronemes. However, this would be contrary to biological evidence that suggested that micronemes developed as membrane-bound vesicles from the endoplasmic reticulum and rudimentary Golgi apparatus (*Bannister et al., 2003, Schrevel et al., 2007, Carruthers and Tomley, 2008*). A recent study suggested that a member of the LCCL/lectin-adhesive like protein (LAP/CCp) family was found in crystalloids of developing *P. berghei* ookinetes (*Carter et al., 2008*). It has been shown that this family of proteins functioned as surface adhesins during *P. falciparum* sexual stage development (*Pradel et al., 2006, Scholz et al., 2008, Simon et al., 2009*). This may implicate crystalloids in the storage and formation of parasite surface-associated proteins. However, these same studies demonstrated that expression of LAP/CCp proteins declined after *P. falciparum* zygote development, and they were not found in ookinete crystalloid. These conflicting data implied that there may be species-specific differences in LAP/CCp expression and function. In conclusion, the function(s) of ookinete crystalloids remain to be elucidated.

These electron micrographs allow detailed ultrastructural analyses of the ookinete apical end which have not been previously demonstrated for *Plasmodium* ookinetes.

Figure 2.6
Ultrastructure of the *P. falciparum* ookinete apical end.
A. Tangential section of the apical end of an ookinete showed microtubules (→) that converged at the polar ring (pr). The narrow ducts of micronemes tracked toward the apical end (*→). Scale bar: 200 nm.
B.Midline longitudinal section of an ookinete apical end showed the central apical pore (cap), micronemes and microtubules (→). Scale bar: 200 nm.
C. Midline longitudinal section of an ookinete showed the central apical pore (cap) between two electron-dense regions. Scale bar: 200 nm.
D. Midline longitudinal section of a lysed ookinete clearly showed underlying cytoskeletal and membranous features of the pellicle: the plasma membrane (pm) and the inner membrane complex (IMC). Electron dense structures seen were cross sections of the polar rings (pr) and were found beneath the central apical pore (cap). Scale bar: 200 nm.
E. Transverse section near the apical end of an ookinete showed circumferential subpellicular microtubules (→). Scale bar: 200 nm.
F. High magnification image of ookinete micronemes showed that they are membrane-bound organelles. Scale bars: 200 nm.
G. High magnification image of a crystalloid body showed that it is highly organized. Scale bar: 200 nm

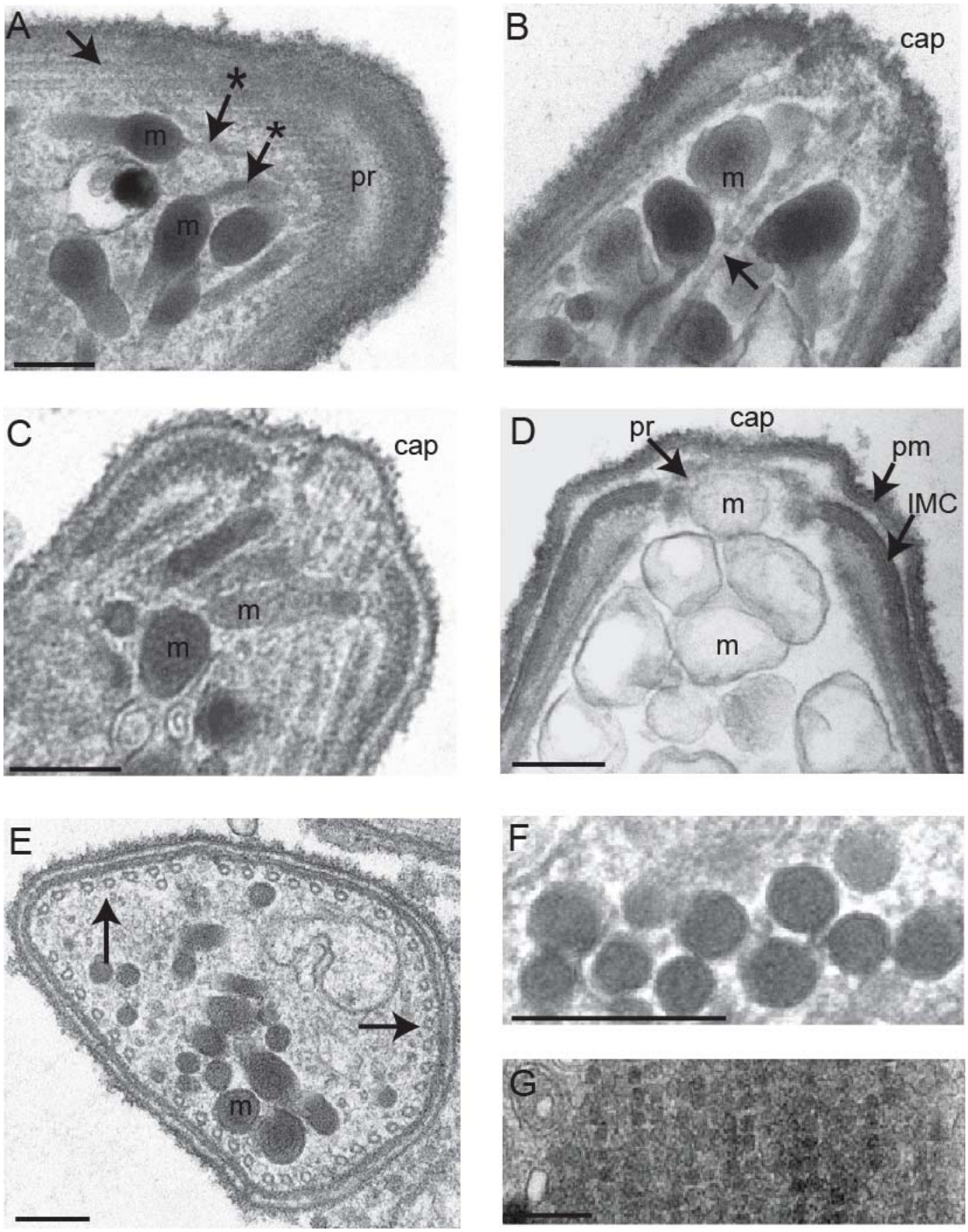

Figure 2.6

Ultrastructure of the *P. falciparum* ookinete apical end.

Ultrastructural analysis of *P. falciparum* macrogametes

Macrogametes and zygotes, both round cells, were difficult to distinguish from each other in electron micrographs. However, transverse sections of macrogametes could be distinguished from ookinetes by the absence of crystalloid, micronemes, or subpellicular microtubules (Figure 2.7). Macrogametes were often found with more than one mitochondrion, which was consistent with mitochondrial replication during gametocyte development (*Okamoto et al 2009*). Whether these mitochondria represented separate, individual entities or syncytial structures could not be determined by electron microscopy (*Sinden, 2009*). Also present in some parasites was a spindle pole body, which has been shown to facilitate chromosome segregation during nuclear replication and division (Figure 2.7). The presence of a spindle pole body suggested that round parasites were zygotes, which may undergo nuclear replication, and not macrogametes.

Clusters of adjacent macrogametes and/or zygotes by TEM were found to have an electron-dense interface between adjacent cell membranes. This was suggestive of protein-specific interactions. Adherent clusters of macrogametes were often found in Leukostat-stained thin smears. Additionally, in gametogenesis assays, emerged macrogametes associated with other macrogametes but not with uninfected red blood cells (data not shown). One could postulate that these macrogamete-macrogamete interactions were mediated by the proteinaceous material observed by TEM. Although it would be tempting to speculate that these interactions were mediated by adhesive LCCL-domain containing proteins, previous studies demonstrated that LCCL-mediated adhesion of macrogametes declined over a period of 6-12 hours (*Simon et*

al., 2009). Adherent macrogamete clusters in these sexual stage cultures persisted for up to 72 hours. It could not be determined if macrogamete adherence was an artifact of parasite densities specific to this culture method or if adherence was physiologically normal within the mosquito midgut. For instance, adherence might minimize individual macrogamete exposure to proteolytic enzymes or could enhance fertilization by microgametes still attached at an exflagellation center.

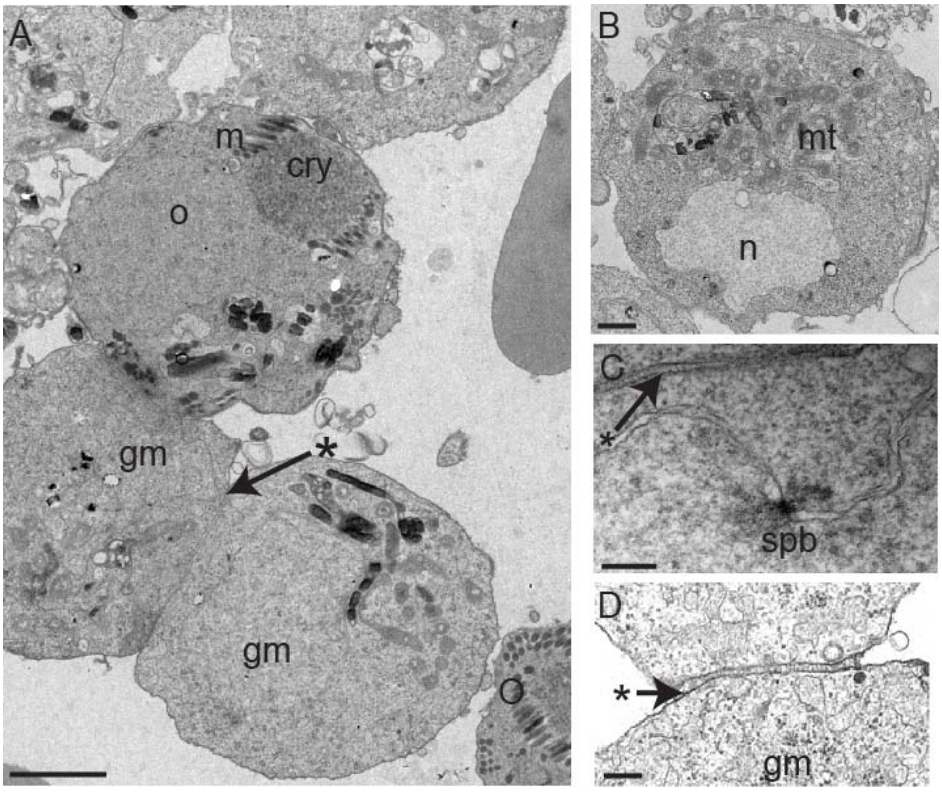

Figure 2.7
Ultrastructural analysis of *P. falciparum* macrogametes.
A. Transverse section through a cluster of adherent parasites demonstrated macrogametes (gm) and an ookinete (o). Ookinetes were distinguished from macrogametes by the presence of a crystalloid body (cry) and micronemes (m) Scale bar: 1 μm.
B. Transverse section through a parasite lacking ookinete-specific structures showed the nucleus and either multiple or syncytial mitochondria. Scale bar: 500 nm.
C. A residual spindle pole body could be seen as an electron dense structure that crossed the double membrane of the nuclear envelope (spb).
D. Examination of adjacent macrogametocytes showed electron dense material (*→) at the cell interface. Scale bar: 200 nm.

Conclusion

The malaria parasite is transmitted by the *Anopheles* mosquito vector, and *Plasmodium* infection of the mosquito vector is dependent on the successful development of parasite sexual forms. Knowledge of *Plasmodium* sexual stage forms is based on studies of animal-infecting *Plasmodium* because large quantities of *P. falciparum*, sexual stage zygotes and ookinetes could not be generated. A method to consistently produce sexual stage parasites was developed to address this shortcoming. Four- to five-fold higher levels of gametocytemia than previously reported were achieved, with the vast majority of gametocytes able to progress into the gamete stage of development (*Berry et al., 2009*). Successful transformation into zygotes and ookinetes was dependent on the presence of mature micro- and macrogametocytes, which was achieved by mixing cultures at different stages of gametocyte maturation. Analysis of these parasites by TEM definitively confirmed that the elongated parasites produced were ookinetes. Furthermore, these studies provided ultrastructural insights into *Plasmodium* ookinete biology which had not previously been demonstrated to this level of detail for *P. falciparum.*

The method presented here was the first to consistently produce large quantities of sexual stage forms of the human malaria parasite *P. falciparum*. This advance provides the basis of studying *P. falciparum* sexual stage parasites by proteomic, biochemical, and genetic analyses which have previously not been completed due to insufficient parasite material. Currently, standard methods for consistently generating *P. falciparum* zygotes and ookinetes involved feeding gametocytes to *Anopheles* mosquitoes followed by dissection of these sexual stage

forms from mosquito midguts. Unlike the dissection of sporozoites from salivary glands, which has routinely yielded sporozoites on the order of 10^4 per gland that have been viable for days, one midgut typically yielded 10^1 to 10^2 ookinetes, which were only viable for a period of 6-12 hours (*Weiss and Vanderberg 1976, Vermeulen et al., 1985, Beier et al., 1992*). Few facilities are equipped to experimentally infect mosquitoes with *P. falciparum* compared to the number of laboratories capable of generating *P. falciparum* gametocyte cultures. Thus, as an alternative, this optimized *in vitro* sexual stage culture method will promote new avenues of research on this neglected stage of parasite biology.

In vitro sexual stage parasites are an incredible advantage for understanding sexual development as this process has been otherwise difficult to observe and manipulate within the mosquito midgut. Conventional membrane feeding assays used to test potential transmission blocking vaccine targets rely on infecting mosquitoes with gametocytes, then confirming the presence of oocysts by dissection of mosquito midguts. To determine the stage at which transmission is interrupted – gamete emergence and fusion, zygote formation, ookinete development or ookinete invasion of the midgut barrier – it is often necessary to use *in vitro* sexual stage parasites from animal-infecting *Plasmodia*. This *P. falciparum* sexual stage culture protocol will allow researchers to focus their studies on *P. falciparum* without the need to employ animal-infecting *Plasmodium spp*. for follow-up studies.

This protocol facilitates the study of sexual stage parasites of the human disease-causing agent, *P. falciparum*. The parasites generated from this method can be used to enhance transmission blocking studies in an effort to understand the

mechanisms underlying transmission-blocking interventions, these parasites can be used to perform organism-wide proteomic studies to gain a global understanding of *P. falciparum* sexual stage biology. This method can also be used as a stepping stone for the mosquito-free, *in vitro*-generation of sporozoites, which would enhance attenuated-sporozoite vaccine research. This improved production of *P. falciparum* sexual stage parasites is a significant step towards understanding *P. falciparum* sexual stage biology, and its optimization comes at a critical juncture in *Plasmodium* transmission-blocking vaccine research.

Chapter 2 will be submitted for publication as: Bounkeua, V., Li, F., Vinetz, J.M. “Biological features of *in vitro*-cultivated *Plasmodium falciparum* sexual stage parasites”. The dissertation author is the primary author and investigator of this paper.

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CHAPTER 3

EX VIVO CULTIVATION OF *PLASMODIUM VIVAX* OOKINETES

Abstract

Over 500 million people are diagnosed with malaria annually, and *Plasmodium vivax* is responsible for over half of the malaria cases in the Americas and Southeast Asia. *Plasmodium* transmission depends on successful completion of its sexual development in the mosquito vector. A modified *P. vivax* sexual stage culture protocol was developed and generated up to 10^6 macrogametocytes, 10^4 zygotes and 10^3 ookinetes per 10 ml of *P. vivax*-infected patient blood. Zygote and ookinete production did not correlate with either gametocyte density or the presence of micro- and macrogametocytes.

Introduction

Plasmodium vivax is responsible for 70-80 million annual cases of malaria worldwide and accounts for over half of malaria cases in the Americas and Southeast Asia (*Mendis et al, 2001, Guerra et al., 2006, Hay et al., 2004, Price et al., 2007*). In the Amazon basin region of Peru, *P. vivax* incidence has increased since 1991 and remains the predominant cause of malaria in the Iquitos region where it is hypoendemic (*Branch et al., 2005, Roshanravan et al., 2003*).

Successful transmission of the *Plasmodium* parasite is dependent on completion of sexual development in the mosquito midgut. Indeed, a recent field study determined that the most critical period influencing *P. vivax* transmission to mosquitoes was gametogenesis and fertilization, which occurs in the midgut (*Zollner et al., 2006*). Molecular interventions to block sexual development in the mosquito and thus prevent people from infecting mosquitoes, termed transmission-blocking

vaccines, are an emerging field of malaria research (*Saul 2007, Gwadz 1976, Lavazec and Bourgouin 2008*). The discovery of new transmission-blocking vaccine targets for *P. vivax* will be accelerated by the ability to generate large numbers of sexual stage parasites for genetic, proteomic and biochemical analyses.

Infectious *P. vivax* gametocytes cannot be generated in culture, necessitating the use of *P. vivax*-infected patient samples to obtain parasite material for study. Only one report detailing *P. vivax* ookinete production *in vitro* has been published, and one report described the incidental observation of *P. vivax* ookinetes (*Suwanabun et al., 2001, Hummert, 1994*). A method was developed from previous protocols to produce *P. vivax* sexual stage parasites *ex vivo* (*Al-Olayan et al 2002, Chapter 2, Methods, Westenberger et al., submitted*).

Methods

Human subjects

Human subjects were recruited from local health clinics (Bellavista/Nanay, Progresso, Masusa, Moronacocha, and San Juan) and regional hospitals (Hospital Apoyo Iquitos and Hospital Regional Loreto) in July, 2009. Twenty-one adult (>18 year old) subjects with a self-reported history of fever and a diagnostic Giemsa-stained blood smear that was confirmed positive only for *P. vivax* malaria by at least two independent microscopists were recruited (Table 3.A1). Patients were enrolled in this study after they gave verbal and written informed consent as approved by institutional review boards at the University of California San Diego, Johns Hopkins Bloomberg

School of Public Health, and ethical review boards at the Peruvian Ministry of Health and Asociacion Beneficia PRISMA.

Approximately 10-15 ml of venous blood was collected into heparin-anticoagulated tubes and transported from the clinic to the laboratory in a 37^{o}C water bath within 1 hour of blood draw. After blood was drawn, patients were then provided with curative doses of primaquine and chloroquine according to Peruvian Ministry of Health guidelines and as described in the approved study protocol. Diagnostic blood smears were further analyzed to quantify asexual and sexual stage parasite densities as well the presence of micro- and macrogametocytes. Slides were analysed by at least two independent microscopists and determined to contain *P. vivax*-infected blood. No samples with mixed *P. falciparum* infection were detected, and no *P. falciparum* gametocytes were seen on the diagnostic blood smears.

Production of *P. vivax* sexual stage parasites from patient blood samples

P. vivax-infected patient blood was depleted of white blood cells (WBC) using a CF-11 column as follows. Autoclaved glass wool and CF-11 powder was packed into a sterile 20 ml syringe until the packed volume of CF-11 was 10 ml. The plunger was removed and a 21G needle was placed on the end of the syringe. The column was equilibrated with 10 ml of 37^{o}C suspended animation solution (SA: 10 mM Tris, 170 mM NaCl, 10 mM glucose, pH 7.4) (*Carter and Nijhout 1997*). Parasitized patient blood was pelleted by centrifugation, resuspended in 3-volumes of pre-warmed SA and then gravity filtered over the CF-11 column in a 37^{o}C incubator. Flow-through was collected and centrifuged to pellet WBC-depleted parasitized red blood cells.

Gametogenesis was induced by resuspending the pellet in 21^{o}C heat-inactivated AB^{+} human serum or 21^{o}C gametogenesis solution (10 mM Tris, 170 mM NaCl, 10 mM glucose, 25 mM $NaHCO_3$, 100 mM xanthenuric acid, 20% FCS, pH 8.4) for 60 minutes at 21^{o}C (*Billker et al., 1998, Garcia et al., 1998*). Parasites were then centrifuged and resuspended to 20% hematocrit in freshly made, filter-sterilized 21^{o}C ookinete medium (RPMI 1640, 25 mM HEPES, 2 mM L-glutamine, 2 g/l $NaHCO_3$, 50 mg/l hypoxanthine, 15-20% heat-inactivated AB^{+} human serum, 100U penicillin/ml, 100 µg streptomycin/ml pH 8.2 - 8.4 with NaOH), (*modified from Al-Olayan et al., 2002*). Cultures were purified after 24-36 hours. All centrifugations were done at 800 x g for 3 minutes.

Purification of sexual stage parasites

The majority of uninfected erythrocytes were removed from sexual stage parasite cultures using density gradient centrifugation (Lympholyte-H, Cedarlane Laboratories, Burlington, NC USA) as described (*Chapter 2, Methods*). Parasites were collected from the gradient interface, washed twice in ookinete medium and further purified by selection on a MidiMACS magnetic separator using an LD-50 column (Miltenyi Biotec, Bergisch Gladbach, Germany) (*Ribault et al., 2008*). The column was placed on the magnet and equilibrated with 5 ml of ookinete medium. The collected parasites were resuspended in 1 ml of ookinete medium and allowed to pass through the column by gravity flow, after which the magnetic column was washed with 5 ml of ookinete medium. Parasites were eluted with 3 ml of ookinete medium by

removing the column from the magnetic stand and gently forcing the medium through with a plunger, according to manufacturer's instructions.

Quantification of sexual stage parasites

Eluted parasites were centrifuged then quantified by light microscopic examination of Giemsa-stained thin smears. A minimum of 10 fields at 100X were counted using an Olympus BX51 microscope (Olympus, Center Valley, PA, USA); pictures were taken with an Olympus DP71 camera (Olympus, Center Valley, PA, USA). Statistical analysis was done using student's t-test assuming unequal variance.

RNA isolation and RT-PCR

RNA was extracted from *P. vivax*-infected patient blood using Trizol reagent (Invitrogen, Carlsbad, CA, USA), and DNA contamination was removed using DNA-free (Ambion, Austin, TX, USA) according to manufacturer's instructions. Species-specific primers for *P. falciparum* Pfs25 (F: 5' – TCT TGT ACA TTG GGA ACT TTG CCT – 3'; R: 5' – TGC GAA AGT TAC CGT GGA TAC TG –3'). SuperScript III first-strand synthesis system (Invitrogen, Carlsbad, CA, USA) was used to generate cDNA according to manufacturer's instructions. PCR on resulting cDNA was done using Platinum PCR SuperMix High Fidelity (Invitrogen, Carlsbad, CA, USA) with 250 nM gene-specific primers; 35 cycles were run with an annealing temperature of 55^{o}C and an extension temperature of 68^{o}C (Table 4.A1). Amplified product was electrophoresed on a 1% agarose gel, stained with ethidium bromide and visualized with an ultraviolet transilluminator (Bio-Rad, Hercules, CA, USA).

Results and Discussion

Microscopic evaluation of *P. vivax*-infected patient samples

Giemsa-stained thick and thin blood smears of 21 *P. vivax*-infected patient samples were analyzed and determined to be free of *P. falciparum* asexual parasites and gametocytes of any stage by and at least two independent expert microscopists: the technician at the local health post and a malaria technician working at an Iquitos malaria diagnostic referral laboratory. These Giemsa-stained thick smears were analyzed to determine the number and type of gametocytes detected in a minimum of 10, 100x-powered fields (Table 3.A1).

Only patient samples with microscopically detectable gametocytes were subsequently used for *ex vivo* sexual stage parasite cultures (Figure 3.1). Eleven of 21 patients had 1000-7000 gametocytes / µl of blood; three patients had more than 7000 gametocytes / µl of blood, and seven patients had less than 1000 gametocytes / µl of blood. Four samples had readily detectable micro- and macrogametocytes, while only microgametocytes were detected in six samples and only macrogametocytes were detected in 11 samples; one sample contained gametocytes whose type could not be determined.

P. falciparum was not detected by RT-PCR in *P. vivax*-infected patient samples

RNA isolated from *P. vivax*-infected patient blood was amplified using primers for *P. falciparum* Pfs25 (Figure 3.1). Only *P. vivax*-specific primers resulted in RT-PCR amplification of patient samples (data not shown), confirming the absence of mixed *P. falciparum* infection (Figure 3.2).

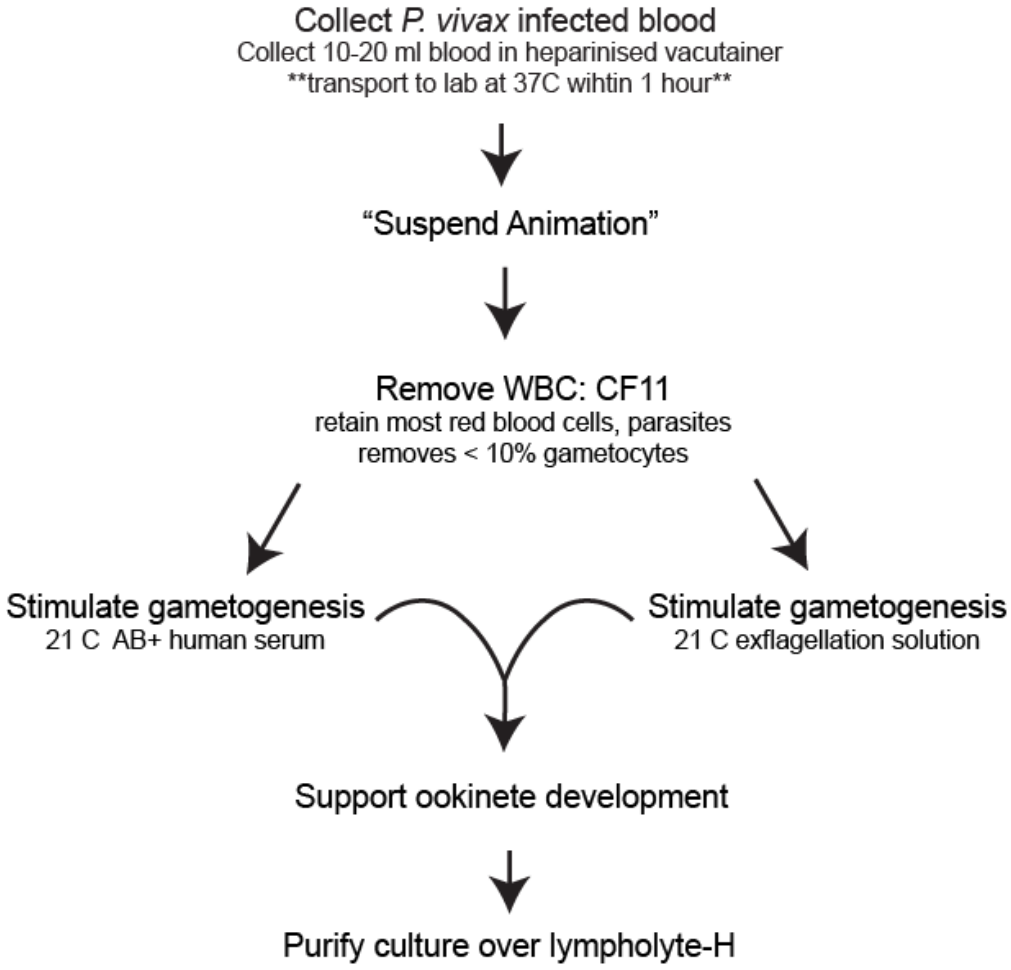


Figure 3.1
Schematic representation of *P. vivax in vitro* sexual stage cultivation protocol.
P. vivax-infected patient blood was collected and gametogenesis was reversibly inhibited by diluting samples in SA buffer. During this time, WBCs were removed by gravity filtration through a sterile CF-11 column. Gametocytes were stimulated to become gametes which fertilized into zygotes. Zygotes were resuspended in ookinete culture medium to promote ookinetes development. Cultures were maintained for 24-36 hours at 21^{o}C at which point sexual stage parasites were purified, fixed on glass slides, stained with Leukostat and quantified by light microscopy.

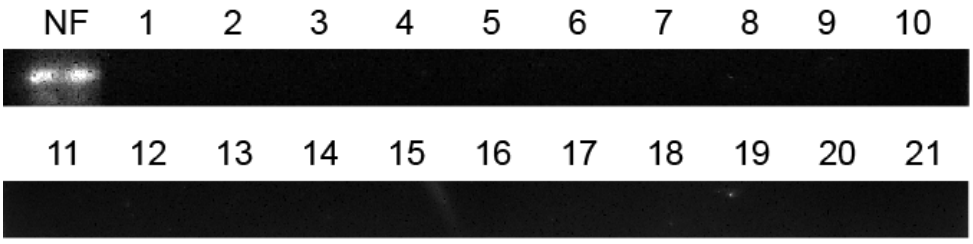


Figure 3.2
***P. falciparum* mixed infections were not detected by RT-PCR.**
RNA from *P. vivax*-infected patient blood was not amplified using *P. falciparum* species-specific primers.

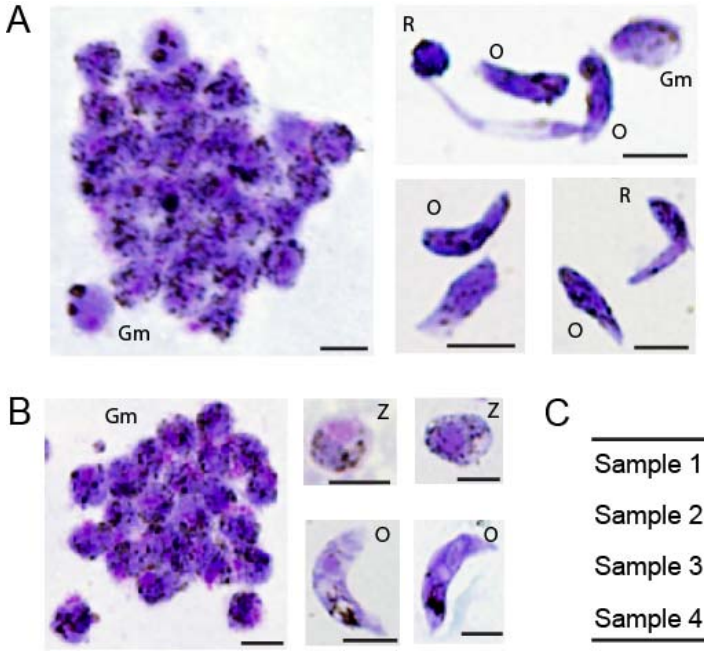
Effect of gametogenesis protocol on *P. vivax* ookinete production *ex vivo*

There have been only two previous reports of *in vitro*-generated *P. vivax* ookinetes. One was a case study that described the incidental finding of *P. vivax* ookinetes in a sample of blood left at room temperature overnight (*Hummert 1994*). In the second report, *Suwanabun et al.* generated *P. vivax* ookinetes using xanthurenic acid to stimulate gametogenesis (*Suwanabun et al., 2001*).To determine whether gametogenesis using serum or xanthurenic acid resulted in improved ookinete production, four samples were divided equally and each was treated with two different gametogenesis solutions: exflagellation solution which contained xanthurenic acid (GS), or heat-inactivated AB^{+} human serum (HS). One hour after gametogenesis, parasites were centrifuged and resuspended in ookinete medium for 24 hours and subsequently examined for production of sexual stage parasites (Figure 3.2).

The GS gametogenesis protocol resulted in the production of macrogametes only (samples 1 and 3), macrogametes and zygotes only (sample 2) and all forms including ookinetes (sample 4). The HS gametogenesis protocol resulted in similar

production of sexual stage parasite forms, except for sample 3, where zygotes and ookinetes were produced in addition to macrogametes. Although it has been well established that xanthurenic acid enhances gametogenesis, likely through calcium mediated pathways, a recent study suggested cGMP is an independent activator of gametogenesis, and empiric evidence demonstrated that serum alone was sufficient for gametogenesis (*Billker et al., 1998, Muhai et al., 2001, McRobert et al., 2008, Carter et al., 1993, Arai et al., 2001*) and has been successfully used to generate *P. falciparum* ookinetes *in vitro* (*Chapter 2*).

Both gametogenesis protocols resulted in the production of *P. vivax* ookinetes in sample 4 (Figure 3.3). However, GS sample 4 contained a high proportion of retort forms, which are incompletely developed ookinetes, and ookinetes with non-characteristic morphology (Figure 3.3). In contrast, HS sample 4, which was derived from the same patient sample, had few retorts, and ookinetes had characteristic morphology (Figure 3.3). The altered ookinete morphology of GS sample 4 compared to HS sample 4 was surprising because they were derived from the same patient sample and the only difference in handling occurred during gametogenesis. Ookinete development occurred in the subsequent 16-28 hours, when both GS sample 4 and HS sample 4 were incubated in ookinete medium. The high proportion of retorts in GS 4 compared to HS 4 may have reflected either lagging ookinete development using the GS gametogenesis protocol or a block in mature ookinete development secondary to low levels of a necessary co-factor present in human serum, the identification of which was beyond the scope of this study. Based on these results, the remaining samples in this study were cultured using HS to stimulate gametogenesis.



	GS	HS
Sample 1	M	M
Sample 2	M, Z	M, Z
Sample 3	M	M, Z, O
Sample 4	M, Z, O	M, Z, O

Figure 3.3
Leukostat-stained light micrographs of *ex vivo*-cultivated *Plasmodium vivax* sexual stage parasites with different gametogenesis protocols.
A. Sexual stage parasite cultures generated by GS gametogenesis protocol exhibited retort forms (R) and ookinetes (O) with abnormal morphology. Scale bar: 5 µm
B. *P. vivax* sexual stage parasite cultures generated by HS gametogenesis protocol. Macrogametes (Gm), zygotes (Z) and ookinetes (O) exhibited typical morphology.
C. Effect of gametogenesis protocol on *P. vivax* sexual stage parasite production. Both GS and HS protocols resulted in the production of macrogametes (Gm), zygotes (Z) and ookinetes (O). Sample 3 only generated macrogametes using the GS protocol but generated zygotes and ookinetes using the HS protocol. Scale bars: 5 µm.

Production of *P. vivax* sexual stage parasites *ex vivo*

Of the 21 blood samples collected for this study, 8 (38%) were cultivated through to ookinetes, 8 (38%) were cultivated through to zygotes, 5 (24%) only developed into macrogametes (Table 3.A1). Macrogamete, zygote and ookinete yields as high as 10^6, 10^4 and 10^3 parasites per 10 ml of whole blood, respectively, were

achieved. Macrogametes constituted the majority of sexual stage parasites found in these cultures (Table 3.1).

Table 3.1
***Plasmodium vivax* sexual stage parasite yields for 3 representative cultures**
Three representative *ex vivo*-generated *P. vivax* sexual stage parasite cultures are shown. Yields are indicated as the number of parasites per 10 ml of infected patient blood.

Culture	3	6	22
Macrogamete (x 10^6)	160	1.4	0.1
Zygote/Retort (x 10^3)	17	29	16
Ookinete (x 10^3)	6.8	1.4	0.1

The *P. vivax* sexual stage parasite forms produced in these cultures could be distinguished from each other by light microscopy. *P. vivax* macrogametes and zygotes, both round forms, could be microscopically distinguished from gametocytes by their dispersed hemozoin and lack of surrounding erythrocyte membrane. Round macrogametes had one small nucleus while round zygotes and retort forms had one, occasionally two, large nuclei. Surprisingly, gametocytes were not detected in any of the sexual stage cultures at the end of the culture period. Ookinetes had a characteristic elongated form with one large nucleus and multiple cleared spots, which may have constituted vacuoles (Figure 3.4).

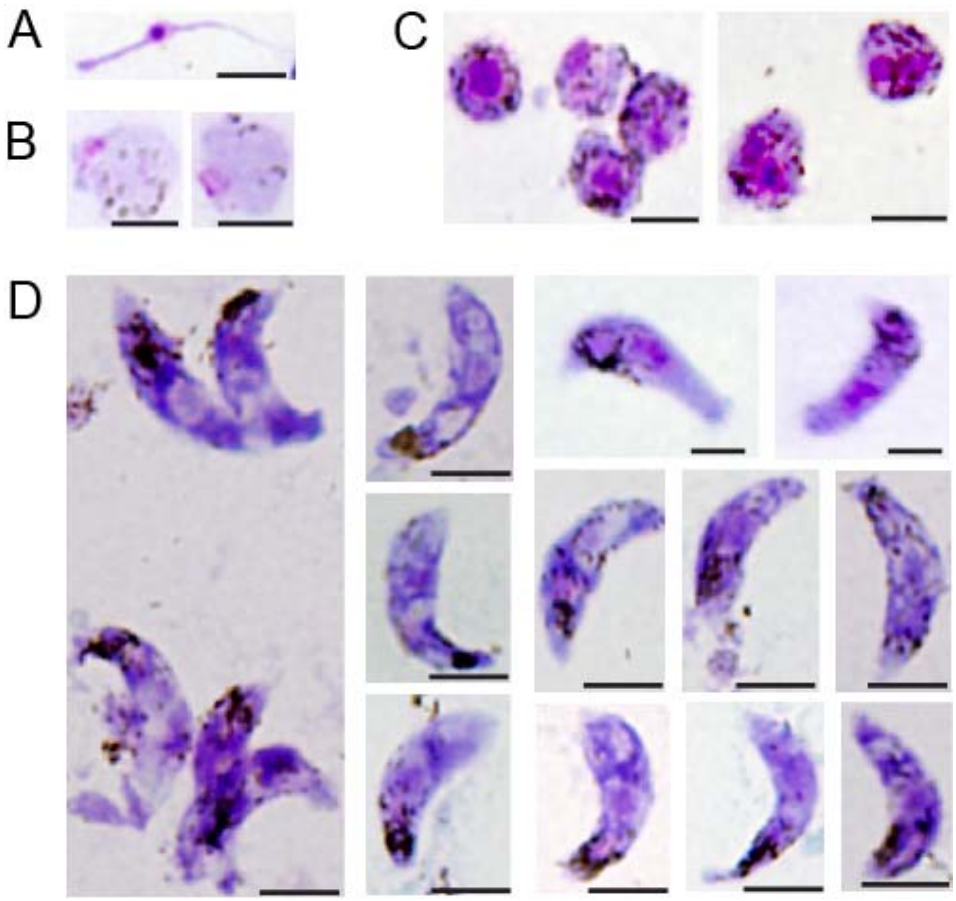


Figure 3.4
Leukostat-stained light micrographs of *ex vivo*-cultivated *Plasmodium vivax* sexual stage parasites.
A. Microgamete with eosinophilic nucleus. Scale bars: 5 µm
B. Macrogamete, each with a single small nucleus. Scale bars: 5 µm
C. Zygotes and retorts with one or two large nuclei. Scale bars: 5 µm
D. Ookinetes with one or two nuclei. Scale bars: 5 µm

P. vivax ookinete production did not correlate with gametocyte density or the presence of micro- and macrogametocytes in peripheral blood smears

Ookinete development could not be correlated to gametocyte density or the presence of micro- and/or macrogametocytes in peripheral blood, parameters that can be quantified from a diagnostic blood smear. Zygote and ookinete development were

not correlated with microscopically determined parasitemia or gametocytemia (Figure 3.5). Samples with detectable presence of both micro- and macrogametocytes were more likely to produce zygotes and ookinetes, but this was not statistically significant (Figure 3.5). Although this finding was consistent with previous reports, data from *in vitro*-cultivated *P. falciparum* ookinetes suggested that ookinete development was strongly associated with gametocyte maturity and microgametocyte to macrogametocyte sex ratios (*Zollner et al., 2006, Kligler and Mer 1977, Eyles et al., 1948, Sattabongkot et al., 1991*, *Chapter 2*).

The maturity of *P. vivax* gametocytes was impossible to determine on microscopic examination. Unlike *P. falciparum*, which has morphologically distinct gametocyte developmental stages, *P. vivax* gametocytes have been thought to be morphologically similar during development and could only be distinguished as micro- and macrogametocytes at maturity. Additionally, the low parasite and gametocyte densities common in *P. vivax* infection may have resulted in an underestimation of the micro- or macrogametocytes present. Indeed, of 6 patient samples with undetectable macrogametocytes, all generated macrogametes and 4 generated zygotes and/or ookinetes. Of 11 patient samples with undetectable microgametes, 8 generated zygotes and/or ookinetes. As these forms could not have been generated without the presence of both micro- and macrogametocytes in culture, this clearly indicated that microscopy was inadequate for the accurate detection and quantification of *P. vivax* gametocytes in peripheral patient blood samples. Molecular approaches to quantification, such as quantitative real-time PCR analysis of stage-specific gametocyte genes, such as *pvs28* and *pvs230*, as well as microgametocyte-

specific genes, such as *alpha tubulin II*, and macrogametocyte-specific genes, such as *pvg377*, would likely improve estimates of gametocyte maturity, sex ratio and density (*Coleman et al., 2006, Nicastri et al., 2009, Parekh et al.,2007*).

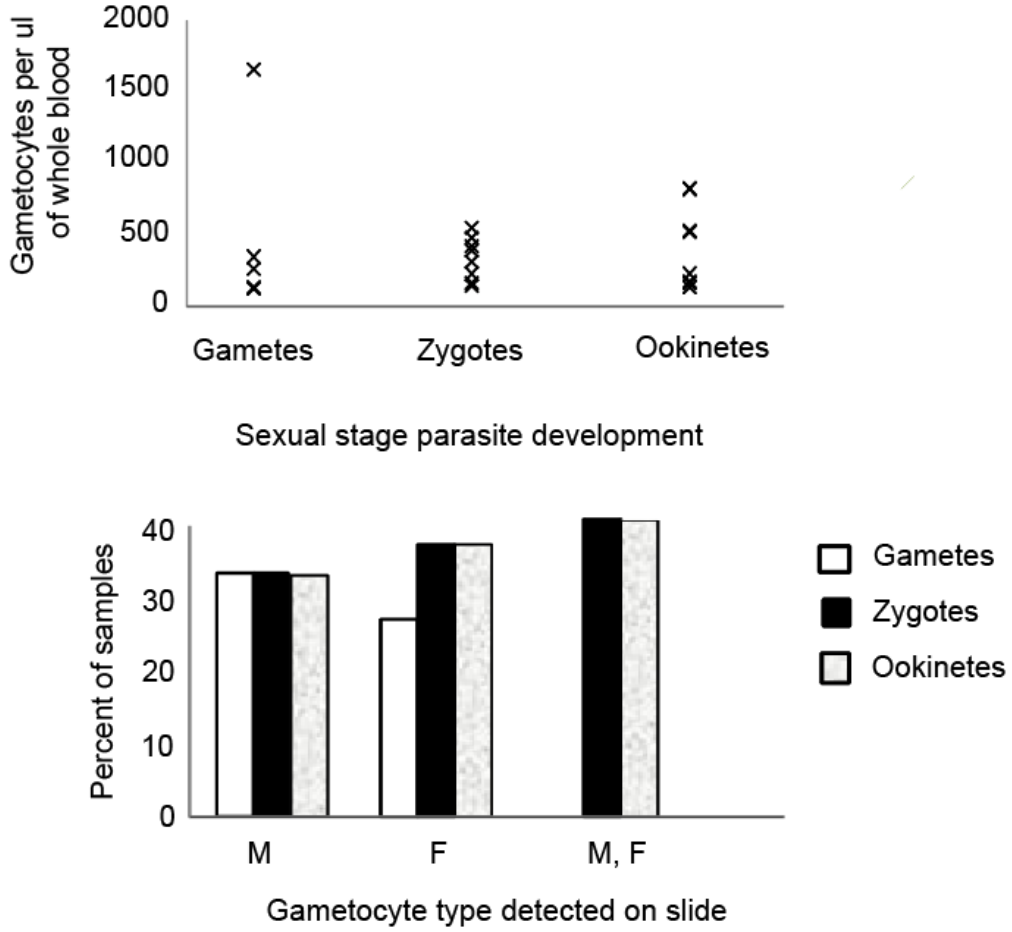


Figure 3.5
***Ex vivo Plasmodium vivax* zygote and ookinete production did not correlate with mature gametocyte density or the presence of micro- and macrogametocytes as determined by microscopic examination.**
A. Zygote and ookinete production did not correlate with microscopically determined gametocytemia.
B. Patient samples that contained both microgametocytes and macrogametocytes detectable by blood smear produced zygotes and ookinetes. However, zygotes and ookinetes were also produced from patient samples that contained only one type of gametocyte detectable by microscopy. The detection of both types of gametocytes in a diagnostic smear did not positively correlate with zygote and ookinete production as determined by student's t-test (*p*-value 0.36).

Conclusions

The *in vitro P. vivax* sexual stage parasite cultivation method described generated up to 10^6 macrogametocytes, 10^4 zygotes and 10^3 ookinetes per 10 ml of *P. vivax*-infected patient blood. Of the 21 patient samples that were collected and processed using this method, all yielded macrogametes and 16 samples generated zygotes and/or ookinetes. Surprisingly, the production of zygotes and ookinetes was not significantly correlated to estimates of gametocyte density or the detection of micro- and macrogametocytes. However, this was likely due to the low sensitivity associated with microscopic examination of *P. vivax* peripheral blood smears and this finding indicated that a molecular approach, such as quantitative real-time PCR, may have provided a more accurate quantification of gametocyte density and sex ratio.

This protocol built on previous work and contributed to the continued efforts at *in vitro* production of *P. vivax* sexual stage parasites. The availability of *P. vivax* sexual stage parasites will allow in-depth investigations into the mechanisms underlying successful transmission-blocking interventions. This cultivation method will further promote our understanding of sexual stage parasite development, especially in this neglected human disease-causing agent, by facilitating genetic and biochemical analyses of sexual stage parasites.

Chapter 3 will be submitted for publication: Bounkeua, V., Li, F., Abeles, S.R., McClean, C.M., Vinetz, J.M. "*in vitro* development of *Plasmodium vivax* ookinetes is positively correlated to gametocyte maturity and sex ratio as determined by qRT-PCR". The dissertation author is the primary author and investigator of this paper.

Table 3.A1
Summary of *Plasmodium vivax*-infected patient samples, continued on next page. Parasite and gametocyte density were calculated by determining the number of parasites per 200 WBC. The sex of the gametocytes detected was determined when possible. The sexual stage parasite forms detected in culture are also listed. Abbreviations: M (Microgametocyte), F (Macrogametocyte), ND (Not Determined), Mg (Macrogamete), Zygote (Z), Ookinete (O)

ID	Parasites / µl	Gametocytes / µl	Sex	Cultured forms
1	5.6×10^3	1.3×10^3	M, F	Mg, Z
2	28×10^3	3.2×10^3	F	Mg, Z
3	3.7×10^3	7.2×10^3	M	Mg, Z, O
4	9.1×10^3	0.8×10^3	M, F	Mg, Z, O
5	17×10^3	0.4×10^3	F	Mg
6	14×10^3	4.2×10^3	F	Mg, Z, O
7	10×10^3	3.8×10^3	F	Mg, Z
8	1.4×10^3	4.3×10^3	M	Mg, Z, O
9	12×10^3	16×10^3	M	Mg
10	2.4×10^3	2.1×10^3	M, F	Mg, Z
11	1.5×10^3	0.6×10^3	M, F	Mg, Z, O
12	11×10^3	2.9×10^3	M	Mg, Z
13	3.1×10^3	0.3×10^3	M	Mg
14	1.6×10^3	0.7×10^3	M	Mg, Z
15	2.4×10^3	1.3×10^3	F	Mg, Z, O
16	3.6×10^3	2.4×10^3	F	Mg
17	1.9×10^3	0.5×10^3	F	Mg, Z
18	5.9×10^3	1.6×10^3	F	Mg
19	17×10^3	4.5×10^3	M, F	Mg, Z
20	8.6×10^3	7.3×10^3	F	Mg, Z, O
21	3.9×10^3	0.4×10^3	ND	Mg, Z, O

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CHAPTER 4

PLASMEPSIN X FACILITATES *PLASMODIUM* TRANSMISSION TO THE MOSQUITO VECTOR

Abstract

Plasmodium invasion of the mosquito midgut is a population bottleneck of the *Plasmodium* life cycle. Recent malaria vaccine research efforts have focused on blocking this crucial step in the *Plasmodium* transmission cycle and have found that specific enzymes are necessary for this process. Inhibiting the activity of these enzymes can decrease or block parasite transmission to mosquitoes. Transcriptomic and proteomic data suggested that a family of *Plasmodium* aspartic proteases, called plasmepsins (PM), were expressed during *Plasmodium* development in the mosquito midgut. To date, most work done on plasmepsins, including PM I, II, IV, V and HAP, have demonstrated a critical role in blood stage parasites. A novel plasmepsin, PM X, was found to be expressed in sexual stage parasites of *P. falciparum*. Furthermore, the presence of antibodies to PM X reduced *P. falciparum* infectivity to *Anopheles gambiae* mosquitoes; this signified that this protease was functionally important for *P. falciparum* transmission in the mosquito vector.

Introduction

Plasmodium sexual development in the mosquito midgut is a neglected area of malaria research, and the molecular interactions between *Plasmodium* and its mosquito vector have only recently begun to be understood (Figure 1.1). Understanding the mechanisms underlying these *Plasmodium*-mosqito interactions is essential to the development of a transmission-blocking vaccine. The ookinete is of particular interest in transmission biology. The ookinete is a motile form of the *Plasmodium* parasite that is responsible for invasion of the mosquito midgut barrier

(Figure 1.1). After successful ookinete invasion of the mosquito midgut, it becomes an oocyst and supports development of sporozoites, the form of the parasite that infects humans. One ookinete that succesfully penetrates the midgut barrier has the potential to generate thousands of sporozoites. For this reason, transmission-blocking vaccine strategies have focused on preventing either ookinete develoment or subsequent penetration of the midgut barrier. Multiple enzymes secreted by the ookinete were shown to be essential for parasite transmission and may be potential transmission-blocking vaccines targets (*Huber et al., 1991, Vinetz et al., 2000, Vinetz et al., 2004* for review). Recent transcriptomic and proteomic data suggested that *Plasmodium* aspartic proteases, plasmepsins, were expressed during mosquito stage development. *P. falciparum* expresses 10 members of this aspartic protease family. Plasmepsins I, II, IV and HAP were shown to be important for hemoglobin metabolism in the food vacuole of blood stage parasites (*Coombs et al., 2001* for review), and Plasmepsin V was recently shown to cleave localize to the endoplasmic reticulum of blood stage parasites (*Klemba and Goldberg, 2005*). Plasmepsin VI has been shown to be important for sporogonic development, particularly early oocyst development (*Ecker et al., 2008*). However the function of the remaining four plasmepsins, including Plasmpesin X (PM X), have yet to be determined.

Transcriptomic data demonstrated that *P. falciparum PM X* mRNA is expressed during gametocytogenesis and in sexual stage zygotes and ookinetes, this suggested that PM X may be required for sexual stage development and/or ookinete invasion of the midgut barrier. PM X, similar to other members of this protease family, was found to contain a predicted signal peptide and was expressed as a

zymogen with a pro-enzyme domain. The presence of a signal peptide suggested that this protease was processed through the endoplasmic reticulum and could be packaged into micronemes, the ookinete secretory organelle. PM X expression in *Plasmodium* ookinetes may facilitate *Plasmodium* invasion of its mosquito vector, and the presence of PM X antibodies in an infectious bloodmeal reduced *Plasmodium* transmission to its mosquito vector.

Methods

Parasites and Mosquitoes

P. falciparum strain NF54 used in this study was provided by Dr. Stephen Hoffman (Sanaria, Rockville, MD USA). Parasites were maintained in asexual culture according to standard protocol (*Read and Hyde, 1993*). Gametocytes as well as sexual stage macrogametes, zygotes and ookinetes were cultured and purified as described (Chapter 2, Methods).

Anopheles gambiae (*A. gambiae*) used in this study were a generous gift from Dr. Anthony James (University of California Irvine, Irvine, CA USA). Mosquitoes were maintained in an enclosed, humidified insectary with an automated 12-hour light-dark cycle according to standard CDC protocol (*Gerberg et al., 1994*). Mosquitoes used in this study were closely monitored and controlled according to the protocol for non-vertebrate animal subjects approved by the UCSD Institutional Animal Care and Use Committee (IACUC).

DNA/RNA isolation and RT-PCR

Plasmodium parasites were either generated *in vitro* or isolated *ex vivo* from infected mosquitoes. For *ex vivo*-isolated mosquito-stage parasite samples, midguts from mosquitoes were dissected and homogenized in groups of five midguts. Genomic DNA was isolated using NucleoSpin Blood (Macherey-Nagel, Bethlehem, PA, USA). Total RNA was isolated using RNeasy (Qiagen, Valencia, CA, USA) and contaminating DNA was removed using DNA-free (Ambion, Austin, TX, USA) according to manufacturer's instructions. Reverse transcription was completed using gene-specific primers (Table 4.A1) for PM X and Pfs25 with SuperScript III first-strand synthesis system (Invitrogen, Carlsbad, CA, USA) according to manufacturer's instructions. PCR on resulting cDNA was done using Platinum PCR SuperMix High Fidelity (Invitrogen, Carlsbad, CA, USA) with 250 nM gene-specific primers; 35 cycles were run with an annealing temperature of 55^{o}C and an extension temperature of 68^{o}C (Table 4.A1).

Production of recombinant PM X in *E. coli*

P. falciparum PM X was amplified from genomic NF54 DNA to generate a gene that lacked the signal peptide. PCR products were gel purified on a 0.8% agarose gel using the PureLink gel extraction kit (Invitrogen, Carlsbad, CA, USA), ligated into pCR 4.0 (Invitrogen, Carlsbad, CA, USA), transformed into Top10 competent cells (Invitrogen, Carlsbad, CA, USA) and sequence-verified (Eton Bioscience, San Diego, CA, USA). *PM X* was then cloned into expression vector pGEX 4T-1 (GE Healthcare, Piscataway, NJ, USA) using EcoRI.

The rPM X GST fusion protein (rPM X-GST) was expressed in Rosetta (Merck/Novagen, Darmstadt, Germany) and SHuffle (New England Biolabs, Ipswich, MA, USA) competent cells according to standard protocol (*LaVallie, 1995*). Briefly, competent cells were transformed with 250-500 ng of purified plasmid DNA, streaked on LB agar plates embedded with 100 μg/ml ampicillin (LB-amp) and allowed to grow overnight at 37^{o}C. Fresh colonies were inoculated into a 5 ml liquid starter culture of LB-amp and grown to confluence. Starter cultures were used to inoculate 500 ml cultures which were grown to OD_{600} 0.5-1. Protein expression was then induced with 0.3 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) for 0.5 to 18 hours at 18^{0}C to 37^{0}C.

rPM X-GST was isolated from either the soluble fraction or from inclusion bodies using BugBuster extraction reagent (Merck/Novagen, Darmstadt, Germany). Inclusion bodies were purified and solubilized in both 4-8 M urea and 4-6 M guanidine hydrochloride overnight. Solubilized protein was then refolded in Tris buffer, pH 7 – 10 in the presence or absence of dithiothreitol (DTT), 2-mercaptoethanol, arginine, glutathione, ethylenediaminetetraacetic acid (EDTA), polyethylene glycol (PEG) $MgCl_2$, and/or $CaCl_2$. Refolded proteins were visually scanned for the presence of precipitates.

Refolding conditions which did not result in protein precipitation were selected (modified from *Luker et al., 1996,* and *Shenai et al., 2000*). In protocol 1, inclusion bodies were solubilized in 6 M guanidine hydrochloride, 20 mM Tris, 250 mM NaCl, pH 8.0 overnight at 4^{0}C. Soluble protein was refolded by rapid dilution in 100 mM Tris, 1 mM EDTA, 250 mM L-arginine, pH 8.0 and incubated overnight at 4^{o}C. In

protocol 2, inclusion bodies were solubilized in 8 M urea, 100 mM Tris 1 mM glycine, 1 mM EDTA, pH 8.0 overnight at 4^{o}C. Soluble protein was diluted 1:1000 in 20 mM Tris, pH 8.0. Refolded protein was dialyzed with Slide-A-Lyzer Dialysis cassettes (Thermo Fisher Scientific, Waltham, MA, USA) with 4 buffer changes and concentrated using Centricon columns (Millipore Corp., Billerica, MA, USA). Refolded proteins and proteins purified from the bacterial lysate soluble fraction were assayed for activity using QuantiCleave fluorescent substrate kit (Thermo Fisher Scientific, Waltham, MA, USA) according to manufacturer's instructions.

Production of monoclonal antibodes against PM X

Peptide monoclonal antibodies (mAb) directed against three regions of PM X were generated in mice (A&G Pharmaceuticals, Columbia, MD, USA) and the resulting hybridoma cell supernatants were screened against the same recombinant peptides used to generate mAb as well as full length rPM X using enzyme linked immuno-sorbant assay (ELISA) and western immunoblot, respectively. Hybridoma cells were grown in Dulbecco's modified eagles medium (DMEM, CellGro, Herndon, VA, USA) supplemented with 10% FCS. Antibodies were used as either concentrated in hybridoma supernatant or purified mAb in PBS (A&G Pharmaceuticals, Columbia, MD, USA). Additionally, monoclonal antibodies 1C3 and 4B7 against *P. falciparum* chitinase (*Langer et al., 2002*) and surface protein 25 (Malaria Research and Reference Reagent Resource Center, Manassas, VA, USA deposited by Dr. D.C. Kaslow, *Barr et al, 1991*.), respectively, were also used.

Immunofluorescence assay

IFA were completed as described (Chapter 2, Methods).

Immunolocalization of Plasmepsin X by electron microscopy

P. falciparum sexual stage parasites were prepared and purified by density gradient centrifugation (Lympholyte-H, Cedarlane Laboratories, Burlington, NC USA). Two different methods were used for the initial overnight fixation: 4% paraformaldehyde and a mixture of 4% paraformaldehyde and 0.5% glutaraldehyde. Samples were cryoprotected in sucrose, frozen in liquid nitrogen and cut into 80 nm sections with a Leica Ultracut UCT microtome equipped with an FCS cryoattachment at -100^{o}C. Sections were incubated with antibodies against PM X or Ig isotype control antibodies overnight at 4^{o}C, followed by incubation with 10-nm gold-conjubated anti-mouse for 2 hours at room temperature. Sections were adsorption stained as previously described and examined on a JEOL 1200 EX-II electron microscope (*Orlando et al., 2001*).

Western immunoblot analysis

P. falciparum parasites were prepared and completed for western immunoblot analysis as described (Chapter 2, Methods). Western immunoblots were probed with PM X mAb as well as PM X mAb that were incubated with 100 μg/ml of the peptides used to generate antibodies for at least one hour at room temperature.

Mass spectrometry

For rPM X protein verification, a Coomassie-stained SDS-PAGE gel slice was excised and processed for mass spectrometry (MS) analysis (The Scripps Research Institute, La Jolla, CA, USA). Gel slices were destained and proteins were reduced with 10 mM DTT, alkylated with 55 mM iodoacetamide and digested in-gel with trypsin as previously described (*Rosenfeld et al., 1992, Hellman, et al., 1995*). Samples were analyzed by mass spectrometry, and identified peptides were searched by BLAST against the *P. falciparum* genome.

Membrane feeding assays

Female *Anopheles gambiae* mosquitoes were selected 3-7 days post-emergence, put into cartons containing 40-60 mosquitoes each, and starved overnight for use in membrane feeding assays with *P. falciparum* gametocytes. Mature *P. falciparum* gametocytes were examined for their ability to undergo gametogenesis (*Carter and Alano, 1993, Chapter 2, Methods*). Cultures with exflagellation- and emergence-competent gametocytes were combined, mixed with antibodies and fed to *Anopheles gambiae* using water-jacketed glass membrane feeders as previously described (*Li et al., 2005*). To control for dilutional effects of adding antibody, the control (infected blood alone) was diluted with the appropriate amount of PBS. 20 minutes after the start of the membrane feeding assay, membrane feeders were removed from the mosquitoes, and unengorged mosquitoes were removed from cartons. *P. falciparum*-infected mosquitoes were kept in a secured incubator separate

from non-infected mosquitoes. Infected mosquitoes were fed with 8% fructose/0.05% p-aminobenzoic acid twice daily (*Sinden et al., 1984*).

After 7-11 days, mosquito midguts were dissected, stained with mercurochrome and examined for the presence of oocysts (*Sinden et al., 1984*). All manipulations were done in accordance with UCSD IACUC-approved protocol for non-vertebrate research animals. Statistics were calculated using Mann-Whitney test for statistical significance. Samples were considered to be statistically significant if the p-value ≤ 0.01.

In vitro-growth of *P. falciparum* sexual stages in the presence of antibodies to PM X

P. falciparum gametocytes were incubated with antibodies to PM X or Ig isotype control antibody and stimulated to undergo gametogenesis in small scale cultures. Micro- and macrogametes were quantified by light microscopy, and parasites were then put into ookinete medium in the presence of antibody and allowed to mature into zygotes and ookinetes for 48 hours. Sexual stage parasite cultures were stained with Leukostat and quantified by light microscopy.

PM X knockout strategies for *Plasmodium falciparum*

A single cross-over recombination strategy was used to knock out *PM X*. A 1 kb region of *PM X* was PCR amplified from *P. falciparum* genomic DNA, sub-cloned into pCR 2.1 (Invitrogen, Carlsbad, CA, USA), transformed into Top10 cells (Invitrogen, Carlsbad, CA, USA) and sequence-verified (Eton Biosciences, San Diego, CA, USA). Sequence-verified *PM X* fragments were then cloned into pHDWT vector

(Malaria Research and Reference Reagent Resource Center, Manassas, VA, USA deposited by Dr. D.C.Fidock and T.E. Wellems, *Fidock et al., 1998*) using EcoRI. A complementation plasmid containing the full-length *PM X* coding region as well as the upstream and downstream untranslated regions of *PM X* was generated. This construct contained 800 nucleotides of the 5' region upstream of the translation start site, the *PM X* coding region with a 6-HIS tag and a hemagglutin (HA) tag was well as 700 of the 3' region downstream of the stop codon. This construct contained the BSD resistance gene from pCAM-BSD (*Sidhu et al., 2005*).

For transfection, *P. falciparum* cultures were sorbitol-synchronized 48 hours prior to transfection. Ring stage parasites at 5-10% parasitemia were electroporated using the Bio-Rad Gene Pulser (Bio-Rad, Hercules, CA, USA) or Nucleofector technology (Lonza/Amaxa, Basel, Switzerland) using 100 µg or 10 µg of purified, endotoxin-free plasmid DNA resuspended in cytomix (120 mM KCl, 0.15 mM $CaCl_2$, 5mM $MgCl_2$, 2 mM ethylene glycol tetraacetic acid (EGTA), 10 mM K_2HPO_4/KH_2PO_4, 25 mM HEPES) or 100 µl T-cell solution (Lonza/Amaxa, Basel, Switzerland), respectively (*Fidock and Wellems, 1997*). The Gene Pulser II was set to 0.31 kV, 950 µF capacitance; the Amaxa Nucleofector was set to program U-33. Electroporated parasites were immediately transferred to warm, complete medium (RPMI1640, 25 mM HEPES, 2 mM L-glutamine, 2.4 g/L $NaHCO_3$, 50 mg/L hypoxanthine, 10% heat-inactivated AB^+ human serum).

Drug pressure was applied using the anti-folate drug WR99210 at 2.5 nM starting 24-48 hours after transfection and continued daily; 50 µl of blood was added to cultures under selection every 3 days and cultures were split every 10 days until

parasite replication rates maintained wild-type levels for one week (*Fidock et al., 1998*). Transfected parasites were then selected with on-off drug pressure: cycles of drug pressure for two weeks followed by two weeks without drug pressure. When parasite replication rates under selection were equal to replication rates off of drug selection, parasites were cloned by limiting dilution and maintained under drug pressure for an additional two weeks. DNA was extracted from cloned, transfected parasites for PCR and southern blot analysis of knockout vector integration.

Southern blot analysis of transformed parasites

For Southern blot, probes A and D (Table 4.2) were labeled with digoxigenin (DIG) by PCR using the PCR DIG Probe synthesis kit (Roche Applied Sciences, Indianapolis, IN, USA) according to manufacturer's instructions. 5 µg of genomic DNA from transformed parasites and 100 ng of plasmid DNA from the knockout construct were digested with Afl II or Kpn I, run on a 1% agarose gel and visualized with ethidium bromide. DNA was then equilibrated in depurination solution (2 N HCl), denaturation solution (0.5 M NaOH, 1 M NaCl), neutralization solution (0.65 M Tris, 1.5 M NaCl, pH 7.4) and 20x sodium citrate/sodium chloride (SSC, 3M NaCl, 300 mM sodium citrate) for 10 minutes each. DNA was transferred from the agarose gel to a nylon membrane by capillary flow in 20x SSC. After transfer, nylon membranes were UV-crosslinked and allowed to air dry. Blots were blocked in Pre-Hybe (Roche Applied Sciences, Indianapolis, IN, USA) buffer at 55^{o}C on a rotator for 2 hours. DIG-labeled PCR probe was boiled for 10 minutes and added to pre-warmed Hybridization buffer (Roche Applied Sciences, Indianapolis, IN, USA). Blots were

switched to Hybridization buffer and allowed to incubate overnight at 50°C on a rotator. Blots were rinsed in low stringency wash buffer (2x SSC/0.1% SDS) for three 15 minutes washes followed by a wash in high stringency wash buffer (0.1x SS/0.1% SDS). All washes were performed at 65°C. Blots were then incubated in DIG Blocking buffer (Roche Applied Sciences, Indianapolis, IN, USA) for 30 minutes. Anti-DIG antibody (Roche Applied Sciences, Indianapolis, IN, USA) was diluted 1:10,000 in blocking buffer. Blots were transferred to antibody in blocking buffer and incubated for an additional 30 minutes. Following antibody binding, blots were incubated in wash buffer (Roche Applied Sciences, Indianapolis, IN, USA) for 15 minutes then developed using CPD-star (Roche Applied Sciences, Indianapolis, IN, USA).

Results and Discussion

Plasmepsin X mRNA is transcribed in *P. falciparum* sexual stage parasites

RT-PCR of RNA isolated from *P. falciparum* sexual stage parasites demonstrated that *PM X* mRNA was transcribed in gametocytes and ookinetes (Figure 4.1). Pfs25, an ookinete surface protein with mRNA expression in gametocytes and sexual stage parasites, was was found to be amplified by RT-PCR, indicating transcription of Pfs25 in gametocytes and sexual stage parasites. As a control for genomic DNA contamination, PCR on RNA samples that were not treated with reverse transcriptase was done. Lack of amplification in these control samples demonstrated that RNA was not contaminated with genomic DNA.

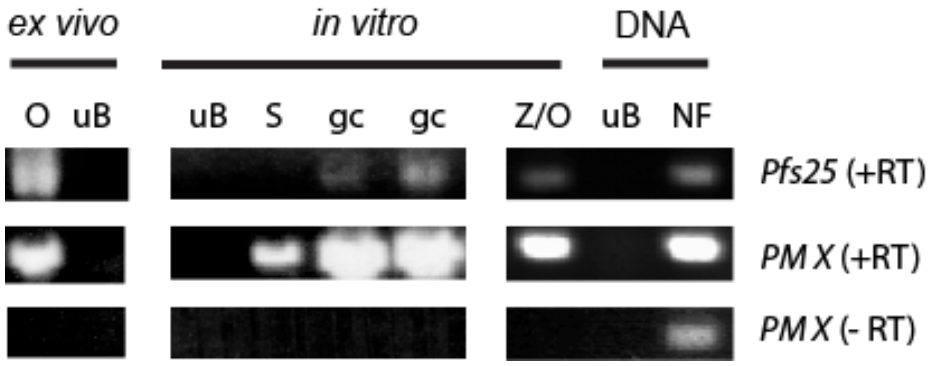


Figure 4.1
***PM X* mRNA was detected in *P. falciparum* sexual stage parasites.**
Total RNA isolated *ex vivo* from dissected mosquito midguts 20 hours after a blood-meal with *P. falciparum* gametocytes (O) or uninfected human blood (uB), *in vitro*-cultivated asexual schizonts (S), gametocytes (gc) and zygotes plus ookinetes (Z/O), as well as DNA from *P. falciparum* (NF) was isolated. Samples were reverse-transcribed and amplified using primers specific for *pfs25* and *PM X* (+RT). Samples that were not reverse transcribed (– RT) but amplified with PM X-specific primers demonstrate that RNA samples were not contaminated with DNA.

Production of monoclonal antibodies

Mouse monoclonal antibodies were generated to three peptides, two located in the catalytic domain and one located in the pro-enzyme domain (Figure 4.2). The antibody secreted from isolated spleen hybridoma cells were screened against BSA-conjugated peptides and recombinant protein. Of 31 hybridomas screened, two lines were western immunoblot positive against recombinant *P. falciparum* PM X. One antibody (PD) was directed against the pro-enzyme domain, and the other antibody (CD2) was directed against the second peptide in the catalytic domain (Figure 4.2). The PD mAb is directed against a peptide region that is not conserved among *P. falciparum* plasmepsins or *Plasmodium* PM X orthologues. The CD2 mAb is directed against a peptide region that is moderately conserved with PM IX. These antibodies were used for western immunoblots, IFA and mosquito membrane feeding assays.

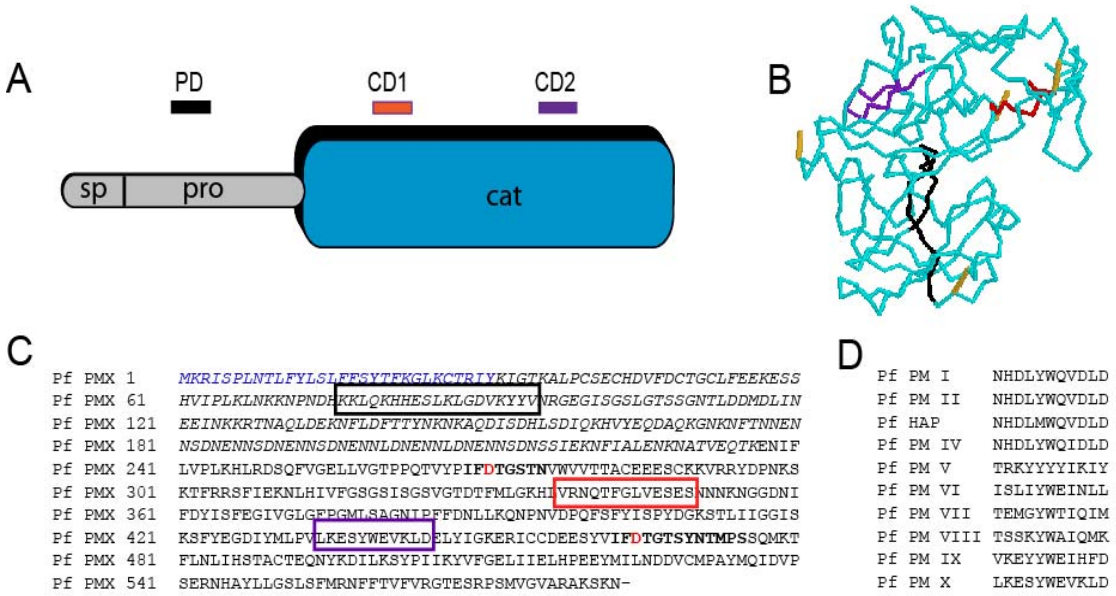


Figure 4.2
Peptide monoclonal antibodies directed against Plasmepsin X.
A. Schematic representation of PM X showing the predicted signal peptide (sp), pro-enzyme domains (pro), and catalytic domain (cat). Peptide monoclonal antibodies designed against three regions of PM X are designated PD (black), CD1 (red) and CD2 (purple).
B. Predicted three-dimensional structure of PM X (modeled against PM IV) with monoclonal antibody target peptides.
C. Plasmepsin X predicted signal peptide (blue) and the predicted pro-enzyme domain (italics) are shown; the two active aspartic acid residues (red) are found within conserved regions (bold). mAb targets are boxed according to Figure 4.2A.
D. Alignment of CD2 peptide target between all *P. falciparum* plasmepsins. Moderate conservation is seen between PM X and PM IX at this site.

Plasmepsin X protein is expressed in *P. falciparum* sexual stage parasites

IFA of *P. falciparum* sexual stage parasites demonstrated that both CD2 and PD recognized protein in zygotes and ookinetes (Figure 4.3). In *P. falciparum in vitro*-cultivated ookinetes, CD2 and PD antibody localization was diffuse and cytoplasmic, similar to what has been demontstrated for chitinase (Figure 2.2), an enzyme shown to be localized to ookinete micronemes (*Li et al., 2004*). To determine the subcellular

localization of PM X, *P. falciparum* parasites were prepared for immunoelectron microscopy. Unfortunately, ookinete fixation for immunolocalization could not be achieved despite parasite fixation for morphological preparations (Figure 2.6). Gametocytes could not be definitively distinguished from ookinetes, and subcellular organelles could not be identified (Figure 4.3).

Western immunoblot of *in vitro*-cultivated *P. falciparum* sexual stage parasites demonstrated PM X expression in macrogametes, zygotes and ookinetes but not gametocytes (Figure 4.3). The PD antibody recognized a 55-60 kDa protein and a 17-25 kDa protein, consistent with the predicted size of full length PfPM X and the PfPM X pro-enzyme domain. The CD2 antibody also recognized a 55-60 kDa protein and a 32-40 kDa protein, consistent with the predicted size of full length PfPM X and the catalytic domain. These bands were detected in both macrogamete/zygote and ookinete samples but not in gameotcyte samples.

Of note, both PM X antibodies and the Ig isotype control antibody recognized two bands not specific to parasite lysate, one at 42-55 kDa and the second at 27-30 kDa. It is possible that these bands represented cross-reactivity against other *Plasmodium* proteins. For example PM IX, as the CD2 mAb peptide target was moderately conserved between these two proteins. It is also possible that these bands indicated further proteolytic processing of PM X which affected neither the PD nor CD2 antibody recognition sites. However, the PD mAb whose target was not homologus to other Plasmepsins, and the Ig isotype control also recognized these bands, making these two explainations less likely.

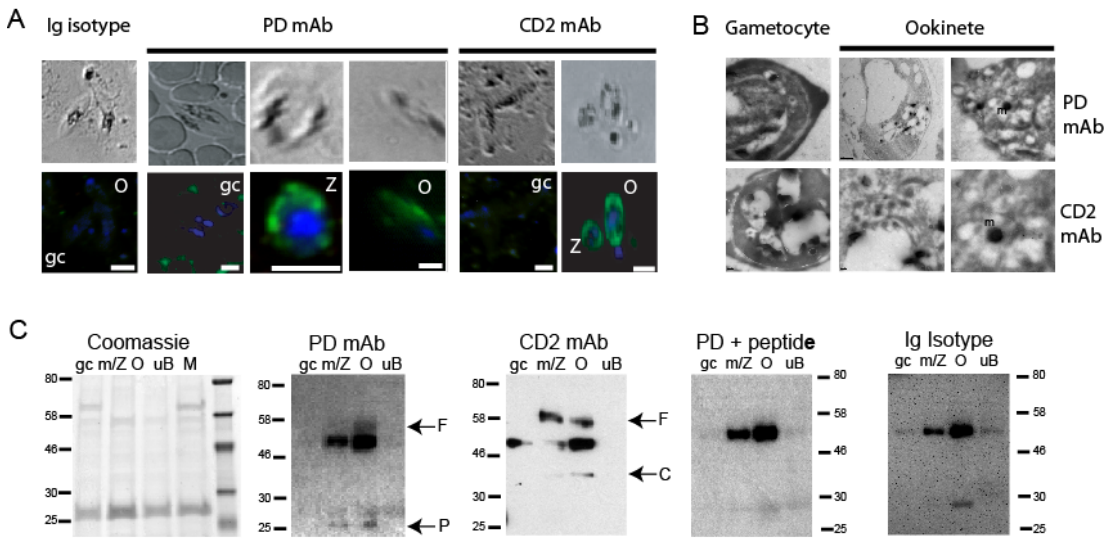


Figure 4.3
PM X was expressed in *P. falciparum* zygotes and ookinetes by IFA and western immunoblot.
IFA of *in vitro*-cultivated parasites was completed using primary antibodies to PM X which were then labeled with FITC-labeled anti-mouse antibodies (green) and nuclei are visualized with DAPI (blue).
A. IFA of *in vitro*-cultivated *P. falciparum* confirmed PM X expression in zygotes (Z) and ookinetes (O) but not gametocytes (gc).
B. Immunolocalization of PM X by TEM in *Plasmodium falciparum* sexual stage parasites. Parasites contained large vacuoles, membranes were distorted, organelle morphology could not be ascertained, and gametocytes could not be distinguished from ookinetes. Primary antibodies to PM X were probed with immunogold secondary (•). Scale bars: 200 nm.
C. Western immunoblot analysis of total protein isolated from *P. falciparum* sexual stage parasites. Both PD and CD2 antibodies demonstrated PM X protein expression in gametes and zygotes (m/Z) and ookinetes (O) but not gametocytes (gc). The predicted size of PfPM X without the signal peptide is 61 kDa (←F); the predicted catalytic domain is 36 kDa (←C), and the predicted proenzyme domain is 23 kDa (←P). Ig isotype control and PD + peptide did not recognize the 61 kDa or 23 kDa bands on western immunblots.

To ensure that the bands recognized at 55-60 kDa and 17-25 kDa were specifically recognized by PM X antibody, peptide competition assays were performed. PM X peptides were mixed with antibodies and then used to probe immunoblots of *P. falciparum* lysate. With the addition of the competing peptide,

these three bands failed to be recognized, but the bands at 42-55 kDa and 27-30 kDa still were recognized, and the blots were similar to those where non-specific Ig were used as the primary antibody (Figure 4.3). This indicated that although these bands were non-specifically recognized by PM X antibodies, the recognition did not occur at the antibody's antigen binding site.

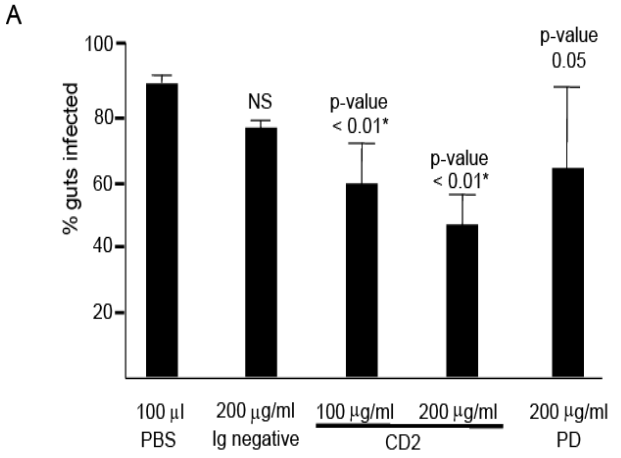
Antibodies to PM X in decreased *P. falciparum* transmission to *A. gambiae* but did not affect *in vitro* sexual stage development

Plasmodium transmission to the mosquito vector is dependent on sexual development and ookinete invasion of the mosquito midgut barrier. In a membrane feeding assay, infectious *Plasmodium* gametocytes were mixed with antibodies or small chemical inhibitors and fed to female mosquitoes via glass feeders. Engorged mosquitoes are maintained in strictly controlled humidified incubators. Gametogenesis and zygote fertilization occurs in the midgut within 20-60 minutes after mosquito feeding. Ookinete maturation proceeds over the following 16-20 hours, and ookinete invasion is thought to occur up to 48 hours after engorgement. Oocysts develop on the basal side of the mosquito midgut over the next 10-14 days. Mosquito midguts are dissected 8-10 days after infection and examined for the presence of oocysts, which are quantified by light microscopy.

To determine if antibodies against PM X could affect *P. falciparum* transmission to its mosquito vector, *A. gambiae*, membrane feeding assays were done. The presence of antibodies to PM X in an infectious bloodmeal significantly reduced *P. falciparum* transmission to mosquitoes (Figure 4.4). At higher concentrations of

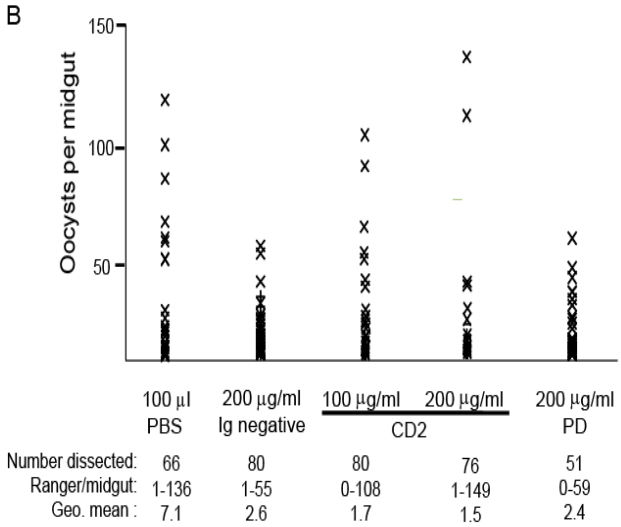
CD2 mAb (> 100 µg/ml), the proportion of infected mosquitoes fed a mixture of gametocytes and mAb was reduced by 20-40% compared to infected blood alone or infected blood with Ig isotype control antibody (*p*-value <0.01). The mean oocyst load was reduced by 30-40% in infected mosquitoes when fed an infectious bloodmeal with CD2 mAb compared to controls (Figure 4.4). Neither PD antibody at any concentration nor CD2 antibody at < 100 µg/ml affected transmission compared to controls (data not shown). IFA and western immunoblot analysis demonstrated that the full length enzyme was present in ookinetes. However, PD binding to PM X may not have been sufficient to inhibit enzyme function. Alternatively, PD may have bound to previously cleaved pro-enzyme domain which was no longer associated with the catalytic domain. This PD antibody would thus have been rendered ineffective at inhibiting PM X function.

The presence of PM X mAb reduced but did not completely block *P. falciparum* transmission to *A. gambiae*. It was possible that PM X mAb were not present at sufficient levels to saturate and block the function of all PM X molecules, possibly because these mAb had a low affinity and/or avidity for PM X. It was also possible that the high levels of mosquito infection achieved in the laboratory setting (up to 50-200 oocysts per infected mosquito midgut) compared to natural infections in the wild (1-10 oocysts per infected mosquito midgut) masked significant effects of the PM X mAb, as has been previously demonstrated (*Li, unpublished*). Alternatively, the loss of PM X function might have been compensated for by the action of other proteases with a shared function, such as a PM IX, which had a similar transcription profile as PM X.



C

	None	Ig neg	CD2	PD
Exflag. centers:	28	34	44	30
Microgametes:	9%	14%	33%	32%
Macrogametes:	91%	86%	67%	68%
Gametocytes:	8%	1%	0	5%
Zygotes:	14%	10%	12%	10%
Ookinetes:	77%	89%	88%	85%



	100 µl PBS	200 µg/ml Ig negative	100 µg/ml CD2	200 µg/ml CD2	200 µg/ml PD
Number dissected:	66	80	80	76	51
Ranger/midgut:	1-136	1-55	0-108	1-149	0-59
Geo. mean :	7.1	2.6	1.7	1.5	2.4

Figure 4.4
Membrane feeding assays in the presence of antibodies directed to PM X reduced *P. falciparum* transmission to *A. gambiae* mosquitoes.
Averages of three independent experiments are shown.
A. The presence of CD2 but not PD antibodies in a *P. falciparum* infected bloodmeal reduced transmission to *Anopheles gambiae* mosquitoes.
B. The presence of CD2 but not PD antibodies in a *P. falciparum* infected bloodmeal reduced oocyst burden in infected mosquito midguts compared to control.
C. *in vitro P. falciparum* gametogenesis assays done in the presence of antibodies to PM X did not significantly affect exflagellation or emergence of gametes. The relative ratios of micro- to macrogametes are shown as percentages.
D. *in vitro* sexual development assays demonstrated that the presence of CD2 or PD antibodies did not affect gametogenesis, fertilization or ookinete maturation. Parasite forms are listed as percentages of total parasites counted.

Currently, the precise mechanism(s) of transmission blocking antibodies are not well understood, but the effect of the transmission-blocking antibody is thought to be directly related to inhibiting the function of the target protein (Figure 4.5). This would include inhibition of parasite-secreted enzymes as well as prevention of parasite cell-surface receptors from recognizing and/or binding to targets. Other mechanisms that do not affect protein function include aggregation of parasites, complement-mediated lysis of antibody-bound parasites and antibody-dependent cellular toxicity. It was possible that a single molecule of polyvalent antibodies, IgM and IgA, bound to multiple ookinetes, decreased parasite motility and thus limited ookinete invasion of the midgut barrier. If active complement was present in the membrane feed, it was also possible that complement bound to antigen-associated IgG and/or IgM and mediated lysis of targed cells. This mechanism has been demonstrated for other transmission-blocking antibodies that prevent exflagellation, however, would be unlikely as serum used in membrane feeding assays was heat-inactivated to denature components of the complement system (*Scholtz et al., 2008*). The possibility that antibody binding recruits leukocytes for cell-mediated killing has also been described (*Ranawaka et al., 1994*). Few leukocytes were present after 2-3 weeks of *P. falciparum* gametocyte culture. However leukocytes were not specifically depleted for the membrane feeding assays, and human leukocyte Fc receptors have been shown to bind murine IgG; thus this possibility could not be formally excluded (*Unkeless et al., 1988 for review*). The precise mechanism of PM X mAb could be determined with *in vivo* membrane feeding assays by dissecting sexual stage *P. falciparum* parasites mosquito midguts, but this would be a tedious and inefficient process.

To determine whether the decreased mosquito transmission in the presence of PM X mAb was due to a defect in gametogenesis, fertilization, ookinete development or antibody-mediated aggregation of ookinetes, processes that could not be easily studied in the mosquito midgut, PM X mAb were added to *in vitro P. falciparum* sexual stage cultures. The presence of antibodies in *P. falciparum* sexual stage cultures did not significantly affect gametogenesis (Figure 4.4). Microgamete exflagellation and macrogamete emergence was similar in the presence or absence of antibodies recognizing PM X; each culture had an approximately 1-2 ratio of microgametes-to-macrogametes after gametogenesis. At the end of the cultivation period, zygotes and ookinetes were present in sexual stage cultures at levels comparable to control (Figure 4.4). Additionally, the presence of PM X mAb did not affect the distribution of zygotes and ookinetes; they were not found in clusters of parasites. This suggested that PM X mAb did not cause aggregation of parasites in membrane feeding assays. The comparable levels of zygotes and ookinetes in the presence or absence of PM X mAb implied that complete-mediated or cellular-mediated lysis of sexual stage parasites did not occur *in vitro.* This demonstrated that PM X mAb affected *Plasmodium* transmission at a point after ookinete maturation, possibly during ookinete invasion of the midgut barrier or early oocyst formation.

Although this *in vitro* sexual stage culture facilitated sexual quantification of parasite sexual development through ookinete maturation, it could not be used to visualize other aspects important for *Plasmodium* transmission to mosquitoes. These limitations included the inability to visualize ookinete motility, real-time examination of ookinete invasion of the midgut barrier, and early oocyst development. Ookinete

motility was not observed with time-lapse microscopy despite the use of polylysine coated slides and incubation of *in vitro*-generated ookinetes in the presence of dissected mosquito midguts. Early attempts to visualize ookinete penetration of the mosquito migut *ex vivo* used lipophilic membrane dyes to stain cultivated ookinetes and dissected mosquito midgut explants (*Vlachou et al., 2004*). However, because of the lack of ookinete motility in the presence of dissected midguts, this assay was not attempted. To facilitate the visualization of early oocyst development, a *P. falciparum* line that constitutively expresses a fluorescent protein is needed. Mosquito midguts infected with a GFP-expressing strain of *P. berghei* could be dissected and maintained *ex vivo* for three to five days (data not shown, *Sultan et al,. 1999*). A similar system could be used to monitor early oocyst development in *P. falciparum* using a recently developed line of NF54 that constitutely expresses GFP throughout the parasite life cycle (*Falkard and Fidock*). This GFP-expressing *P. falciparum* could also be used in conjunction with *Anopheles stephensi* expressing dsRed in midgut epithelia to study ookinete invasion of the mosquito midgut barrier *ex vivo* (data not shown). These assays were not available at the time of this writing and generation of active, recombinant protein and a PM X knockout parasite was attempted to fully elucidate the role of PM X in *Plasmodium*-to-mosquito transmission.

It is important to note that relatively high concentrations of mAb against PM X, $\geq$ 100 µg/ml, were necessary before significant decreases in transmission could be appreciated. Although antibody concentrations of > 300 µg/ml have been achieved by immunization of mice against *Plasmodium* proteins, human antibody concentrations in the range of 0.5 – 20 µg/ml have observed after vaccination against *Haemophilus*

influenza type b and *Streptococcus pneumoniae* (*Kubler-Kielb et al., 2006, Wiertsema et al., 2004, Rao et al., 1995, Tarrago et al., 2005*). Based on these data, PM X was not a strong candidate as a transmission-blocking vaccine target as high levels of antibody would be necessary before significant transmission effects could be demonstrated. However, they do indication that PM X function is important for *P. falciparum* transmission to *A. gambiae*.

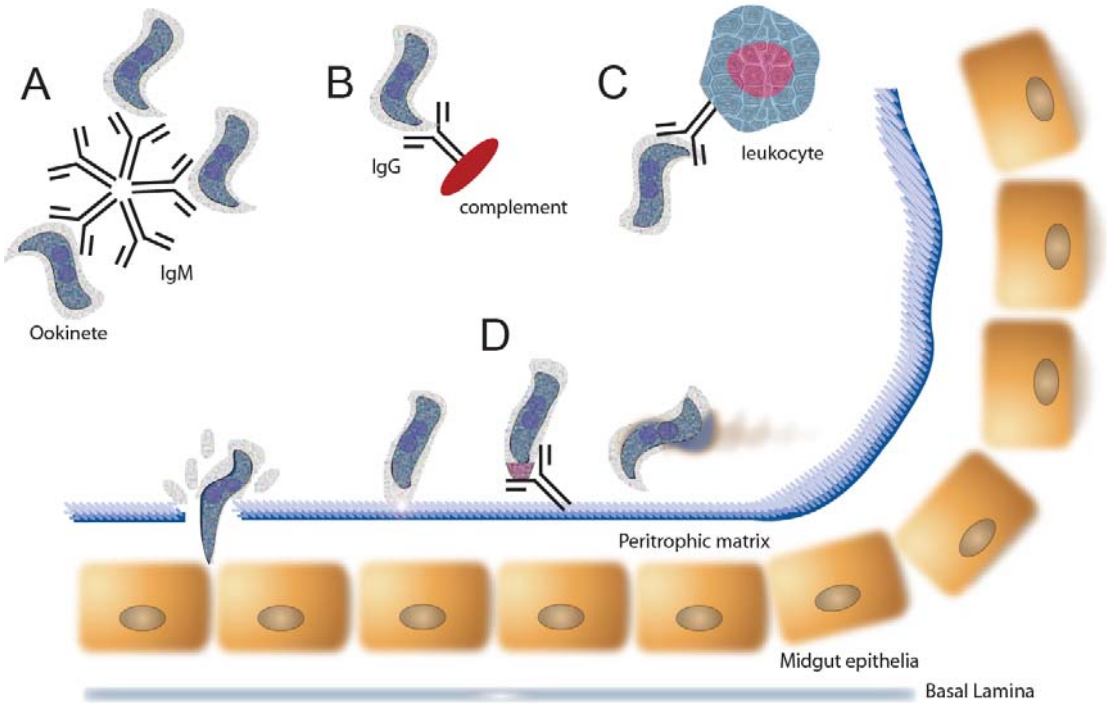


Figure 4.5
Potential mechanisms of transmission-blocking antibodies
A. Polyvalent antibodies IgM and IgA might block parasite transmission by binding to multiple ookinetes and inhibiting ookinete motility.
B. Active complement, if present in the blood meal, can bind to the Fc region of antigen-bound IgG and IgM to mediate lysis of the targed cell.
C. Leukocytes, if present in the blood meal, may recognize the parasite-bound antibody and mediate parasite lysis or phagocytosis.
D. Antibody can directly interfere with the function of a parasite secreted or surface associated protein to inhibit ookinete invasion.

Solubilization and refolding of recombinant PM X fails to generate active protease

To determine whether PM X facilitated mosquito transmission by cleaving and activating PM X within the parasite or by cleaving proteins specific to the mosquito, active, recombinant PM X is needed. Generation of active, recombinant PM X was attempted using multiple expression systems and multiple expression protocols, none of which resulted in active protease. The *PM X* gene from *P. falciparum* was cloned into the pGEX 4T-1 expression vector and expressed rPM X-GST fusion protein in Rosetta bacteria. This strain of *E. coli* was derived with a plasmid to generate tRNAs for codons which are rare in *E. coli.* Protein expression was induced using 0.03 mM to 1 M concentrations of IPTG to induce expression at growth temperatures between18^{o}C to 37^{o}C. Under all conditions tested, the majority of rPM X-GST was produced as inclusion bodies.

In an attempt to improve production of rPM X-GST as a soluble protein in *E. coli*, the gene encoding *PM X* was *E. coli*-codon optimized. The *E. coli*-codon optimized *PM X* was expressed in Rosetta bacteria as well as SHuffle bacteria. SHuffle was engineered with an integrated gene cassette of enzymes promoting formation of disfulide bonds. Expression of *E. coli*-codon optimized genes in Rosetta or SHuffle did not significantly improve yields of soluble protein.

Protein isolated from inclusion bodies have the advantage of relative purity, consisting mostly of recombinant protein, and ease of isolation. rPM X-GST was isolated from inclusion bodies, solubilized and refolded using protocols previously shown to be successful for PM II and PM IV (*Gulnik et al., 2002, Luker et al., 1996, Hill et al., 1994, Wyatt et al., 2002, Shenai et al., 2000, Li et al., 2004*). Although the

majority of rPM X-GST produced was isolated from inclusion bodies, a small fraction of rPM X-GST could be purified from the soluble fraction using GST-conjugated sepharose beads. This purified soluble fraction was dialyzed and concentrated. Both refolded and soluble protein was assayed for activity using the Pierce fluorometric Quanticleave kit (Figure 4.6). These preparations of rPM X-GST did not exhibit significant activity compared to trypsin positive control. The resulting activity in refolded fractions, though greater than activity from GST negative control samples, was below threshold activity of trypsin positive control.

To ensure that the 25-27 kDa GST tag did not block the catalytic active site, refolded and soluble protein was cleaved with thrombin to remove the GST tag. Thrombin-cleaved rPM X-GST failed to exhibit significant activity compared to control. This may be due to the continued presence of the pro-enzyme domain. The pro-enzyme domain was known to be important for aspartic protease processing and facilitates proper protein folding (*Horimoto et al., 2009*). Protease expression as a zymogen is also a well characterized mechanism of post-translational protein regulation. The acidic food vacuole plasmepsins, PM II and PM IV, are known to exhibit auto-catalytic activity in their native, low pH environment (*Kim et al., 2006*), resulting in cleavage of the proenzyme domain. PM X appears to be important in the invasion of the mosquito midgut, which is a slightly basic environment of pH 8.0 – 9.5 (*Billker et al., 2000, del Pilar Corena et al., 2005*). To determine if buffer pH could enhance protease activity by promoting autocatalysis of recombinant rPM X, a range of pH refolding buffers were tested from pH 7.0 to 10, but this did generate active rPM X. To determine if cleavage of the pro-enzyme domain from rPM X-GST could

enhance activity, refolded rPM X-GST was cleaved with 0.1 ng of tcpk-trypsin and assayed, but no activity could be demonstrated (Figure 4.6).

The production of recombinant, active *Plasmodium* enzymes in *E. coli* has been typically fraught with difficulty. Improvements in the production of properly folded, recombinant protein has been achieved by synthesizing *E. coli*-codon optimized genes (*Yadava and Ockenhouse, 2003, Flick et al., 2004*) or using a cell-free wheat germ transcription and translation system (*Tsuboi et al., 2008, Takeo et al., 2009*). Neither of these alternatives resulted in the production of active, recombinant PM X (data not shown).

Figure 4.6
Recombinant PM X produced in *E. coli* did not exhibit activity.
Various activation schemes to generate active recombinant PM X. Activity was measured in absorbance units (AU). Although rPM X activity may have been higher relative to GST controls, the absolute activity levels were below threshold and were too low for use in biochemical assays.
A. rPM X-GST was expressed in Rosetta bacteria, purified from inclusion bodies, run on SDS-PAGE and stained with Coomassie blue to visualize proteins. The predicted size of rPM X-GST was ~90 kDa. A band of this size was excised and MALDI-TOF fingerprint identification confirmed the protein as PM X. Signal peptide (blue) and MALDI-TOF identified peptides (red) are shown.
B. PM X was expressed in SHuffle bacteria, purified from the soluble fraction and assayed for activity.
C. PM X was expressed in SHuffle bacteria, purified from the inclusion body fraction, solubilized and refolded at varying pH (7.0-10.0). After refolding, a precipitate formed. The soluble (refolded) and insoluble (precipitate) fractions were separately assayed for activity as shown.
D. PM X was expressed in SHuffle bacteria, purified from the inclusion body fraction, solubilized and refolded at pH 8.5. The GST tag was cleaved with thrombin, and the resulting protein was assayed for activity as shown.
E. PM X was expressed in SHuffle bacteria, purified from the inclusion body fraction, solubilized and refolded at pH 8.5. The GST tag was cleaved with thrombin, and the resulting protein was incubated with 0.1 ng of trypsin (tcpk) in an attempt to cleave the pro-enzyme domain and activate the enzyme. Tcpk-cleaved protein was then assayed for activity as shown.

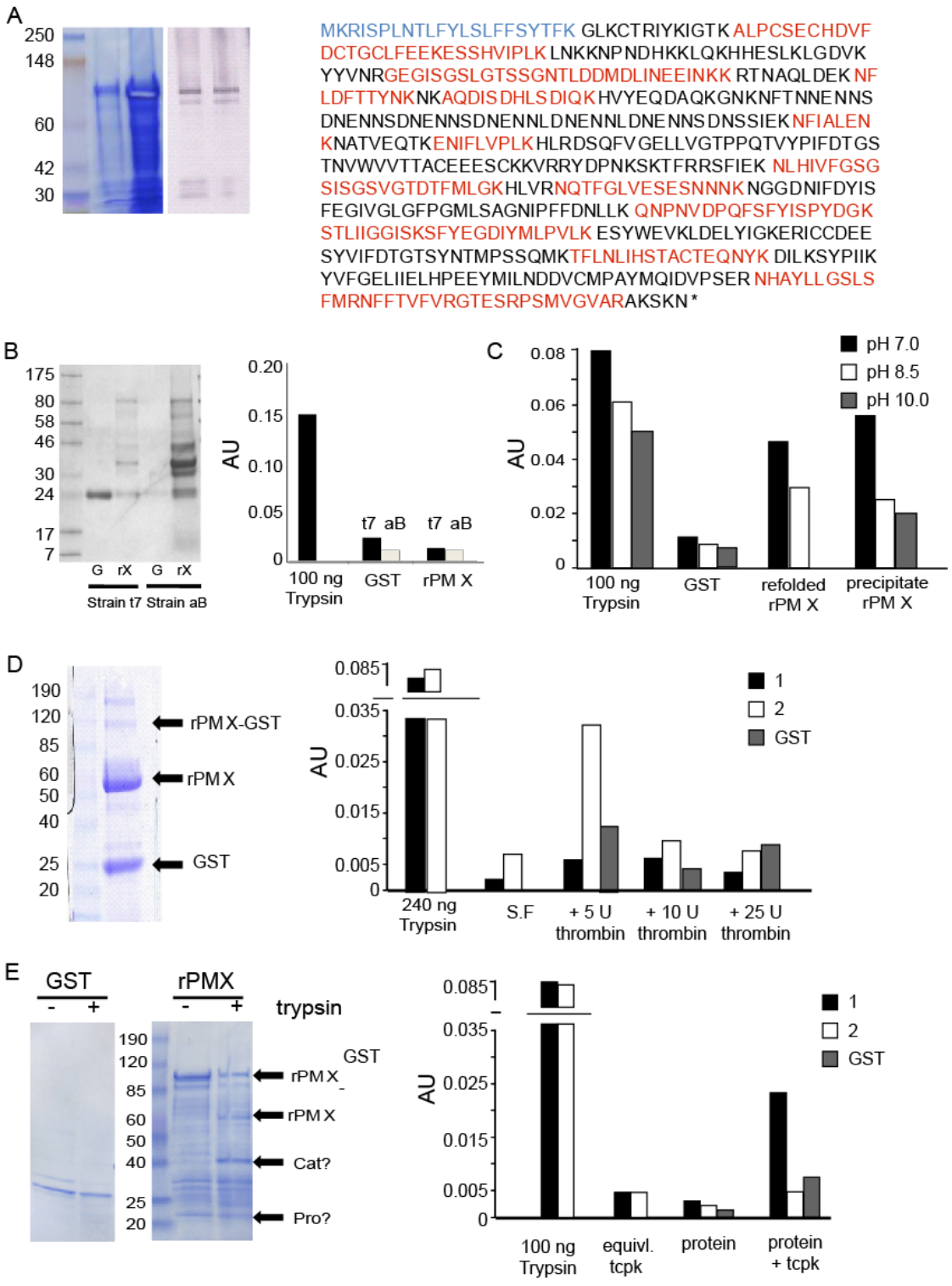


Figure 4.6
Recombinant Plasmepsin X produced in *Escheria coli* did not exhibit activity.

Plasmepsin X appears to be essential for *P. falciparum* asexual development

To determine whether gene disruption of PM X would demonstrate a defect in sexual stage development or ookinete invasion of the mosquito midgut similar to that seen in transmission-blocking assays, disruption of *PM X* in *P. falciparum* were attempted using single cross-over recombination. *P. falciparum* knockout construct pHDWT, which confered resistance to pyrimethamine and WR99210, was used for genetic disruption experiments. A 1 kb region of *PM X* was cloned into pHDWT to generate knockout construct *PM X*-pHDWT. The sequence of the construct was verified, and plasmid DNA was purified and resuspended in either cytomix or sterile, nuclease-free water. Parasites were transformed using two electroporation systems, the Bio-Rad Gene Pulser or the Amaxa Nucleofector system. Electroporation using both systems was attempted on three different occasions. Three to five parasite samples were transformed using each electroporation system on each occasion that resulted in 24 total attempts. Continuous drug pressure using WR99210 was applied for 4-8 weeks followed by 2-week cycles of on-off drug selection for an additional 6-8 weeks. Of 24 initial parasite lines, only three (1.2, 3.1 and 3.3) resulted in WR99210-resistant parasites; two of these lines were electroporated using the Bio-Rad system, and the third was electroporated using the Nucleofector system.

Transformed parasites could have acquired drug resistance in three ways: retention of plasmid DNA as an episomally replicating form, targeted integration of the knockout construct into the gene of interest or non-targeted integration of the knockout construct into a gene locus for the plasmodium promoters present on the construct. During asexual replication, circular episomes could be unequally segregated

to daughter cells (*van Dijk et al., 1997*). Continuous drug pressure promoted the growth of resistant parasites with episomes as well as integrated resistance genes. Cycles of on-off drug pressure have been shown to significantly reduce or eliminate parasites with episomes while promoting growth of parasites with resistance genes integrated into the parasite genome (*van Dijk et al., 1996, Crabb and Cowman, 1996, O'Donnell et al., 2001*). PCR was used to determine if any drug resistant parasites which grew out under cyclic on-off drug pressure had correctly integrated the *PM X*-pHDWT construct into the *PM X* gene (Figure 4.7). One of the three lines, 3.1, appeared to contain parasites with correctly integrated knockout construct. The other two lines, 1.2 and 3.3, were not PCR positive for correctly integrated knockout constructs. However, correct targeting may have occurred in a minority of parasites in these other two lines. All three lines were sub-cloned by limiting dilution and analysed by Southern blot analysis to determine correct integration.

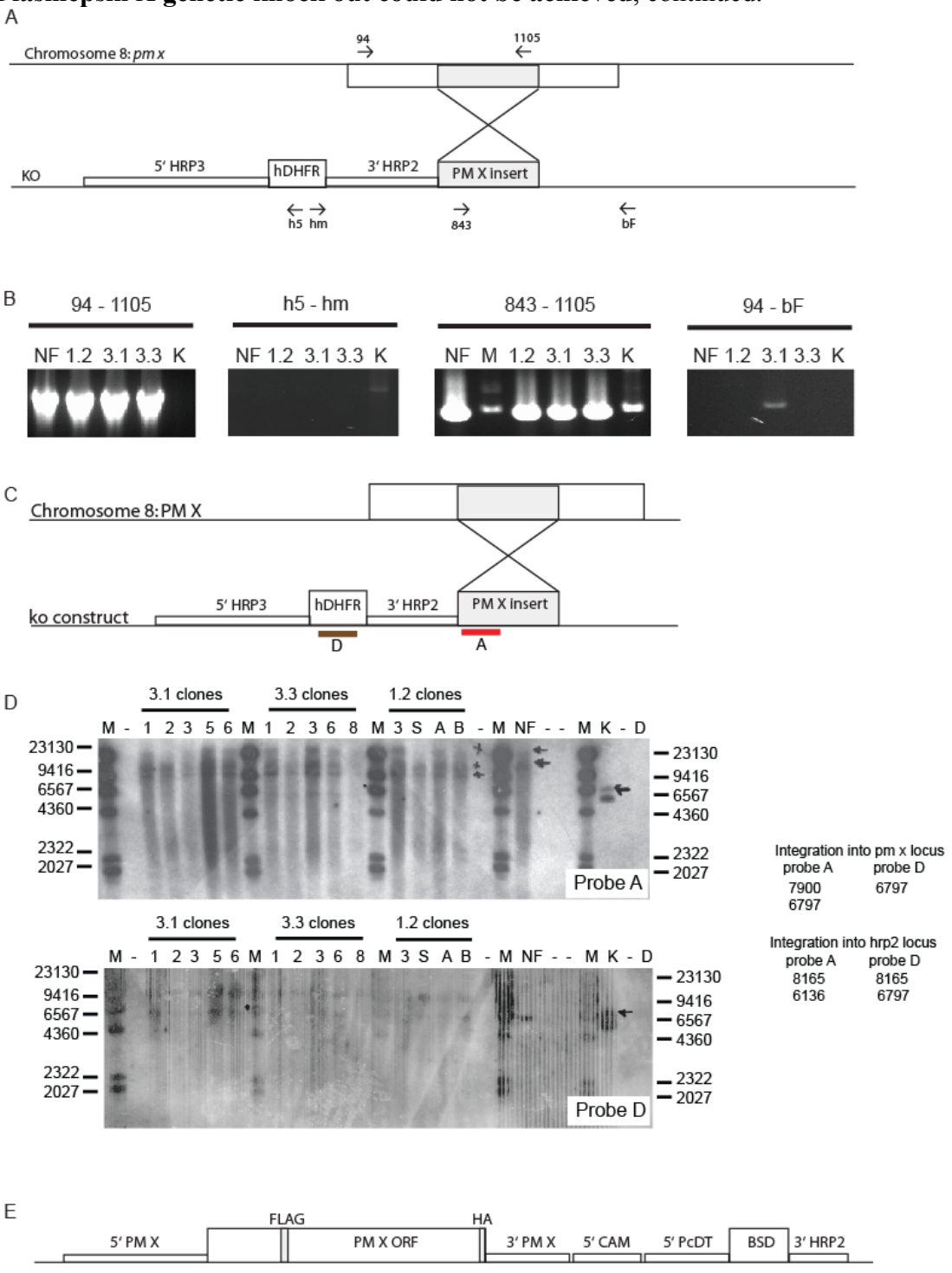
Transformed lines 3.1 and 3.3 each generated 12 sub-cloned lines while transformed line 1.2 generated 8 sub-cloned lines. Representative results for 5 of the 12 subcloned lines from 3.1 and 3.3 as well as 4 of the 8 subcloned lines from 1.2 are shown; lines that are not shown had similar results to those that are shown. Sub-cloned parasites appeared after an additional 2-3 weeks of culture. Genomic DNA from each line was extracted and digested with AflII then subjected to Southern blot analysis. Two probes, A and D, specific for *PM X* and the *hDHFR* resistance gene, respectively, were hybridized to membrane-bound DNA fragments (Figure 4.7). None of the sub-cloned lines integrated into the *PM X* locus. These data, suggested that *P. falciparum PM X* is essential for blood stage parasite survival (Figure 4.7).

To address this, simultaneous transfection of the knockout construct with a complementation plasmid that contained the gene of interest was attempted (Figure 4.7). Double transfection experiments were attempted on two separate occasions with two samples on each occasion. After transfection, parasites were selected using both blasticidin and WR99210. No parasite outgrowth was detected from the four transfected cultures that were monitored for up to 12 weeks post transfection.

P. berghei is a rodent malaria parasite which is particularly amenable to genetic manipulation because of efficient genomic integration of the knockout construct and short periods required for generation of drug-resistant parasite clones (*Sultan et al., 1998, Janse and Waters, 2005*). Two separate attempts (8 transfections per attempt) to generate a *P. berghei PM X* knockout parasite using construct pD.-D., which confers resistance to pyrimethamine, were unsuccessful (data not shown). Subsequent knockout experiments by another group also confirmed the inability to knockout *P. berghei PM X* (*Ecker et al., 2008*).

Figure 4.7
Plasmepsin X genetic knock out could not be achieved.
A. Schematic diagram of *PfPM X* knockout and PCR primers used to determine knockout construct integration. Primer set 94-1105 amplified a section of an uninterrupted *PM X* gene. Primer set h5-hm only amplified circular plasmid. Primer set 843-1105 amplified a region within the insert of the knockout construct. Primer set 94 – bF only amplified a product if the knockout construct is correctly integrated.
B. PCR of *Plasmodium falciparum* lines that grew out after 4-5 weeks of continuous drug selection followed by 4-8 weeks of cyclic drug pressure. Of three WR99210-resistant lines (1.2, 3.1, 3.3), only 3.1 appeared to contain some parasites with correctly integrated knockout construct. All lines contained parasites with wildtype *PM X*. Lanes include parental parasites (NF), marker (M) and knockout plasmid (K).
C. Schematic diagram of *PfPM X* knockout and DIG-labeled probes, A and D. Predicted fragments recognized by each probe after AflII digestion are listed.
D. Southern blot analysis of *Plasmodium falciparum* clones that survived continuous and cyclic drug pressure for 3 months demonstrated that sub-cloned lines are genomically integrated, but they are integrated into the hrp2 locus (8100 base pairs). Lanes are labeled for marker (M, in base pairs), sub-clone number (1, 2, 3, 5, 6, 8, S, A, B), parental NF54 (NF), knockout construct (K) and pHDWT empty vector (D).
E. Schematic diagram of *PfPM X* complementation plasmid simultaneously transfected with the *PfPM X* knockout construct.

Figure 4.7
Plasmepsin X genetic knock out could not be achieved, continued.



The failure to generate correctly integrated genetic disruptions of *PM X*, in conjunction with transcriptomic and proteomic data that demonstrated Plasmepsin X expression in asexual stage parasites, suggested that PM X is essential for the asexual blood stage life cycle (Figure 4.8). Genetic knockout of essential blood stage development genes is not possible using single or double-cross over recombination techniques. Adaptation of inducible knockout strategies, such as the Tetracycline-On or Tetracycline-Off systems, was not readily adaptable to *Plasmodium*. For the study of knockout phenotypes in sexual stage parasites, a temperature-sensitive inducible system would be ideal, as gametogenesis could be stimulated in part by a drop in temperature, and sexual development occurs at 19-21^{o}C, compared to asexual development which occurs at 37^{o}C.

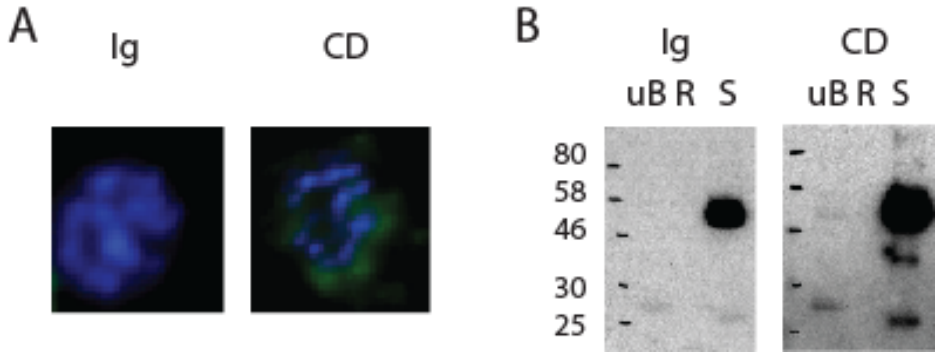


Figure 4.8
Plasmpesin X protein was detected in *Plamsodium falciparum* blood stage schizonts.
A. IFA of *P. falciparum* schizonts using CD2 antibody (green) demonstrated protein expression. DAPI (blue), was used to visualize nuclear material.
B. Western immunoblot analysis of *P. falciparum* schizonts using CD2 antibody demonstrated protein expression

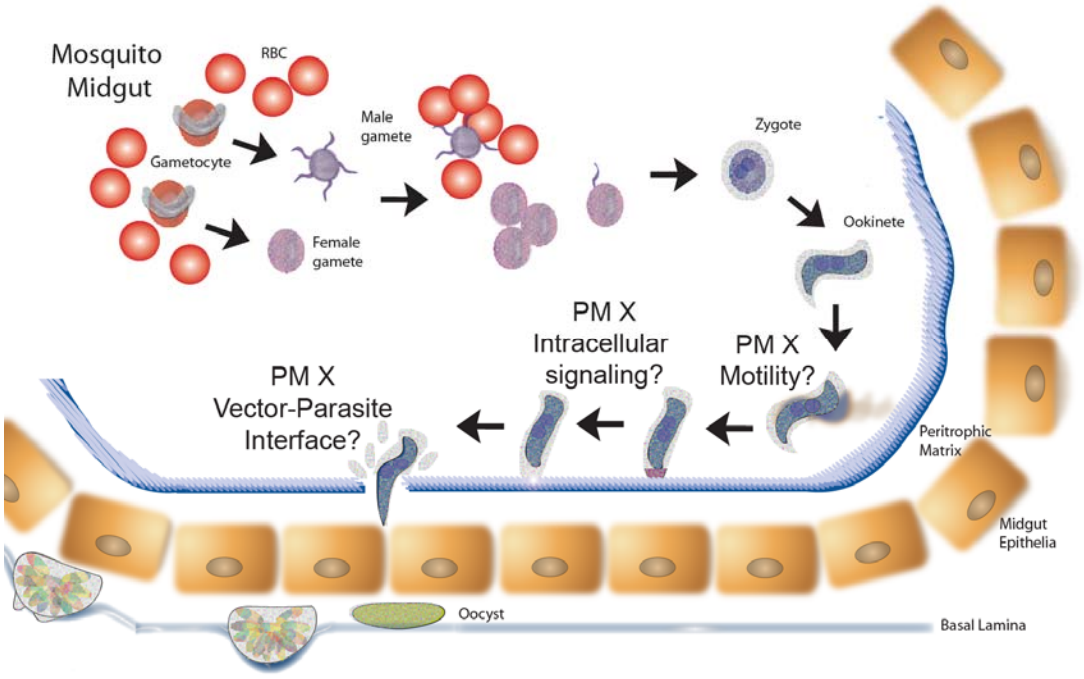


Figure 4.9
Model for the role of Plasmepsin X in ookinete invasion of the mosquito midgut barrier.
Plasmepsin x mRNA was expressed in *P. falciparum* gametocytes, but PM X protein was not expressed until *Plasmodium* zygote and ookinete maturation. Antibodies to Plasmepsin X did not inhibit ookinete development and maturation *in vitro* but decreased ooocyst formation *in vivo. In vitro* sexual development assays demonstrated that antibodies to PM X did not affect gametocyte escape from the red cell during gametogenesis. Similarly, gamete fertilization to generate zygotes and zygote maturation into ookinetes was not affected. The presence of antibodies to PM X did not result in the aggregation of zygotes or ookinetes, which suggested that the mechanism of action was not a non-specific mass effect (Figure 4.5). Instead, PM X may be important for ookinete motility or recognition of the midgut barrier or subsequent intracellular signaling. Alternatively, Plasmepsin X may be secreted to facilitate ookinete penetration of the peritrophic matrix, possibly by degrading components of the peritrophic matrix or facilitating passage through the midgut epithelia.

Conclusion

Plasmepsin X was demonstrated to be expressed in sexual stage parasites. This study illustrated that antibodies to this protease decrease *Plasmodium* transmission to

its mosquito vector but do not affect ookinete maturation. This demonstrated that PM X is important for ookinete invasion of the midgut barrier (Figure 4.9). This was the first time that a non-food vacuole Plasmepsin has been shown to be important for ookinete invasion of the mosquito midgut. Efforts to generate a *PM X*-knock out parasite were unsuccessful. Although initially disappointing, this suggested that PM X function was essential for blood stage development; indeed, PM X was expressed in blood-stage schizonts. It is likely that PM X function was conserved in *Plasmodium* invasive stages, including merozoite invasion of the red blood cell and sporozoite invasion of the salivary gland, as suggested by transcriptomic data show *PM X* mRNA upregulation in these stages. These results indicated that further analysis of PM X in other invasive stages is warrented.

Chapter 4 will be submitted for publication: Bounkeua V., Pettersen, K.D., Vinetz, J.M. “Plasmepsin X facilitates *Plasmodium* transmission to the mosquito vector”. The dissertation author is the primary author and investigator of this paper.

Table 4.A1
Primers used for RT-PCR and genetic constructs.

Primer name	Sequence
Pfpmx KO Xho F	5’ – caactagtgcgttaccgtgttctgagtgtcacg – 3’
Pfpmx KO Spe R	5’ – caactagtgccctcataaaacgatttactaatcccacc – 3’
PfPMX RT F	5’ – gtgatgaagaaagttacgttatatttgacacagg – 3’
PfPMX RT R	5’ – gctcttgctactccaaccatagaagg – 3’
Pfs25 RT R	5’ – tcttgtacattgggaactttgcct – 3’
Pfs25 RT F	5’ – tgcgaaagttaccgtggatactg –3’
rPfPMX no SP F	5’ – gggttaaaatgcaccagaatatataaaatcgg – 3’
rPfPMX stop R	5’ – ttagtttttacttttgctcttgctactccaaccatagaaggtc – 3’

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CHAPTER 5

PROTEOMIC ANALYSIS OF *PLASMODIUM FALCIPARUM* SEXUAL STAGE PARASITES AND *PLAMSODIUM VIVAX* MACROGAMETES

Abstract

Since the complete annotation of the *P. falciparum* genome in 2002, global proteomic analyses have been completed for the asexual blood stage and mosquito sporogonic stage developmental cycles. These data, in conjunction with transcriptomic data, have provided invaluable insights into *Plasmodium* biology that have resulted in the investigation of novel chemo-therapeutics. Notably absent from these analyses, however, is the global proteomic analysis of *P. falciparum* sexual stage zygotes and ookinetes. Increasingly, vaccine-based strategies to control malaria have focused on transmission-blocking interventions that target the sexual stages of the malaria parasite. A recently optimized method to generate *P. falciparum* sexual stage parasites was used to generate parasites for global proteomic analysis of *P. falciparum* zygotes and ookinetes. Furthermore, this method was adapted to generate *P. vivax* macro-gametes for proteomic analysis. These analyses identified an as yet uncharacterized set of potential transmission-blocking vaccine candidates. Examination of stage-specific protein expression in conjunction with previously published transcriptomic data allowed for the identification of novel motifs that may be improtant *P. falciparum* translational repression.

Introduction

The first whole-organism proteomic analysis of *P. falciparum* was published in 2002 in conjunction with the complete annotation of the *P. falciparum* genome (*Lasonder et al., 2002, Florens et al, 2002*). Since these landmark papers, major advances in basic parasite biology have facilitated the proteomic analysis of asexual

blood stage parasites and sporogonic mosquito stage parasites (*Hall et al., 2005, Lasonder et al., 2008*). These studies have provided global transcriptomic evidence for stage-specific protein expression (*Le Roch et al.,2003, Le Roch et al., 2004*, *Gardner et al., 2002, Hall et al., 2005, Mair et al., 2006*). Proteomic analysis of *P. falciparum* gametocytes and macrogametes has been completed (*Lasonder et al., 2002*). Global proteomic analysis of *P. falciparum* zygotes and ookinetes has not been attempted because of the hitherto lack of available parasite material, and zygote and ookinete proteomic data is only available for animal-infecting *Plasmodium spp.* (*Ecker et al., 2008, Patra et al., 2008, Lal et al., 2009*). Proteomic analysis of any stage of the *P. vivax* life cycle has yet to be completed.

Recently, a method was optimized to cultivate sexual stage parasites *in vitro* (Chapter 2). With this method, quantities of *P. falciparum* zygotes and ookinetes suitable for proteomic analysis were produced; these parasites were purified by flow cytometry. Furthermore, this method was adapted and combined with others to generate *P. vivax* sexual stage parasites (*Suwanabun et al., 2001, Westenberger et al., submitted,* Chapter 2). This was the first proteomic analysis of *P. falciparum* sexual stage zygotes and ookinetes as well as the first analysis of *P. vivax* macrogametes.

Methods

In vitro production of *P. falciparum* sexual stage parasites

P. falciparum strain NF54 used for these studies was obtained from Dr. Stephen Hoffman (Sanaria, Rockville, MD USA). Gametocytes and sexual stage parasites were cultivated *in vitro* as previously described (*Chapter 2, Methods*).

Ex vivo production of *P. vivax* macrogametes

Human subjects involved in this study consisted of 10 adult (>18 years old) volunteers that presented to local health clinics in Iquitos, Peru as previously described (*Chapter 3, Methods*). Patients provided written informed consent to participate in these studies, which were approved by the Institutional Review Boards of the University of California San Diego, Johns Hopkins Bloomberg School of Public Health and the Peruvian Ministry of Health, Asociacion Beneficia PRISMA, and Universidad Peruana Cayetano Heredia, Lima, Peru. *P. vivax*-infected patient blood was processed for sexual stage parasite cultivation *in vitro* (*Chapter 3, Methods*).

Purification of *P. falciparum* sexual stage parasites and *P. vivax* macrogametes

A minimum of 4 independent *P. falciparum* parasite cultures were generated and pooled for each stage of the *P. falciparum* sexual stage life cycle analyzed: gametocytes, macrogametes, zygotes and ookinetes. Approximately 10^7 purified *P. falciparum* gametocytes, 10^8 purified *P. falciparum* macrogametes, 5 x 10^6 purified *P. falciparum* zygotes and 10^7 purified *P. falciparum* zygotes plus ookinetes were used in this study. Ten *P. vivax* sexual stage cultures were generated, pooled and purified to yield approximately 10^7 *P. vivax* macrogametes for this study.

Removal of uninfected erythrocytes

Most uninfected erythrocytes were removed from sexual stage parasite cultures by density gradient centrifugation (Lympholyte-H, Cedarlane Laboratories, Burlington, NC USA). Parasites were collected from the gradient interface, washed

twice in ookinete medium and further purified by positive selection on a magnetic separator (Miltenyi Biotec, Bergisch Gladbach, Germany) as previously described (*Fivelman et al., 2007, Chapter 3, Methods*). Purification of each of the sexual stage parasite forms was done at 19-21^{o}C using ookinete medium.

Purification of *P. falciparum* gametocytes

P. falciparum gametocyte cultures were purified by magnetic column at 37^{o}C using complete culture medium. Gametocytes eluted from magnetic columns were released from infected erythrocytes using saponin lysis buffer (7 mM K_2HPO_4, 1 mM NaH_2PO_4,11 mM $NaHCO_3$, 58mM KCl, 56mM NaCl, 1mM $MgCl_2$,14 mM glucose, 0.02 mM saponin, pH 7.5) and washed by 5-8 times in PBS (*Prieto et al., 2008*).

Purification of *P. falciparum* and *P. vivax* macrogametes

Sexual stage parasites depleted of uninfected erythrocytes were then purified over 6%-11%-16% Nycodenz (Sigma-Aldrich, St. Louis, MO, USA) gradient to separate clusters of adherent macrogametes from ookinetes, zygotes, non-adherent macrogametes and untransformed gametocytes (*Vermeulein et al., 1985*). 400 µL of 6% Nycodenz, 400 µL of 11% Nycodenz and 400 µL of 16% Nycodenz were layered in a 1.5 ml microcentrifuge tube. Parasites were resuspended in 200 µL ookinete media, layered on top of the three step gradient, and centrifuged at 10,000 x g for 10 minutes at 4^{o}C, no brake. Parasites from the 6%-11% and 11%-16% interfaces were collected separately. Clusters of adherent macrogametes were collected from the 6-11% interface were washed three times with serum-free ookinete media, followed by

three washes in phosphate buffered saline, pH 8.0. For *P. falciparum* cultures, parasites from the 11-16% interface were used for flow cytometric sorting.

Purification of *P. falciparum* zygotes and ookinetes by flow cytometric sorting

Mixed sexual stage parasites consisting of zygotes, ookinetes, gametocytes and non-adherent macrogametes were collected from 11%-16% Nycodenz gradient interface, washed three times with ookinete medium and stained with Vybrant DyeCycle Green stain (Molecular Probes, Invitrogen, Carlsbad, CA USA) according to manufacturer's instructions. Approximately 10^7 - 10^8 parasites were then passed through a 30 μm filter to remove adherent cells and sorted based on fluorescence intensity using the MoFlo high speed sorter (Dako, Glostrup, Denmark). Sorted parasites were washed three times in serum-free ookinete media, followed by three washes in phosphate buffered saline, pH 8.0. A small aliquot of parasites were stained with Leukostat and examined by light microscopy. A minimum of 10 fields at 100X were counted using an Olympus BX51 microscope; pictures were taken with an Olympus DP71 camera.

Multidimensional protein identification technology (MudPIT)

Parasites were disrupted by three cycles of freeze-thaw lysis in digestion buffer (4 M urea, 0.4% Triton X-100, 50 mM Tris, 5 mM EDTA, 10 mM EDTA, 10 mM $MgSO_4$, pH 8.0) with Complete protease inhibitors (Roche Applied Sciences, Indianapolis, IN, USA). Parasites were processed by 3 cycles of freeze-thaw lysis followed by sonication on ice for 15 minutes in 1 minute bursts using a Misonix

Sonicator 3000 with an output setting of 6-7 (Misonix, Farmingdale, NY, USA). The lysate was then centrifuged at 10,000 x *g* for 20 minutes at 4^{o}C to remove hemozoin (*Prieto et al., 2008*). Proteins were precipitated by methanol-chloroform extraction, solubilized in Invitrosol (Invitrogen, Carlsbad, CA, USA), a MudPIT compatible solvent, and digested with trypsin. The digested peptide mixture was analyzed by MudPIT analysis using high-resolution liquid chromatography followed by tandem mass spectrometry (LS/MS/MS). Strong cation exchange liquid chromatography with Partisphere silica matrix (Whatman, Piscataway, NJ USA) followed by reversed-phase liquid chromatography using C-18 coated beads to separate peptides in a 2-dimensional liquid phase. Isolated peptides were then subjected to collision-induced dissociation on custom made silica tips using a Quartz micropipette puller P-2000 (Sutter Instrument Company, Novato, CA USA) and recorded by tandem mass spectrometry on a Finnigan LTQ (Thermo Scientific, Waltham, MA USA).

The resulting MS/MS spectra were analyzed with SEQUEST (*Eng et al., 1994*) to identify amino acid sequences matching the identified peptide masses and fragment ions. Peptides identified using SEQUEST were then analyzed using DTASelect 2.0 (*Cociorva et al., 2006*), which used a quadratic discrimination analysis to dynamically set XCorr and DeltaCN thresholds for the data set. Minimum and maxiumum charge states accepted were 1 and 16, respectively. The false positive rate was determined to be 1% for *P. falciparum* gametocyte, macrogamete, zygote and ookinete samples and 3-5% for mixed *P. falciparum* sexual stage parasite samples and *P. vivax* macrogametes. The false positive rate was estimated by the program from the number and quality of spectral matches to internal standards and a decoy database. Ambiguous

peptides that could have been derived from multiple proteins were excluded from analysis. The proteins were searched against PlasmoDB version 6.0 (*Aurrecoechea et al., 2008*) Only proteins with a minimum of two spectra were further analyzed.

In silico analysis of identified proteins

All proteins identified were manually classified into functional groups based on GO annotation and major protein domain homologies (*Aurrecoechea et al., 2008*). The presence of signal peptides and/or transmembrane domains was predicted by SignalP (*Bendtsen et al., 2004*) and TMHMM (*Krogh et al., 2001*), respectively. Comparisons against microarray gene expression data were done using previously published databases (*Young et al., 2005*, *Zhou et al., 2008*).

Results and Discussion

Purification of *in vitro*-cultivated *P. falciparum* sexual stage parasites

Sexual stage cultures were produced as mixtures of uninfected red blood cells, gametocytes, macrogametes, zygotes and ookinetes. The purification scheme employed utilized density gradient centrifugation and magnetic separation to remove > 98% of uninfected red blood cells (Figure 5.1). A discontinuous density gradient was then used to separate adherent macrogametes which were determined to be >98% pure. This was then followed by flow cytometric sorting of zygotes and ookinetes by DNA content which were shown by light microscopy to contain <5% contamination from other parasite stages. Purity was determined by examination of Leukostat-stained thin smears by light microscopy.

Separation of gametocytes and macrogametes

The NF54 strain of *P. falciparum,* which produced mature gametocytes at levels of 5-10%, was used to generate sexual stage parasite cultures with yields of zygotes and ookinetes ranging from 5-40% of total parasites in culture. Mature gametocytes were positively selected using a magnetic column at 37^{o}C to minimize gametogenesis, which could be stimulated by a drop in temperature (*Carter et al., 1993, Vermeulen et al., 1985*). Of note, magnetic purification of parasites in this technique is mediated by non-specific positive selection of hemozoin-containing parasites. This isolation scheme resulted in >95% purity of gametocytes (Figure 5.1).

Macrogametes were separated from sexual stage parasites using density gradient centrifugation as described above. *In vitro*-cultivated macrogametes were observed to associate in adherent clusters of cells, which may have been mediated by protein-protein interactions (*Chapter 2*). This phenotype was exploited to purify adherent macrogametes from untransformed gametocytes, non-adherent macrogametes, zygotes and ookinetes with a discontinuous Nycodenz gradient (*Vermeulen et al., 1985, Carter et al., 1993*). *P. falciparum* and *P. vivax* macrogametes isolated using this method were found to contain <2% contamination from other sexual stage parasites (Figure 5.1).

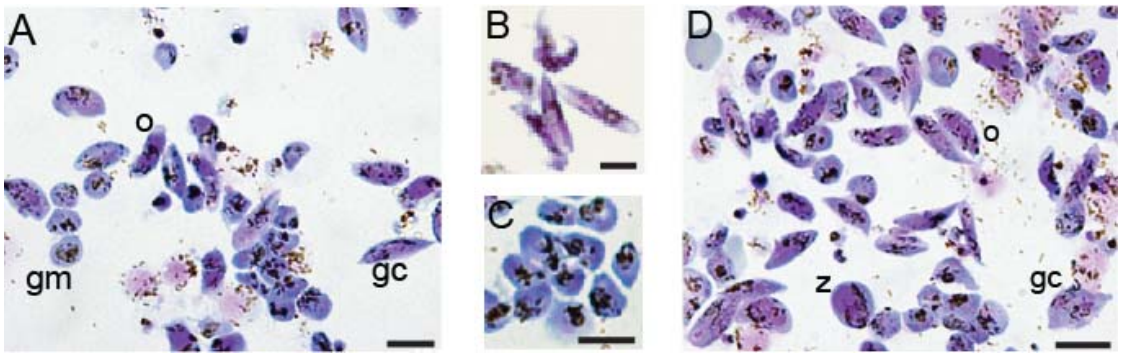

Figure 5.1
Purification of *in vitro*-cultivated *Plasmodium falciparum* sexual stage parasites and *Plasmodium vivax* macrogametes.
Leukostat-stained thin smears of purified sexual stage parasite cultures demonstrated purification of gametocytes (gc), macrogametes (gm) and zygotes (z) and ookinetes (o) from uninfected erythrocytes.
A. More than 97% of uninfected red blood cells were removed using a combination of Lympholyte-H density gradient and magnetic column purification.
B. Mature, *P. falciparum* gametocytes were purified from uninfected red blood cells at 37^{o}C using magnetic column purification
C. Adherent *P. falciparum* and *P. vivax* macrogametes were purified from other sexual stage parasites by discontinuous gradient centrifugation over a 6%-11%-16% Nycodenz gradient. Macrogametes were collected from the 6-11% interface.
C. Zygotes and ookinetes could not be purified using density gradient centrifugation because these parasites sediment to the same interface as untransformed gametocytes and non-adherent macrogametes.

Flow cytometric sorting of sexual stage parasites based on DNA content

Zygotes and ookinetes are the only sexual stage parasite forms that are diploid, suggesting that they can be separated from other sexual stage parasites by DNA content. Studies of DNA content in *P. berghei* sexual stage parasites demonstrated that zygote and ookinetes contained 2-4 times as much DNA as gametocytes, and *P. falciparum* ookinetes have been observed to have two nuclei (*Canning and Sinden, 1973, Janse et al., 1986, Chapter 2*). Flow cytometry has previously been used to isolate transgenic *Plasmodium* expressing fluorescent proteins, to analyze DNA content, and to separate asexual stage parasites (*Sultan et al., 1999, Janse et al.,* 2006,

Janse et al., 1987, Hall et al., 2005). This technique was used to isolate *P. falciparum* zygotes and ookinetes based on DNA content. Gametocytes, non-adherent macrogametes, zygotes and ookinetes were found to collect at the same interface layer on density gradients and could not be separated by centrifugation. All of the sexual stage parasite forms contained sufficient amounts of hemozoin to be positively selected on a magnetic column, thus this selection modality could not be used to separate the sexual stage parasites from each other.

Sexual stage parasite *P. falciparum* cultures were stained with Vybrant DyeCycle Green, a cell-permeant fluorescent dye which has been found to stoichiometrically and preferentially bind double-stranded DNA. Stained parasites were then analyzed by flow cytometry and found to contain three distinct fluorescence regions (Figure 5.2). These regions were sorted as indicated (R3-R6), stained with Leukostat and examined by light microscopy. R3 demonstrated enrichment for gametocytes, and R4 was enriched for macrogametes. The finding that macrogametes could be separated from gametocytes by DNA content was striking, but consistent with previous fluorometric studies (*Janse et al., 1985*). Vybrant DyeCycle Green has been shown to preferentially bind double-stranded DNA, but it is still able to bind RNA (*Zhao et al., 2009*). The increased fluorescence seen in macrogametes may be a reflection of higher levels of RNA present as a result of increased transcription and translation in this form (*Sinden et al., 1978, Sinden, 1983, Janse et al., 1985*). Region R5 consisted of parasites with 2-4 times the fluorescence of R3 (gametocytes) and was found to contain a mixture of zygotes, ookinetes, erythrocytes infected with two gametocytes, and macrogametes in clusters of 2-3 cells. R6 was enriched for a mixture

of zygotes and ookinetes. The majority of zygotes and ookinetes had at least four times as much DNA as gametes, consistent with previous studies that suggested that meiotic replication occurs shortly following gamete fusion (*Sinden and Hartley, 1985, Janse et al., 1986, Reininger et al., 2009*).

The R6-sorted zygote plus ookinete samples used for proteomic analysis were derived from a mixed population of 30-40% ookinetes and 60-70% zygotes (Figure 5.2). Zygotes and ookinetes could be separated from gametocytes and macrogametes by DNA content. However, zygotes were not easily separable from ookinetes using any of the purification methods described. Zygote samples were obtained by collecting sexual stage parasites cultured 4-8 hours after gametogenesis, before complete ookinete maturation, which takes at least 20 hours. Ookinete-specific proteins are thus described as proteins identified in the ookinete sample but absent from the zygote samples. Otherwise, the proteome derived from R6-sorted is described as the zygote plus ookinete proteome.

It is important to note that asexual schizonts co-purified with zygotes and ookinetes using this purification scheme (*Ribaut et al., 2008*). Typically, very low levels of asexual parasites (less than 1%) remained in late stage *P. falciparum* gametocyte cultures and even fewer survived in ookinete medium. However, schizonts could have co-purified with sexual stage parasites during density gradient and magnetic separation. They could then be enriched during flow cytometric sorting for zygotes and ookinetes, as they have more than 4 times the DNA content of gametocytes. The *P. falciparum* sexual stage parasites prepared for proteomic analysis had < 5% contamination by asexual parasites as determined by light microscopy.

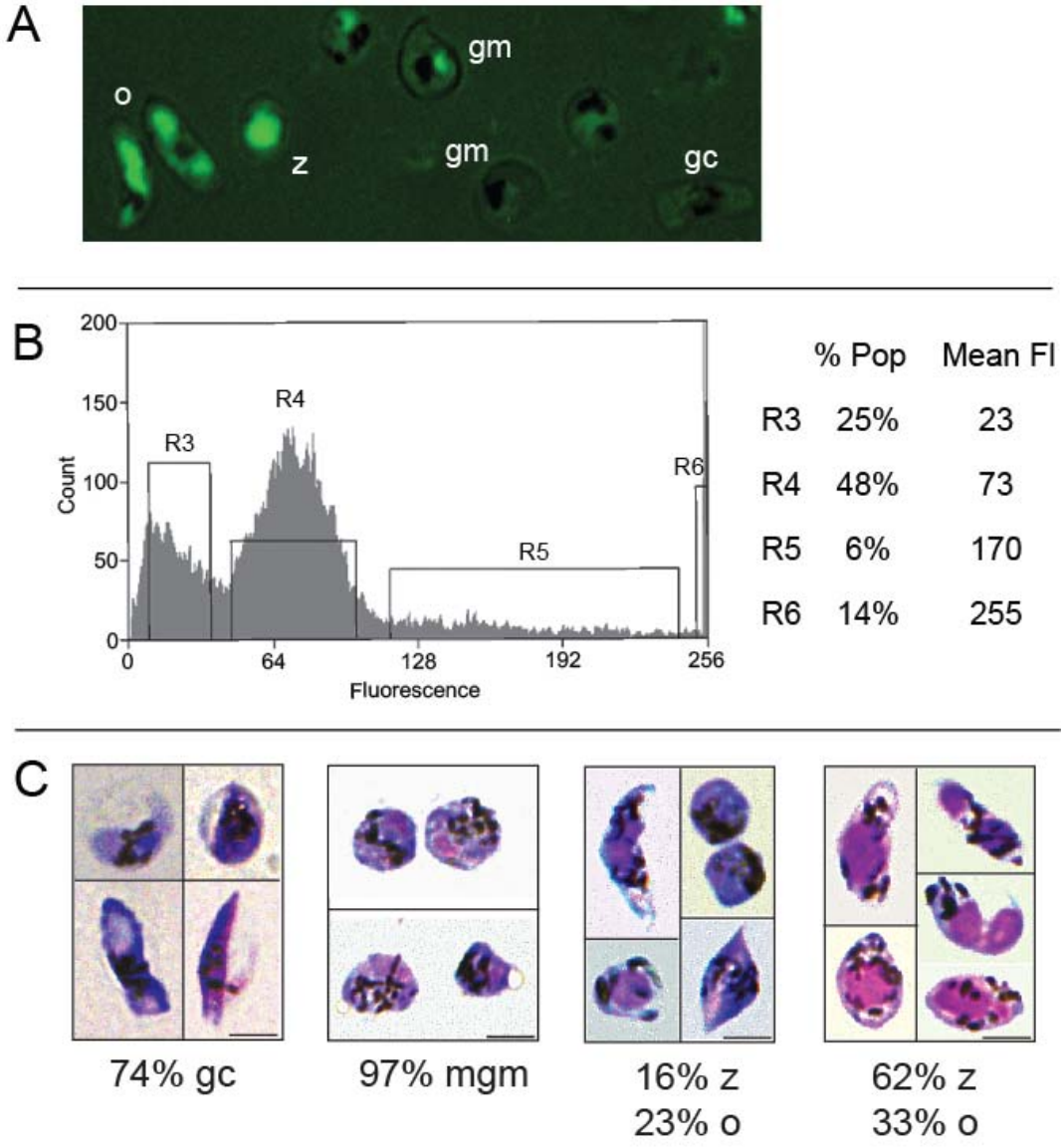

Figure 5.2
Flow cytometric sorting of *Plasmodium falciparum* zygotes and ookinetes based on DNA content.
A. Vybrant Dyecycle Green stained sexual stage parasites demonstrated differential fluorescence of haploid macrogametes (gm) and gametocytes (gc) compared to diploid zygotes (z) and ookinetes (o).
B. Flow cytometry of Vybrant DyeCycle Green-stained sexual stage parasites demonstrated two distinct fluorescent peaks which were divided into 4 populations, R3-R6. No distinct peaks were found in region R5. Percent of cells (% Pop) and mean fluorescence (Mean Fl) for each region are shown.
C. Sorted parasites from each population were stained with Leukostat and examined under light microscopy for quantification of parasite forms. 300-1000 parasites were counted per sorted population. Scale bars are identical for images: 5 µm.

Proteomic survey of gametocytes, macrogametes, zygotes and ookinetes

Proteomic analysis of four stage-specific populations and one mixed population led to the identification of 1505 proteins in sexual stage parasites (Figure 5.3). 775 proteins were found in the gametocyte proteome, 1125 proteins in the *P. falciparum* macrogamete proteome, 736 proteins in the zygote proteome, 877 proteins were found in zygote plus ookinete preparation proteome and 616 proteins were found in *P. vivax* macrogamete proteome. 548 of the *P. falciparum* proteins identified in sexual stage parasites were shared between macrogametes, zygotes and ookinetes; 478 proteins were shared between all *P. falciparum* sexual stages examined, and 444 proteins of this subset were shared with asexual stage parasites. Additionally, 190 proteins identified have not been previously detected in *P. falciparum* parasites by whole-organism proteomic analysis. Of the 616 *P. vivax* macrogamete proteins, 118 were not detected in *P. falciparum* macrogametes (Figure 5.3) and 77 *P. vivax* macrogamete proteins were not detected in any *P. falciparum* sexual stage parasites.

To determine whether proteins expressed in ookinetes could be differentiated from zygote-expressed proteins, the zygote plus ookinete proteome was compared to the zygote proteome. Purified parasites that were pooled to generate the zygote plus ookinete sample contained approximately 30% ookinetes and 70% zygotes. 31% (274 of 877 proteins) of proteins identified in the pooled ookinete sample were not found in the pooled zygote sample and likely represent ookinete-specific proteins, including previously characterized proteins such as: inner membrane complex (IMC) 1-related proteins important for the structure of the ookinete pellicle (*Tremp et al., 2008*); and

the micronemal protein cell-traversal protein for ookinetes and sporozoites (CelTOS) which functions in mosquito midgut invasion (*Kariu et al., 2006*).

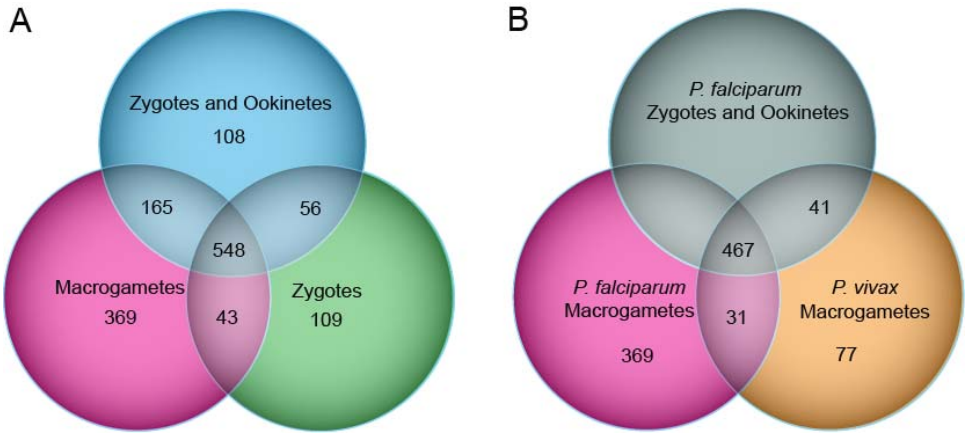

Figure 5.3
Identification of proteins in enriched populations of *P. falciparum* and *P. vivax* sexual stage parasite proteomes.
A. Venn diagram of proteins identified in *P. falciparum* separated sexual stage parasites. 548 proteins were shared between macrogametes, zygotes and ookinetes, of which 478 were shared between all four stages.
B. Venn diagram comparing proteins identified in *P. falciparum* macrogametes and *P. vivax* macrogametes.

Surprisingly, few of the previously characterized micronemal proteins were identified in this proteomic analysis. Notably absent proteins from the zygote plus ookinete proteome included chitinase, von Willebrand factor A domain-related protein (WARP) circumsporozoite protein and thrombospondin-related adhesive protein (CTRP), and secreted ookinete adhesive protein (SOAP), proteins that have all previously been identified in the *P. berghei* and *P. gallinaceum* ookinete proteomes. (*Hall et al., 2005, Patra et al., 2008, Lal et al., 2009*). These enzymes have been shown to be secreted by ookinetes to facilitate ookinete penetration of the mosquito midgut barrier. It was possible that these proteins were not detected due to incomplete

lysis of the parasites during sample preparation such that proteins within subcellular organelles, including micronemes, were lost. However, 69% (226 of 327) of proteins identified in the *P. berghei* microneme proteome were detected in the *P. falciparum* zygote plus ookinete proteome, which indicated that most of the microneme proteins were detected. Additionally, western immunoblot analysis of one of these undetected enzymes, chitinase, demonstrated that it was present in a the lysed zygote plus ookinete preparations (Figure 2.4).

The lack of detection of these enzymes in this global proteomic analysis was likely due to a limit of detection of this technique. It has been typically difficult to confidently detect proteins with lower relative protein abundance. In this proteomic analysis, proteins were collected and analyzed in real time. At any given point of elution from the HPLC column, only proteins with the highest abundance were selected for tandem MS/MS peptide identification, thus proteins with low abundance were lost to detection. This dataset only included proteins that were detected with ≥ 2 spectra. Thus, if lower abundance proteins were detected with only one spectrum, they were excluded from this dataset. Additionally, only unique peptides were included in this analysis; peptides which could have been derived from multiple, homologous proteins were ignored in this analysis.

More than 77% and 88% of all peptides detected in this analysis of the *P. falciparum* gametocyte and macrogamete samples, respectively, were identified as *Plasmodium* proteins. Conversely, only 30% of all detected peptides in the zygote and ookinete samples and 22% of peptides identified in the *P. vivax* macrogamete samples were identified as *Plasmodium* proteins. The majority of non-*Plasmodium* proteins

were identified as hemoglobin or human serum proteins. This was likely a result of residual hemoglobin and serum contamination despite multiple washes in serum-free buffers and careful sedimentation of hemozoin from parasite lysate. This level of contamination in the zygote and ookinete samples may have accounted for the lack of detection of lower-abundance proteins from this data set.

Sexual stage-specific enrichment of protein functional groups

All detected *Plasmodium* proteins were categorized into functional groups based on documented function and gene ontology (GO) annotation (Figure 5.4). Changes in relative contributions of proteins uniquely identified in a specific sexual stage demonstrate that different functional group proteins were expressed in different sexual stages. Over half of the proteins that were detected in all sexual stage proteomes examined (shared proteins) could be categorized into five functional groups: chaperones; DNA/cell cycle; trafficking/cytoskeleton/motility; metabolism and protein synthesis. Approximately 10-20% of all proteins identified in each proteome were unique to that parasite stage (Tables 5.A1 – 5.A4).

Figure 5.4
Functional categorization of identified proteins according to annotated and predicted gene ontology functions
Sexual stage proteins detected were categorized into functional categories based on documented function and GO category. Only one class was assigned per protein. Relative proportions are shown.
A. All proteins identified in *P. falciparum* sexual stages (all proteins) were categorized into functional groups. A subset of proteins which were detected in all *P. falciparum* stages (shared proteins) were shown.
B. Proteins uniquely identified in only one *P. falciparum* sexual stage proteome were categorized into functional groups. A larger proportion of proteins unique to a single sexual stage proteome were uncharacterized compared to proteins identified in all sexual stage proteomes ( ).
C. Proteins that were shared between *P. falciparum* and *P. vivax* macrogametes were categorized into functional groups.
D. Proteins unique to *P. vivax* macrogametes, those that were not detected in the *P. falciparum* macrogamete proteome, were characterized into functional groups.

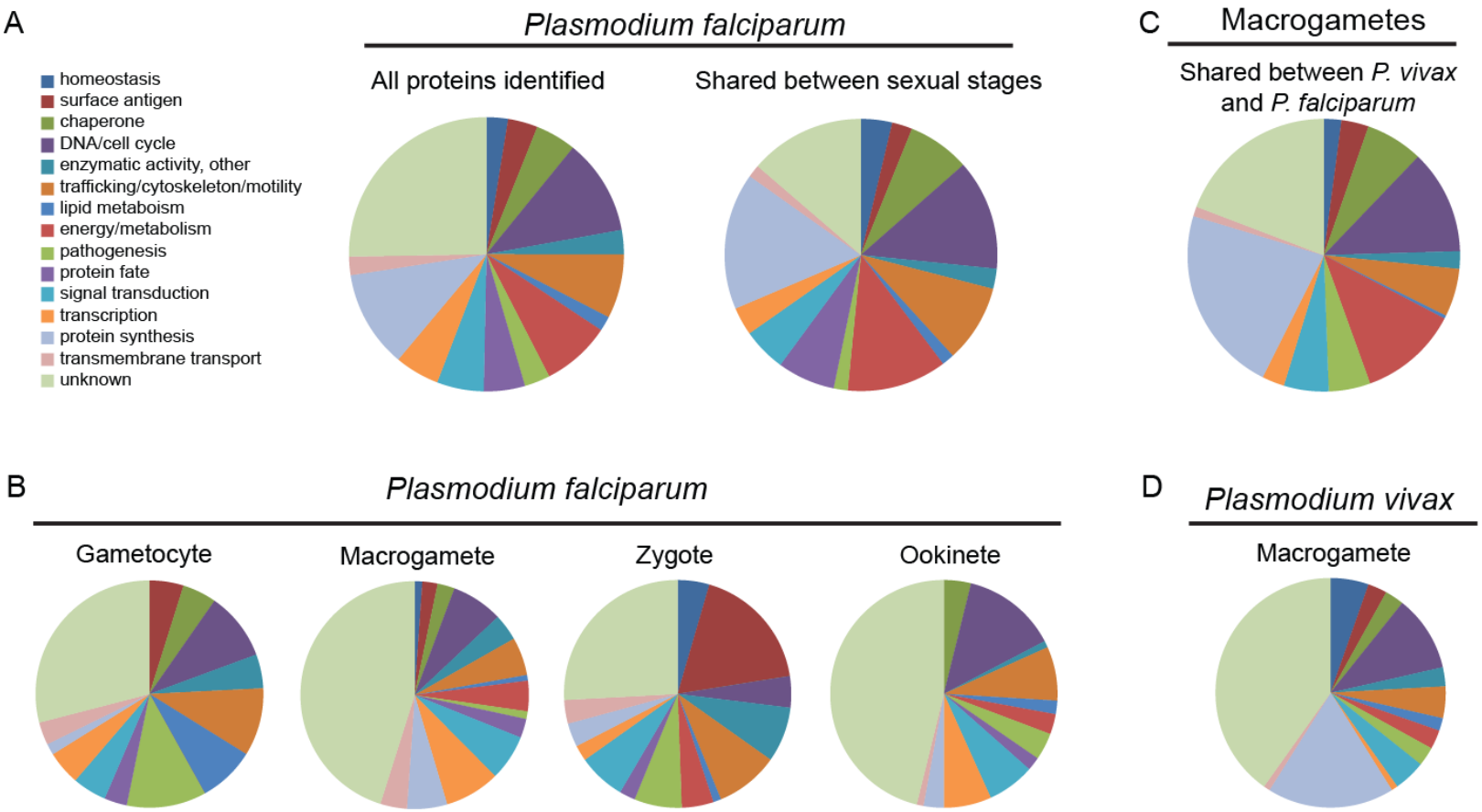

Figure 5.4
Functional categorization of identified proteins according to annotated and predicted gene ontology functions, continued.

Identification of 'hypothetical' proteins in the *P. vivax* macrogamete proteome

In this sexual stage proteomic analysis, uncharacterized proteins (unknown) constituted the largest population of proteins in each stage-specific proteome: 29% of gametocyte-specific proteins, 45% of *P. falciparum* macrogamete-specific proteins, 40% of *P. vivax* macrogamete-specific proteins, 25% of zygote-specific proteins and 46% of ookinete-specific proteins (Figure 5.4). One unknown protein identified from the *P. falciparum* sexual stage proteome was annotated as 'hypothetical', while 151 proteins identified from the *P. vivax* macrogamete proteome were annotated as 'hypothetical'. Examination of the *P. falciparum* orthologues of these *P. vivax* hypothetical proteins, indicates that 25% (38 of 151) of 'hypothetical' proteins may require genome reannotation, as their *P. falciparum* orthologs were characterized, while 75% (113 of 151) have yet to be characterized (Table 5.A5).

Potential transmission-blocking vaccine targets among uncharacterized proteins

Plasmodium sexual development within the mosquito midgut and subsequent invasion of the midgut barrier is a significant population bottleneck in the parasite life cycle (*Saul, 2007*). Proteins considered good transmission-blocking vaccine targets were either expressed on the surface of or secreted from sexual stage parasites. These characteristics allow antibodies access to the target protein during *Plasmodium* development in the mosquito midgut. Surface proteins currently proposed as transmission-blocking vaccine candidates include: surface protein 25/28 (*Vermeulen et al., 1985, Barr et al., 1991, Hisaeda et al., 2000, Arakawa et al., 2003, Kongkasuriyachai et al., 2004, Malkin et al., 2005, Wu et al., 2008, Arakawa et al.,*

2009), surface protein 45/48 (*Kocken et al., 1995, 2001, Ploton et al., 1995, van Dijk et al., 2001*), surface protein 47, (*van Schaijk et al., 2006*), surface protein 230 (*Williamson et al., 1993, Healer et al., 1997, Eksi et al., 2006*) and LCCL/lectin-adhesive like protein (*Delrieu et al., 2002, Claudianos et al., 2002, Trueman et al., 2004, Raine et al., 2007, Ecker et al., 2008, Simon et al., 2008*) and contain at least one transmembrane domain. Generally, secreted proteins contain a signal peptide that mediates protein processing through the endoplasmic reticulum and, presumably, packaging into budding micronemes. Potential secreted transmission-blocking vaccine candidates include: chitinase (*Huber et al., 1991, Vinetz et al., 2000, Tsai et al., 2001, Dessens et al., 2001*), CTRP (*Yuda et al., 1999, Dessens et al., 1999, Yuda et al., 1999, Templeton et al., 2000*), WARP (*Yuda et al., 2001, Li et al., 2004*), SOAP (*Dessens et al., 2003*), membrane attack ookinete protein (MAOP, *Kadota et al., 2004*), CelTOS (*Kariu et al., 2006*), plasmodium perforin-like proteins (PPLP, *Ecker et al., 2007, Ecker et al., 2008*). Current studies in animal models indicate that transmission-blocking vaccine strategies are effective in limiting or completely blocking transmission of parasites to the mosquito vector. However, only a handful of viable transmission-blocking vaccine candidates have been characterized to date.

Of the *P. falciparum* uncharacterized proteins identified in this study, 29% (124 of 424) contained a predicted signal peptide and/or transmembrane domain (Figure 5.5). This is a similar proportion to that seen in characterized *P. falciparum* proteins detected in this study (24%, 252 of 1024). In *P. vivax,* however, the proportion of proteins without characterized *P. falciparum* orthologs that contained either a signal peptide or transmemebrane domain was 44% (47 of 106), which is

twice as many as the proportion seen in characterized *P. vivax* macrogamete proteins (22%, 110 of 500) detected in this study. This novel set of signal peptide- and/or transmembrane domain-containing proteins represented a large pool of potential transmission-blocking vaccine targets which need to be studied further.

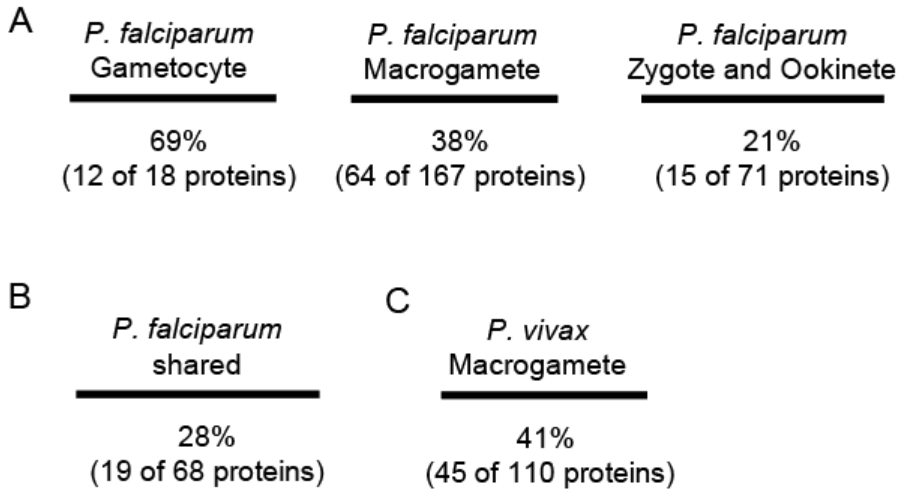

Figure 5.5
Proportion of uncharacterized proteins in *Plasmodium* sexual stage proteomes with either a signal peptide or transmembrane domain.
The proportion of proteins that contained a transmembrane domain and/or signal peptide were identified from uncharacterized proteins:
A. unique to the *P. falciparum* sexual stage proteomes analyzed,
B. shared between all *P. falciparum* sexual stage proteomes analyzed, and
C. analyzed in the *P. vivax* macrogamete proteome.

Comparison of protein and gene expression in sexual stage parasites

Plasmodium protein expression typically follows gene expression during the parasite life cycle, and mRNA expression has been shown to be a good predictor of protein expression (*Le Roch et al., 2004*). One notable exception where protein expression does not follow mRNA expression is during sexual stage parasite development. *Plasmodium* gametocytes are known to harbor mRNA transcripts for

proteins that are not expressed until the mosquito midgut stages: gametes, zygotes and ookinetes. This phenomenon, known as translational repression, was recently studied in *P. berghei* and found to be mediated in part by the RNA helicase development of zygote inhibited (DOZI, *Mair et al., 2006*). Attempts to knockout the DOZI ortholog in *P. falciparum* have failed. Additionally, two pumilio family RNA-binding proteins (Puf) were characterized in *P. falciparum* and found to bind to a 3' untranslated region (UTR) Nanos-response element, which has been shown to mediate translational repression (*Cui et al., 2002*).

In the analysis of *P. falciparum* sexual stage proteomes reported here, 26 DEAD box helicases or RNA binding proteins were detected. These proteins included the *P. falciparum* DOZI ortholog (PFC0915w), and a Puf-domain containing protein (PFE0430w). Of these 26 protiens, six (PFB0730w, PFE0430w, PFE0935c, PF14_0513, PF14_0563, PFC0955w) have not been previously detected by proteomic analyses, and three (PFB0730w, PFE0430w, PFE0935c) had maximal gene expression in gametocytes, as determined by transcriptomic analysis (*Young et al., 2005*). In addition to the *P. falciparum* DOZI-ortholog, other RNA helicases or binding proteins may be important for translational repression in *P. falciparum*. The DEAD box helicases and RNA-binding proteins identified in this analysis may also be important for translational repression in *P. falciparum*.

To determine whether transcript abundance could predict protein expression in gametocytes or later sexual stage parasites, as detected in this proteomic analysis of *P. falciparum* sexual stage parasites, we compared our protein data set to previously published gene expression data sets (*Young et al., 2008, Zhao et al., 2008*). *P.*

falciparum genes were characterized as highly expressed in gametocytes if they met three criteria: 1) absolute expression levels in gametocytes were greater than in asexual blood stages 2) maximal expression percentile of the gene occurred in gametocytes, and 3) absolute expression levels in gametocytes was greater than 300 counts. Of 267 genes that met these criteria, 102 and 46 corresponding proteins were detected in these proteomes of sexual stage parasites using a low threshold (≥ 4 spectra and ≥ 2 sequences detected) and a high threshold (≥ 10 spectra and ≥ 2 sequences detected) cutoff, respectively. Approximately half of the corresponding proteins were detected in the gametocyte proteome; the remainder were not detected in gameotcytes but were found in the macrogamete, zygote and zygote plus ookinete proteomes using both threshold limits (Figure 5.6, Table 5.A5 to 5.A7).

Among the 45 highly expressed gametocyte transcripts detected as proteins in the gametocyte proteome were proteins known to be important for gametocyte development (Figure 5.6). These included: the abundant gametocyte surface protein Pfs230, the macrogametocyte-specific osmophillic body protein Pfg377 (*Alano et al., 1995, Severini et al., 1999, de Koning-Ward et al., 2008*); microgametocyte-specific alpha tubulin II(*Rawlings et al., 1992, Guinet et al., 1996*) and male development gene-1 (*Lal et al., 2009, Ponzi et al., 2009*), and calcium dependent protein kinase 4 (CDPK4) which has been shown to be important for gamete and gametocyte development (*Billker et al., 2004, Ranjan et al., 2009, Kato et al., 2009*). Six of these 45 proteins were also detected in the macrogamete proteome, and 39 of these proteins were detected in all proteomes analyzed: macrogamete, zygote and ookinete.

There were 57 highly expressed gametocyte gene transcripts that were not detected as proteins in the gametocyte proteome but were detected in the macrogamete (41 proteins), and zygote and ookinete proteome (16 proteins). These included: the abundant zygote and ookinete surface protein Pfs25; Pf11-1, a protein implicated in macrogamete emergence (*Scherf et al., 1992a, Scherf et al., 1992b*); structural IMC1-related membrane skeletal proteins (*Tremp et al., 2008*); and the micronemal protein CelTOS (*Kariu et al., 2006*). Of the 41 proteins detected in the macrogamete proteome, 16 proteins were also detected in the zygote and ookinete proteome. An additional 16 proteins were detected in the zygote and ookinete proteome that were not detected in the gametocyte or macrogamete proteome. These data represented a new dataset of putative translationally repressed proteins specific to *P. falciparum*.

These putative translationally repressed proteins were compared to *P. berghei* orthologues that were proposed to be translationally repressed. Two separate datasets of translationally repressed genes have been identified in *P. berghei.* One set of over 200 genes was derived from analyses of genes whose expression was affected by a DOZI knockout (*Mair et al., 2006*). The second set of 9 genes was derived by comparing *P. berghei* gene transcripts isolated from gametocytes to *P. berghei* protein expression in gametocytes and ookinetes (*Hall et al., 2005*). Of the translationally repressed proteins identified in this analysis, 18% (10 of 57) had *P. berghei* orthologs affected in the DOZI knockout parasite compared to only 3% (3 of 45) of the non-translationally repressed proteins (*Mair et al., 2006*). This set of *P. falciparum* translationally repressed proteins was also compared to a *P. berghei* dataset of 9 translationally repressed proteins derived from transcriptomic and proteomic data

(*Hall et al., 2005*). Only one protein identified in this *P. falciparum* data set contained the proposed 47-mer 3' regulatory motif identified in *P. berghei* (*Hall et al., 2005*). This suggested that translational repression regulatory motif(s) may differ between *Plasmodia* species or that they may not be restricted to the 3' UTR.

These putative translationally repressed proteins were compared to a *P. falciparum* dataset derived from transcriptomic data and considered to be important in sexual development (*Young et al., 2008*). Of the putative, translationally repressed genes, 25% (14 of 57) contained one or more 5' motifs previously identified in the *Young et al.* dataset. However, a similar proportion, 24% (11 of 45), of the high transcript genes in gametocytes that were detected in the gametocyte proteome contained these same 5' motifs. This finding suggested that although the *Young et al.* dataset described proteins that are important in sexual development, these proteins may not have been specific to translationally repressed proteins.

To determine whether combined analysis of *P. falciparum* proteomic and transcriptomic data could identify novel translational repression motifs, 1000 base pair regions upstream and downstream of these putative translationally repressed genes were analyzed with Multiple Exception maximum for Motif Elicitation (MEME), (*Bailey and Elkan, 1994*). A modified zero-order background model was user-defined to compensate for the AT-rich *P. falciparum* genome. Nucleotide frequencies of non-coding regions were calculated and input as: 0.32 A, 0.17 C, 0.17 G, and 0.32 T. If this background model was not used, MEME only identified degenerate motifs containing only A and T nucleotides, reflecting the composition of the AT-rich genome. Limits were set to include motifs which were 6-50 nucleotides in length with any number of

occurrences in the untranslated regions. The 5' and 3' UTRs of these genes were analyzed in two groups: macrogamete (41 genes); and zygote and ookinete (16 genes) putative translationally repressed proteins.

Using these parameters, a total of 10 non-AT rich motifs were identified in the 5' UTR – 6 motifs from the macrogamete group and 4 motifs from the zygotes and ookinete group. A total of 6 non-AT rich motifs were identified in the 3' UTR – 2 from the macrogamete group and 4 from the zygote and ookinete group. The 5' UTR motif and 3' UTR motif with the highest levels of representation in these groups are shown (Figure 5.6). The 6-mer 5' UTR motif AAAGGG was found in 37% (15 of 41) of macrogamete and 88% (14 of 16) of zygote plus ookinete putative translationally repressed genes. The 6-mer motif identified in the 3' UTR, CACACA, was found downstream of 85% (35 of 41) of macrogamete and 100% (16 of 16) of zygote plus ookinete putative translationally repressed genes.

More than half, 51% (29 of 57), of the proteins in this novel putative translationally repressed subset have not been characterized. Genetic disruption of DOZI in *P. berghei* prevented parasite maturation into zygotes and ookinetes, presumably because DOZI-regulated genes are essential for sexual stage development (*Mair et al., 2006*). The proteins identified here are likely to be important for sexual stage development and represent a set of proteins specific to *P. falciparum* to be studied. This is the first time that *P. falciparum* translationally repressed genes have been identified using a comparative, global analysis.

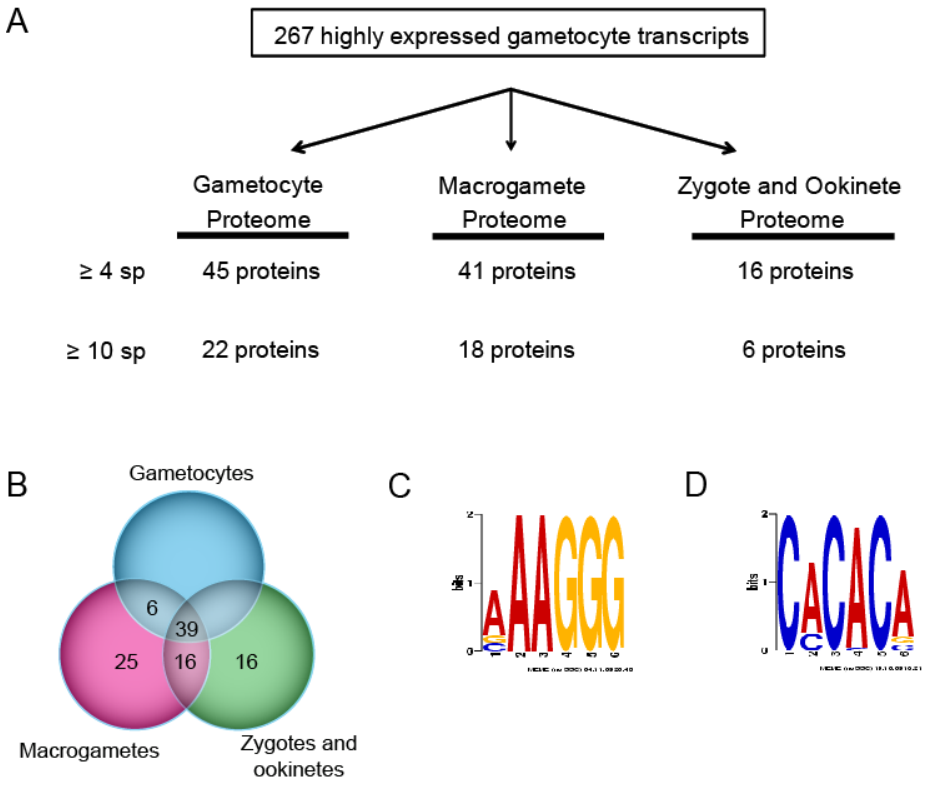

Figure 5.6
Stage-specific protein expression of highly expressed gametocyte transcripts.
A. 267 highly expressed gametocyte gene transcripts were identified in previously published microarray data. Comparison of gene transcript data with protein expression data demonstrated that 45 corresponding gene products were detected in gametocytes while 57 corresponding gene products were detected in macrogametes, zygotes and ookinetes using a low threshold (≥ 4 spectra detected). 22 and 19 corresponding gene products were detected in gametocytes and later sexual stages, respectively, using a high threshold (≥ 10 spectra detected).
B. Venn diagram of highly expressed gametocyte transcripts detected as proteins in this analysis. Of 45 proteins detected in the gametocyte proteome, 6 were also detected in the macrogamete but not zygote/ookinete proteome, and 39 were detected in all proteomes. Of 41 proteins detected in the macrogamete proteome but not the gametocyte proteome, 16 were also detected in the zygote and/or ookinete proteome. 16 different proteins were detected in the zygote and/or ookinete proteome but not the gametocyte or macrogamete proteomes.
C. A 5' UTR and D. 3' UTR regulatory motif identified using this data set of putative translationally repressed proteins.

Conclusion

Data from global proteomic analyses of sexual stage parasites have advanced our understanding of these developmental stages by validating and extending information gathered from global transcriptomic analyses (*Le Roche et al., 2003, Hall et al., 2005*, *Tarun et al., 2008*). Towards this end, this study has confirmed the expression of novel, hypothetical proteins in *P. vivax*, thus refining genome annotation (*Florens et al., 2002, Lasonder et al., 2002, Hall et al., 2005, Young et al., 2005, Lasonder et al., 2008*, *Patra et al., 2008, Taurn et al., 2008*).

Data from proteomic studies of sexual stage parasites provide experimentally validated insights into mechanisms of *Plasmodium* sexual maturation and ookinete development in the mosquito midgut. These proteins may serve as a source of discovery for families of novel drug and/or vaccine targets on a scale not using the classic protein-by-protein approach to drug discovery (*Ecker et al., 2008*). There is growing emphasis on transmission-blocking initiatives that block parasite development in and transmission by the mosquito vector (*Saul, 2007*). This new information can be applied towards the development of novel transmission-blocking initiatives. The data provided for *P. falciparum* zygotes and ookinetes and *P. vivax* macrogametes has not previously been available. This information is critical, especially for the human disease-causing agents, *P. falciparum* and *P. vivax.*

Chapter 5 will be submitted for publication: Bounkeua, V., Li, F., Prieto, J.H., Bright, A.T., Winzeler, E.A., Yates, J.R., Vinetz JM. “Proteomic analysis of *Plasmodium falciparum* sexual stage parasites and *Plasmodium vivax* macrogametes”. The dissertation author is the co-primary author and investigator of this paper.

Table 5.A1

Proteins detected only in the *P. falciparum* gametocyte proteome.

ID	Product
PF11_0088	conserved Plasmodium protein, unknow n function
PF14_0013	DNAJ protein, putative
PFA0110w	DNAJ protein, putative
MAL13P1.221	aspartate carbamoyltransferase
pf07_0047	cell division cycle ATPase, putative
PF11_0077	DEAD/DEAH box helicase, putative
PF11_0172	folate/biopterin transporter, putative
PFL1180w	chromatin assembly protein (ASF1), putative
PFL1920c	hydroxyethylthiazole kinase, putative
PF10_0220	phospholipid scramblase 1, putative
PF13_0032	hydrolase, putative
MAL13P1.285	patatin-like phospholipase, putative
PF11_0197	conserved Plasmodium protein, unknow n function
PFF0290w	long chain polyunsaturated fatty acid elongation enzyme, putative
PFF1375c	ethanolaminephosphotransferase, putative
PFI1180w	patatin-like phospholipase, putative
MAL13P1.176	reticulocyte binding protein 2, homolog b
MAL7P1.27	chloroquine resistance transporter
PF13_0198	reticulocyte binding protein 2 homolog A
PF13_0275	Plasmodium exported protein, unknow n function
PFD0095c	Plasmodium exported protein (PHISTb), unknow n function
PFD1150c	reticulocyte binding protein homolog 4, Rh4
PFE0050w	Plasmodium exported protein, unknow n function
MAL8P1.157	ubiquitin-like protease 1 homolog, Ulp1 homolog, putative
PF10_0170	26s proteasome subunit p55, putative
PFL0585w	polyubiquitin
PF10_0085	nucleolar protein NOP5, putative
PF11_0281	protein phosphatase, putative
PF14_0224	serine/threonine protein phosphatase
PF14_0614	phosphatase, putative
PF11_0509	ring-infected erythrocyte surface antigen, putative
PFB0915w	liver stage antigen 3
PFB0935w	cytoadherence linked asexual protein 2
MAL13P1.231	Sec61 alpha subunit, PfSec61
MAL8P1.121	coatomer epsilon subunit, putative
PF13_0274	MON1 protein
PF14_0548	ATPase, putative
PFC0890w	SNARE protein
PFD0250c	Sec24-like protein, putative

Table 5.A1
Proteins only detected in the *P. falciparum* gametocyte proteome, continued

ID	Product
PFA0450c	mRNA cleavage factor-like protein, putative
PFB0245c	DNA-directed RNA polymerase II 16 kDa subunit, putative
PFI0820c	RNA binding protein, putative
MAL13P1.23	CorA-like Mg2+ transporter protein, putative
PFI0720w	transporter, putative
MAL13P1.271	V-type ATPase, putative
PFL1125w	phospholipid-transporting ATPase, putative
MAL8P1.127	conserved Plasmodium protein, unknown function
MAL8P1.53	conserved Plasmodium protein, unknown function
PF10_0100	conserved Plasmodium protein, unknown function
PF10_0130	conserved Plasmodium protein, unknown function
PF11_0275	conserved Plasmodium protein, unknown function
PF11_0324	conserved Plasmodium protein, unknown function
pf13_0104	conserved Plasmodium protein, unknown function
PF13_0106	conserved protein, unknown function
PF13_0219	conserved Plasmodium protein, unknown function
PF13_0273	conserved Plasmodium protein, unknown function
PF14_0105	conserved Plasmodium protein, unknown function
PFC0355c	conserved Plasmodium protein, unknown function
PFD0720w	conserved ARM repeats protein, unknown function
PFD0940w	conserved Plasmodium protein, unknown function
PFE0680w	conserved Plasmodium protein, unknown function
PFF0335c	probable protein, unknown function
PFI0525w	nucleotide binding protein, putative
PFL2515c	conserved Plasmodium protein, unknown function
PFC0130c	conserved Plasmodium protein, unknown function
PFC0800w	band 7-related protein

Table 5.A2
Proteins detected only in the *P. falciparum* macrogamete proteome.

ID	Product
PF08_0121	peptidyl-prolyl cis-trans isomerase precursor
PF10_0058	DNAJ protein, putative
PF11_0290	conserved Plasmodium protein, unknown function
pf14_0137	DNAJ protein, putative
PF14_0417	HSP90
PF14_0538	conserved Plasmodium protein, unknown function
PFI0220w	prefoldin subunit, putative
PFL0550w	HSP20-like chaperone
PFL1455w	t-complex protein 1, gamma subunit, putative
MAL13P1.216	DNA helicase, putative
MAL13P1.289	mitotic control protein dis3 homologue, putative
MAL13P1.328	DNA topoisomerase VI, B subunit, putative
pf10_0067	conserved Plasmodium protein, unknown function
PF10_0292	conserved Plasmodium protein, unknown function
PF11_0317	structural maintenance of chromosome protein, putative
PF14_0254	DNA mismatch repair protein Msh2p, putative
PF14_0657	conserved Plasmodium protein, unknown function
PFB0730w	DEAD/DEAH box helicase, putative
PFC0610c	zinc finger protein, putative
PFC0955w	ATP-dependent RNA Helicase, putative
PFD0470c	replication factor a protein, putative
PFD0755c	adenylate kinase 1
PFE0430w	ATP-dependent RNA Helicase, putative
PFE1095w	conserved Plasmodium protein, unknown function
PFI0965w	Pyridoxal 5'-phosphate dependent enzyme class III, putative
PFI1420w	guanylate kinase
PFL0075w	XPA binding protein 1, putative
PFL0305c	IMP-specific 5'-nucleotidase
PFL0575w	DNA replication licensing factor MCM5, putative
PFL0815w	DNA-binding chaperone, putative
PFL1335w	cyclin-related protein, Pfcyc-2
PFL1345c	cyclin related protein, putative
PFL2150c	CCCH-type Zn-finger protein, putative
MAL8P1.144	AAA family ATPase, putative
PF11_0226	petidase, M16 family
PF11_0405	AAA family ATPase, putative
PF11_0427	dolichol phosphate mannose synthase
PF14_0616	ATP-dependent protease la, putative
PFI1340w	fumarate hydratase, putative
PFL1620w	asparagine/aspartate rich protein, putative
PFL1635w	Ulp1 protease, putative
PFL2510w	chitinase

Table 5.A2
Proteins only detected in the *P. falciparum* macrogamete proteome, continued

ID	Product
MAL7P1.88	thioredoxin-like protein
PFF1265w	oxidoreductase, short-chain dehydrogenase family, putative
PFI1250w	thioredoxin-like protein 2
PF07_0066	conserved Plasmodium protein, unknown function
PFA0225w	LytB protein
PFD0085c	acyl-CoA synthetase, PfACS6
pf08_0099	acyl CoA binding protein, putative
PF10_0245	glucosamine-fructose-6-phosphate aminotransferase, putative
pf13_0061	ATP synthase gamma chain, mitochondrial precursor, putative
PF13_0327	cytochrome c oxidase subunit 2, putative
PF14_0375	conserved Plasmodium protein, unknown function
PFB0560w	conserved Plasmodium protein, unknown function
PFB0610c	conserved Plasmodium protein, unknown function
PFD0285c	lysine decarboxylase, putative
PFD0835c	LETM1-like protein, putative
PFF0680c	thiamin-phosphate pyrophosphorylase, putative
PFL0630w	iron-sulfur subunit of succinate dehydrogenase
PFL1275c	cof-like hydrolase, had-superfamily, subfamily iib
PFL1940w	3-hydroxyisobutyryl-coenzyme A hydrolase, putative
PF08_0052	perforin like protein 5
pf10_0281	merozoite TRAP-like protein, MTRAP
PF13_0348	rhoptry protein
PF14_0637	rhoptry protein, putative
MAL7P1.19	ubiquitin transferase, putative
MAL8P1.23	ubiquitin-protein ligase 1, putative
PF11_0201	ubiquitin-protein ligase, putative
PF11_0329	ubiquitin-like protein, putative
PF13_0096	Ubiquitin Carboxyl-terminal Hydrolase-like zinc finger protein
pf13_0156	proteasome subunit beta type 7 precursor, putative
pf14_0025	proteosome subunit, putative
PFI0740c	ubiquitin conjugating enzyme, putative
PF08_0009	translation initiation factor EIF-2b alpha subunit, putative
PF08_0092	methyltransferase, putative
PF10_0143	transcriptional activator ADA2, putative
PF10_0179	60S ribosomal protein L30e, putative
PF11_0284	methyltransferase, putative
PF11_0312	60S ribosomal protein L38e, putative
PF11_0471	nucleolar preribosomal assembly protein, putative
PF13_0051	snornp protein gar1 homologue, putative
PF13_0208	exoribonuclease, putative
pf14_0027	40S ribosomal protein S31/UBI, putative
PF14_0327	methionine aminopeptidase, type II, putative
PF14_0360	eIF2A
PF14_0494	ribosome biogenesis protein tsr1, putative
PFC0200w	60S Ribosomal protein L44, putative
PFD0515w	exosome complex exonuclease rrp4, putative
PFE0185c	60S ribosomal protein L31, putative
PFI0455w	exoribonuclease, putative
PFL0335c	eukaryotic translation initiation factor 3, subunit 6, putative
PFL2430c	eukaryotic translation initiation factor 5, putative

Table 5.A2
Proteins only detected in the *P. falciparum* macrogamete proteome, continued

ID	Product
MAL13P1.256	phosphatidylinositol transfer protein, putative
MAL13P1.275	protein phosphatase, putative
MAL8P1.91	phospholipase DDHD1, putative
PF07_0110	protein phosphatase, putative
PF10_0071	rhoGAP GTPase, putative
PF10_0140	conserved protein, unknown function
PF11_0389	calcium-binding protein, putative
PF13_0302	phosphatase 2A regulatory subunit-related protein, putative
PF14_0672	cyclic nucleotide phosphodiesterase, putative
PF14_0699	GTPase activator, putative
PFC0105w	serine/threonine protein kinase, putative
PFC0475c	phosphatidylinositol 3- and 4-kinase, putative
PFD0505c	protein phosphatase, putative
PFE1305c	ADP-ribosylation factor GTPase-activating protein, putative
PFF1370w	protein kinase PK4
PFI0345w	GTPase activator, putative
PFI1005w	ADP-ribosylation factor-like protein
PFL1870c	sphingomyelin phosphodiesterase, putative
PFL2365w	protein phosphatase, putative
MAL13P1.133	conserved Plasmodium membrane protein, unknown function
PF13_0168	CPW-WPC family protein
PFA0280w	asparagine-rich antigen Pfa35-2
PFC0640w	CSP and TRAP-related protein (CTRP)
PFC0895w	CPW-WPC family protein
PFL1215c	erythrocyte membrane protein pfemp3, putative
PFL1395c	Merozoite Surface Protein 9, MSP-9
MAL8P1.146	filament assembling protein, putative
PF08_0033	membrane skeletal protein IMC1-related
PF10_0196	cytoplasmic dynein intermediate chain, putative
PF10_0331	Sec1 family protein, putative
PF11_0119	SNARE protein, putative
PF11_0202	clathrin coat assembly protein, putative
PF14_0569	conserved Plasmodium protein, unknown function
PFA0335w	Rab5c, GTPase
PFB0750w	vacuolar protein-sorting protein VPS45, putative
PFC0582c	vesicle transport v-SNARE protein, putative
PFE0690c	PfRab1a
PFF0675c	myosin E
PFI1700c	sec-1 family protein
PFL0925w	formin 2, putative
PFL1030w	membrane skeletal protein, putative
PFL2415w	Hbeta58/Vps26 protein homolog, putative
MAL13P1.45	U4/U6 small nuclear ribonucleoprotein, putative
MAL8P1.9	u6 snRNA-associated Sm-like protein, putative
PF08_0034	histone acetyltransferase GCN5, putative
PF10_0057	regulator of nonsense transcripts, putative
pf10_0075	transcription factor with AP2 domain(s), putative
PF10_0232	Chromodomain-helicase-DNA-binding protein 1 homolog, putative
PF10_0314	dcp1 homologue, putative
PF11_0053	PfSNF2L
PF11_0091	transcription factor with AP2 domain(s), putative

Table 5.A2
Proteins only detected in the *P. falciparum* macrogamete proteome, continued

ID	Product
PF11_0297	NOT family protein, putative
PF11_0385	UVB-resistance protein UVR8 homologue
PF11_0404	transcription factor with AP2 domain(s), putative
PF14_0174	pseudouridine synthase, putative
PF14_0513	RNA binding protein, putative
PF14_0656	U2 snRNP auxiliary factor, putative
PFE0750c	RNA recognition motif, putative
PFE0840c	transcription factor with AP2 domain(s), putative
PFE0925c	snrnp protein, putative
PFF0165c	conserved Plasmodium protein, unknown function
PFI0475w	small nuclear ribonucleoprotein (snRNP), putative
PFI0685w	pseudouridylate synthase, putative
MAL13P1.206	Na+ -dependent Pi transporter, sodium-dependent phosphate transporter
PF07_0032	Cg8 protein
PF07_0035	cg1 protein
PF07_0115	cation transporting ATPase, cation transporter
PF08_0098	ABC1 family, putative
PF11_0435	conserved Plasmodium membrane protein, unknown function
PF13_0019	sodium/hydrogen exchanger, Na+, H+ antiporter
PF13_0048	NUDIX hydrolase, putative
PFL0655w	conserved Plasmodium membrane protein, unknown function
MAL13P1.124	conserved Plasmodium protein, unknown function
MAL13P1.128	conserved Plasmodium protein, unknown function
MAL13P1.15	conserved Plasmodium protein, unknown function
MAL13P1.182	conserved Plasmodium protein, unknown function
MAL13P1.21	conserved Plasmodium protein, unknown function
MAL13P1.230	conserved Plasmodium protein, unknown function
MAL13P1.240	conserved Plasmodium protein, unknown function
MAL13P1.293	conserved Plasmodium protein, unknown function
MAL13P1.333	conserved Plasmodium protein, unknown function
MAL13P1.347	conserved Plasmodium protein, unknown function
MAL13P1.352	conserved Plasmodium protein, unknown function
MAL13P1.75	conserved Plasmodium protein, unknown function
MAL7P1.126	conserved Plasmodium protein, unknown function
MAL7P1.129	conserved Plasmodium protein, unknown function
MAL7P1.146	conserved Plasmodium protein, unknown function
MAL7P1.35	conserved Plasmodium protein, unknown function
MAL7P1.98	conserved Plasmodium protein, unknown function
MAL7P1.99	conserved Plasmodium protein, unknown function
MAL8P1.135	conserved Plasmodium protein, unknown function
MAL8P1.143	conserved Plasmodium protein, unknown function
MAL8P1.148	conserved Plasmodium protein, unknown function

Table 5.A2
Proteins only detected in the *P. falciparum* macrogamete proteome, continued

ID	Product
MAL8P1.79	conserved Plasmodium protein, unknow n function
MAL8P1.85	conserved Plasmodium protein, unknow n function
PF07_0082	conserved Plasmodium protein, unknow n function
PF07_0097	conserved Plasmodium protein, unknow n function
PF08_0127	conserved Plasmodium protein, unknow n function
PF10_0031	conserved Plasmodium protein, unknow n function
PF10_0061	conserved Plasmodium protein, unknow n function
PF10_0076	conserved Plasmodium protein, unknow n function
PF10_0099	conserved Plasmodium protein, unknow n function
PF10_0106	conserved Plasmodium protein, unknow n function
PF10_0246	conserved Plasmodium protein, unknow n function
PF10_0257	conserved Plasmodium protein, unknow n function
PF10_0287	conserved Plasmodium protein, unknow n function
PF11_0100	conserved Plasmodium protein, unknow n function
PF11_0218	conserved Plasmodium protein, unknow n function
PF11_0229	conserved Plasmodium protein, unknow n function
PF11_0409	conserved Plasmodium protein, unknow n function
PF11_0433	conserved Plasmodium protein, unknow n function
PF11_0479	conserved Plasmodium protein, unknow n function
pf13_0041	conserved Plasmodium protein, unknow n function
PF13_0047	conserved Plasmodium protein, unknow n function
PF13_0081	conserved Plasmodium protein, unknow n function
PF13_0134	conserved Plasmodium protein, unknow n function
PF13_0165	conserved Plasmodium protein, unknow n function
PF13_0210	conserved Plasmodium protein, unknow n function
PF13_0295	conserved Plasmodium protein, unknow n function
PF13_0329	conserved Plasmodium protein, unknow n function
PF13_0355	conserved Plasmodium protein, unknow n function
PF14_0031	conserved Plasmodium protein, unknow n function
PF14_0048	conserved Plasmodium protein, unknow n function
PF14_0069	conserved Plasmodium protein, unknow n function
PF14_0101	conserved Plasmodium protein, unknow n function
PF14_0152	conserved Plasmodium protein, unknow n function
PF14_0188	conserved Plasmodium protein, unknow n function
PF14_0195	conserved Plasmodium protein, unknow n function
PF14_0196	conserved Plasmodium protein, unknow n function
PF14_0217	conserved Plasmodium protein, unknow n function
PF14_0237	conserved Plasmodium protein, unknow n function
PF14_0310	conserved Plasmodium protein, unknow n function
PF14_0365	conserved Plasmodium protein, unknow n function

Table 5.A2
Proteins only detected in the *P. falciparum* macrogamete proteome, continued

ID	Product
PF14_0419	conserved Plasmodium protein, unknown function
PF14_0461	conserved Plasmodium protein, unknown function
PF14_0505	conserved Plasmodium protein, unknown function
PF14_0574	conserved Plasmodium protein, unknown function
PF14_0583	conserved Plasmodium protein, unknown function
PF14_0591	conserved Plasmodium protein, unknown function
PF14_0708	conserved Plasmodium protein, unknown function
PF14_0712	conserved Plasmodium protein, unknown function
PFA0315w	conserved Plasmodium protein, unknown function
PFA0320w	conserved Plasmodium protein, unknown function
PFA0490w	conserved Plasmodium protein, unknown function
PFB0145c	Kid domain containing protein
PFB0190c	conserved Plasmodium protein, unknown function
PFB0425c	conserved Plasmodium protein, unknown function
PFB0555c	conserved Plasmodium membrane protein, unknown function
PFB0655c	conserved Plasmodium protein, unknown function
PFC0100c	regulatory protein, putative
PFC0315c	conserved Plasmodium protein, unknown function
PFC0430w	conserved Plasmodium protein, unknown function
PFC0760c	conserved Plasmodium protein, unknown function
PFC0820w	conserved Plasmodium protein, unknown function
PFC0885c	conserved Plasmodium protein, unknown function
PFC1060c	conserved Plasmodium protein, unknown function
PFD0330w	conserved Plasmodium protein, unknown function
PFD0485w	conserved Plasmodium protein, unknown function
PFD0595w	conserved Apicomplexan protein, unknown function
PFD0605c	conserved Plasmodium protein, unknown function
PFD0795w	conserved Plasmodium protein, unknown function
PFD0875c	conserved Plasmodium protein, unknown function
PFD0905w	conserved Plasmodium protein, unknown function
PFD0945c	conserved Plasmodium protein, unknown function
PFD1065c	conserved Plasmodium protein, unknown function
PFD1115c	conserved Plasmodium protein, unknown function
PFE0250w	conserved Plasmodium protein, unknown function
PFE0645w	conserved Plasmodium protein, unknown function
PFE0670w	conserved Plasmodium protein, unknown function
PFE1025c	conserved Plasmodium protein, unknown function
PFE1055c	conserved Plasmodium protein, unknown function
PFE1140c	G10 protein, putative

Table 5.A2
Proteins only detected in the *P. falciparum* macrogamete proteome, continued

ID	Product
PFE1145w	conserved Plasmodium protein, unknow n function
PFE1165c	conserved Plasmodium protein, unknow n function
PFE1180c	conserved Plasmodium protein, unknow n function
PFE1210c	conserved Plasmodium protein, unknow n function
PFE1335c	conserved Plasmodium protein, unknow n function
PFE1340w	conserved Plasmodium protein, unknow n function
PFE1570c	conserved Plasmodium protein, unknow n function
PFF0175c	conserved Plasmodium protein, unknow n function
PFF0380w	conserved Plasmodium protein, unknow n function
PFF0555w	conserved Plasmodium protein, unknow n function
PFF0575c	conserved Plasmodium protein, unknow n function
PFF0790c	conserved Plasmodium protein, unknow n function
PFF0875w	conserved Plasmodium protein, unknow n function
PFF1055c	conserved Plasmodium protein, unknow n function
PFF1065c	conserved Plasmodium protein, unknow n function
PFF1465w	conserved Plasmodium protein, unknow n function
PFI0435c	conserved Plasmodium protein, unknow n function
PFI0675w	conserved Plasmodium protein, unknow n function
PFI0705w	conserved Plasmodium protein, unknow n function
PFI0745w	conserved Plasmodium protein, unknow n function
PFI1150w	conserved Plasmodium protein, unknow n function
PFI1210w	conserved Plasmodium protein, unknow n function
PFI1265w	conserved Plasmodium protein, unknow n function
PFI1330c	conserved Plasmodium protein, unknow n function
PFI1355w	conserved Plasmodium protein, unknow n function
PFL0130c	conserved Plasmodium protein, unknow n function
PFL0165c	conserved Plasmodium protein, unknow n function
PFL0175c	conserved Plasmodium protein, unknow n function
PFL0530c	conserved Plasmodium protein, unknow n function
PFL0675c	conserved Plasmodium protein, unknow n function
PFL0765w	conserved Plasmodium membrane protein, unknow n function
PFL0915c	conserved Plasmodium protein, unknow n function, could not be ko In pb
PFL0965c	conserved Plasmodium protein, unknow n function
PFL1065c	conserved Plasmodium protein, unknow n function
PFL1080c	A TP-binding protein, putative
PFL1330c	conserved Plasmodium protein, unknow n function
PFL1460c	conserved Plasmodium protein, unknow n function
PFL1610c	conserved Plasmodium membrane protein, unknow n function
PFL1685w	conserved Plasmodium protein, unknow n function
PFL1800w	conserved Plasmodium protein, unknow n function
PFL2320w	conserved Plasmodium protein, unknow n function
PFL2390c	conserved Plasmodium protein, unknow n function
PFL2450c	conserved Plasmodium protein, unknow n function

Table 5.A3
Proteins detected only in *P. falciparum* zygote and ookinete proteomes.

ID	Product
MAL7P1.228	Heat Shock 70 KDa Protein, (HSP70)
PFL0565w	heat shock protein DNAJ homologue Pfj4
PF14_0167	prefoldin subunit 2, putative
PFB0090c	RESA-like protein with PHIST and DnaJ domains
PFE0595w	prefoldin subunit, putative
PFL1770c	conserved Plasmodium protein, unknown function
MAL7P1.206	DNA mismatch repair protein, putative
PF08_0100	ruvB-like DNA helicase, putative
PF10_0114	DNA repair protein RAD23, putative
PFL1745c	clustered-asparagine-rich protein
PFF0865w	histone H3
PFL0145c	high mobility group protein
PFL1500w	conserved Plasmodium protein, unknown function
MAL7P1.21	origin recognition complex subunit 2, putative
PF10_0228	conserved Plasmodium protein, unknown function
PF13_0043	CCAAT-binding transcription factor, putative
PF13_0149	chromatin assembly factor 1 subunit, putative
PF13_0152	transcriptional regulatory protein sir2 homologue (telomere associated?)
PF14_0090	DNA-damage inducible protein, putative
PF14_0148	uracil-DNA glycosylase, putative
PFA0345w	centrin-1
PFB0720c	origin recognition complex subunit 5
PFC0250c	AP endonuclease (DNA-[apurinic or apyrimidinic site] lyase), putative
PFC0340w	DNA polymerase epsilon subunit B, putative
PFC1016w	conserved Plasmodium protein, unknown function
PFC1050w	SMN-like protein
PFF0275c	nucleoside diphosphate kinase, putative
PFF0750w	cyclin-dependent protein kinase, predicted
PFL1295w	proliferating cell nuclear antigen 2
PFL1655c	DNA polymerase epsilon subunit B, putative
PF11_0174	cathepsin C, homolog
PF14_0376	leucine carboxyl methyltransferase, putative
PFB0330c	serine repeat antigen 7 (SERA-7)
PFB0335c	serine repeat antigen 6 (SERA-6)
PFB0345c	serine repeat antigen 4 (SERA-4)
PF10_0230	conserved Plasmodium protein, unknown function
PFI1370c	phosphatidylserine decarboxylase
PF11_0295	farnesyl pyrophosphate synthase, putative
PF14_0060	glycerophodiester phosphodiesterase, putative
PF07_0059	4-nitrophenylphosphatase, putative
PF08_0031	oxoglutarate/malate translocator protein, putative
MAL13P1.61	Plasmodium exported protein (hyp8), unknown function
MAL7P1.171	Plasmodium exported protein, unknown function
PF08_0137	Plasmodium exported protein (PHISTc), unknown function

Table 5.A3
Proteins only detected in *P. falciparum* zygote and ookinete proteomes, continued

ID	Product
PFL0805w	MAC/Perforin, putative
PFL0050c	Plasmodium exported protein (PHISTb), unknown function
MAL7P1.170	Plasmodium exported protein, unknown function
PF14_0752	Plasmodium exported protein (PHISTa), unknown function
PFL0800c	CelTOS, putative
PFC0255c	ubiquitin conjugating enzyme E2, putative
PFC0785c	proteasome regulatory protein, putative
PF10_0330	ubiquitin conjugating enzyme, putative
PFF0305c	ubiquitin conjugating enzyme E2, putative
MAL13P1.245	conserved Plasmodium protein, unknown function
PF08_0079	translation initiation factor SUI1, putative
PF11_0437	60S ribosomal protein L28, putative
PFD1070w	eukaryotic initiation factor, putative
PF10_0149	cysteinyl-tRNA synthetase, putative
PF14_0579	60S ribosomal protein L27, putative
PF13_0126	translation initiation factor EIF-2B subunit related
PF13_0285	inositol-polyphosphate 5-phosphatase
PFL0720w	conserved Plasmodium membrane protein, unknown function
MAL8P1.108	protein phosphatase, putative
PF11_0156	Ser/Thr protein kinase
PF14_0492	protein phosphatase 2b regulatory subunit, putative
PFE1430c	cyclophilin, putative
PF10_0343	S-antigen
MAL13P1.60	erythrocyte binding antigen-140
MAL7P1.176	erythrocyte binding antigen 175
MAL7P1.208	rifin-like protein
PF10_0323	early transcribed membrane protein 10.2, etramp 10.2
PF10_0344	glutamate-rich protein
PF10_0345	merozoite surface protein 3
PF10_0346	merozoite surface protein 6
PF10_0348	erythrocyte membrane protein, putative
PF11_0344	apical membrane antigen 1, AMA1
PF13_0338	cysteine-rich surface protein
PFA0125c	erythrocyte binding antigen-181
PFB0300c	merozoite surface protein 2 precursor
PFD1120c	early transcribed membrane protein 4, ETRAMP4
PF13_0062	clathrin-adaptor medium chain, putative
MAL13P1.205	Rab11b, GTPase
PF10_0039	membrane skeletal protein IMC1-related
PF14_0127	N-myristoyltransferase
PFD0895c	Bet3 transport protein, putative
PFL2225w	myosin A tail domain interacting protein
PFL2460w	coronin

Table 5.A3
Proteins only detected in *P. falciparum* zygote and ookinete proteomes, continued

ID	Product
MAL8P1.46	outer arm dynein light chain 2, putative
PF11_0148	dynein light chain type 2, putative
PFC0185w	membrane skeletal protein IMC1-related
PFE1285w	membrane skeletal protein IMC1-related
PFE1505w	SNARE protein, putative
PFI1080w	dynein intermediate chain 2, ciliary
MAL13P1.120	splicing factor, putative
PFE0415w	transcription factor IIb, putative
PFI1695c	small nuclear ribonucleoprotein (snRNP), putative
PF13_0058	RNA binding protein, putative
PF10_0062	NOT family protein, putative
PF11_0266	small nuclear ribonucleoprotein D1, putative
PF14_0146	ribonucleoprotein, putative
PFB0865w	small nuclear ribonucleoprotein, putative
PFE0160c	Ser/Arg-rich splicing factor, putative
PFF0350w	MYND finger protein, putative
PF13_0238	kelch protein, putative
PF14_0369	copper transporter putative
PFD0275w	secy-independent transporter protein, putative
MAL7P1.204	conserved Plasmodium protein, unknown function
PF11_0057	conserved Plasmodium protein, unknown function
PFD0240c	6-cysteine protein, putative
MAL13P1.329	conserved Plasmodium membrane protein, unknown function
MAL7P1.141	conserved Plasmodium protein, unknown function
PF10_0033	conserved Plasmodium protein, unknown function
PF10_0138	conserved Plasmodium protein, unknown function
PF10_0351	probable protein, unknown function
PF11_0364	conserved Plasmodium protein, unknown function
PF14_0092	conserved Plasmodium membrane protein, unknown function
PF14_0526	conserved Plasmodium protein, unknown function
PF14_0527	conserved Plasmodium protein, unknown function
PFC0435w	conserved Plasmodium protein, unknown function
PFC0910w	conserved Plasmodium protein, unknown function
PFE0155w	conserved Plasmodium protein, unknown function
PFE1015c	conserved Plasmodium protein, unknown function
PFE1045c	conserved Plasmodium protein, unknown function
PFF0095c	conserved Plasmodium protein, unknown function
PFF0410w	conserved Plasmodium protein, unknown function
PFI0610w	conserved Plasmodium protein, unknown function
MAL13P1.151	conserved Plasmodium protein, unknown function

Table 5.A3
Proteins only detected in *P. falciparum* zygote and ookinete proteomes, continued

ID	Product
MAL13P1.177	conserved protein, unknown function
MAL13P1.222	conserved Plasmodium protein, unknown function
MAL13P1.226	conserved Plasmodium protein, unknown function
MAL13P1.229	conserved Plasmodium protein, unknown function
MAL13P1.246	conserved Plasmodium membrane protein, unknown function
MAL13P1.250	conserved Plasmodium protein, unknown function
MAL8P1.60	conserved Plasmodium protein, unknown function
PF07_0055	conserved Plasmodium protein, unknown function
PF08_0026	conserved protein, unknown function
PF10_0048	conserved Plasmodium protein, unknown function
PF10_0101	conserved Plasmodium protein, unknown function
PF10_0205	conserved Plasmodium protein, unknown function
PF11_0056	conserved Plasmodium protein, unknown function
PF11_0213	conserved Plasmodium protein, unknown function
PF11_0360	conserved Plasmodium protein, unknown function
PF11_0527	conserved Plasmodium protein, unknown function
PF13_0139	conserved Plasmodium protein, unknown function
PF13_0189	conserved Plasmodium protein, unknown function
PF13_0243	conserved Plasmodium protein, unknown function
PF13_0339	conserved Plasmodium protein, unknown function
PF14_0032	conserved Plasmodium protein, unknown function
PF14_0062	conserved Plasmodium protein, unknown function
PF14_0085	conserved Plasmodium protein, unknown function
PF14_0121	conserved Plasmodium protein, unknown function
PF14_0129	conserved Plasmodium protein, unknown function
PF14_0305	leucine-rich repeat protein 5, LRR5
PF14_0537	conserved Plasmodium protein, unknown function
PF14_0559	conserved Plasmodium protein, unknown function
PF14_0596	conserved Plasmodium membrane protein, unknown function
PFA0435w	conserved Plasmodium protein, unknown function
PFB0161c	conserved Plasmodium protein, unknown function
PFC0335c	conserved Plasmodium protein, unknown function
PFD0403w	conserved Plasmodium protein, unknown function
PFE0540w	WD-repeat protein, putative
PFE0790c	BolA-like protein, putative
PFE1105c	conserved Plasmodium protein, unknown function
PFF0190c	conserved Plasmodium protein, unknown function
PFF0580w	lsm12, putative
PFF1285w	conserved Plasmodium protein, unknown function
PFF1495w	conserved Plasmodium protein, unknown function
PFI0245c	conserved Plasmodium protein, unknown function
PFI1450c	conserved Plasmodium protein, unknown function
PFI1470c	conserved Plasmodium protein, unknown function
PFL2035c	conserved Plasmodium membrane protein, unknown function

Table 5.A4
Proteins detected in the *P. vivax* macrogamete but not in the *P. falciparum* macrogamete proteome.

ID	Product
PVX_081760	peroxiredoxin, putative
PVX_083155	thioredoxin-2 precursor, putative
PVX_113510	glutaredoxin-like protein, putative
PVX_116850	protein disulfide isomerase, putative
PVX_122195	DnaJ domain containing protein
PVX_123745	endoplasmin precursor, putative
PVX_000650	DNA polymerase alpha, putative
PVX_081240	replication factor c, putative
PVX_085940	CTP synthase, putative
PVX_085985	DNA-damage inducible protein, putative
PVX_087875	DNA mismatch repair enzyme, putative
PVX_088085	cell division cycle ATPase, putative
PVX_092085	proliferating-cell nucleolar antigen p120, putative
PVX_111045	DNA binding protein Myb2, putative
PVX_113495	polypyrimidine tract binding protein, putative
PVX_113665	histone H3, putative
PVX_114020	histone H3, putative
PVX_119520	DNA polymerase delta small subunit, putative
PVX_091405	vivapain-2
PVX_091415	vivapain-2
PVX_118465	allantoate amidinohydrolase, putative
PVX_081250	kinesin, putative
PVX_085210	dynein-associated protein, putative
PVX_110885	dynamin protein, putative
PVX_111560	dynein heavy chain, putative
PVX_122645	formin-binding protein, putative
PVX_082790	1-deoxy-D-xylulose 5-phosphate synthase, putative
PVX_083280	cytidyltransferase domain containing protein
PVX_089165	mitochondrial 2-oxoglutarate/malate carrier protein, putative
PVX_091555	haloacid dehalogenase-like hydrolase, putative
PVX_114245	hypothetical protein
PVX_084235	ubiquitin-conjugating enzyme E2 4, putative
PVX_088170	proteasome subunit alpha type 6, putative
PVX_089655	ubiquitin carboxyl-terminal hydrolase a, putative
PVX_082950	small GTPase rab11b, putative
PVX_089540	protein kinase C inhibitor, putative
PVX_091965	hypothetical protein, conserved
PVX_114695	phosphoinositide phosphatase SAC1, putative
PVX_119565	dual-specificity protein phosphatase, putative
PVX_092030	multiprotein bridging factor type 1, putative
PVX_001835	60S ribosomal protein L30, putative
PVX_002650	40S ribosomal protein S26, putative
PVX_002915	eukaryotic peptide chain release factor subunit 1, putative
PVX_080275	40S ribosomal protein S24, putative

Table 5.A4
Proteins detected in the *P. vivax* macrogamete but not in the *P. falciparum* macrogamete proteome, continued

ID	Product
PVX_082470	elongation factor Tu, mitochondrial precursor, putative
PVX_082970	60S ribosomal protein L18, putative
PVX_084205	hypothetical protein, conserved
PVX_085735	60S ribosomal protein L10, putative
PVX_087825	40S ribosomal protein S29, putative
PVX_091145	60S ribosomal protein L36, putative
PVX_091865	60S ribosomal protein L35, putative
PVX_092775	translation initiation factor eIF-1A, putative
PVX_094315	RNA binding protein, putative
PVX_096265	40S ribosomal protein S5, putative
PVX_096340	60S ribosomal protein L11, putative
PVX_099510	50S ribosomal protein L29, putative
PVX_117420	60S ribosomal protein L27, putative
PVX_119470	40S ribosomal protein S23, putative
PVX_119635	eukaryotic translation initiation factor 3 subunit 11, putative
PVX_122285	60S ribosomal protein L24, putative
PVX_113990	mitochondrial import receptor subunit tom40, putative
PVX_000010	hypothetical protein
PVX_001670	hypothetical protein
PVX_001920	hypothetical protein, conserved
PVX_002795	hypothetical protein, conserved
PVX_003545	hypothetical protein, conserved
PVX_003555	hypothetical protein, conserved
PVX_003995	hypothetical protein, conserved
PVX_079760	hypothetical protein, conserved
PVX_079830	hypothetical protein, conserved
PVX_080260	hypothetical protein, conserved
PVX_080555	hypothetical protein, conserved
PVX_081530	hypothetical protein, conserved
PVX_081830	hypothetical protein
PVX_083190	hypothetical protein, conserved
PVX_083560	hypothetical protein, conserved
PVX_084760	hypothetical protein, conserved
PVX_084950	hypothetical protein, conserved
PVX_085360	hypothetical protein, conserved
PVX_087670	hypothetical protein, conserved
PVX_087865	hypothetical protein, conserved
PVX_088165	hypothetical protein, conserved
PVX_089075	hypothetical protein, conserved
PVX_089485	hypothetical protein, conserved
PVX_089940	hypothetical protein, conserved
PVX_090960	hypothetical protein, conserved
PVX_091440	hypothetical protein, conserved

Table 5.A4
Proteins detected in the *P. vivax* macrogamete but not in the *P. falciparum* macrogamete proteome, continued

ID	Product
PVX_091450	hypothetical protein, conserved
PVX_092420	hypothetical protein, conserved
PVX_092430	hypothetical protein, conserved
PVX_094335	hypothetical protein, conserved
PVX_096260	hypothetical protein, conserved
PVX_098655	hypothetical protein, conserved
PVX_099105	hypothetical protein, conserved
PVX_099970	hypothetical protein, conserved
PVX_101345	hypothetical protein, conserved
PVX_114405	hypothetical protein, conserved
PVX_114905	hypothetical protein, conserved
PVX_115450	hypothetical protein, conserved
PVX_118385	hypothetical protein, conserved
PVX_119390	hypothetical protein, conserved
PVX_121935	hypothetical protein
PVX_122765	hypothetical protein
PVX_123105	hypothetical protein, conserved
PVX_123395	hypothetical protein, conserved
PVX_123690	hypothetical protein, conserved

Table 5.A5
Proteins detected in the *P. vivax* macrogamete proteome annotated as "hypothetical" in the *P. vivax* genome and found to have characterized orthologs in *P. falciparum*.

P. vivax gene ID	P. falciparum gene ID	Product
PVX_083215	MAL13P1.233	nucleic acid binding protein, putative
PVX_083105	MAL13P1.540	heat shock protein 70 (hsp70), putative
PVX_122470	MAL13P1.63	asparagine-rich protein
PVX_087715	MAL7P1.12	erythrocyte membrane-associated antigen
PVX_081792	MAL7P1.162	dynein heavy chain, putative
PVX_088110	MAL7P1.38	regulator of chromosome condensation, putative
PVX_123260	MAL8P1.109	protein phosphatase, putative
PVX_089055	MAL8P1.23	ubiquitin-protein ligase 1, putative
PVX_089170	PF08_0032	DNAJ protein, putative
PVX_094505	PF10_0063	DNA/RNA-binding protein, putative
PVX_092935	PF11_0086	MIF4G domain containing protein
PVX_092415		
PVX_091040		
PVX_091440	PF11_0169	SNO glutamine amidotransferase, putative
PVX_091550	PF11_0189	insulinase, putative
PVX_091965	PF11_0281	protein phosphatase, putative
PVX_092430	PF11_0374	tudor staphylococcal nuclease
PVX_085645	PF14_0159	Root hair defective 3 GTP-binding protein (RHD3) homolog, putative
PVX_084820	PF14_0324	Hsp70/Hsp90 organizing protein, putative
PVX_117795	PF14_0510	p23 co-chaperone, putative
PVX_118650	PF14_0699	GTPase activator, putative
PVX_123260	PF14_0723	LCCL domain-containing protein CCP1
PVX_095400	PFC0730w	HVA22/TB2/DP1 family protein, putative
PVX_097945	PFE0380c	Nuclear pore associated protein (NLP4), putative
PVX_079830	PFE1415w	cell cycle regulator with zn-finger domain, putative
PVX_079860	PFE1505w	SNARE protein, putative
PVX_113275	PFF0105w	MYND finger protein, putative
PVX_114255	PFF1095w	leucyl tRNA synthase
PVX_098710	PFI0260c	dynein heavy chain, putative
PVX_098825	PFI0365w	translation initiation factor SUI1, putative
PVX_098945	PFI0490c	ran-binding protein, putative
PVX_099035	PFI0580c	Falstatin, putative
PVX_087095	PFI1565w	profilin, putative
PVX_084205	PFL0160w	signal recognition particle SRP14, putative
PVX_084310	PFL0280c	histone binding protein, putative
PVX_084340	PFL0305c	IMP-specific 5'-nucleotidase
PVX_123395	PFL0685w	Phosphatidylinositol-glycan biosynthesis class O protein, putative
PVX_123505	PFL0795c	male development gene 1
PVX_123755	PFL1080c	ATP-binding protein, putative
PVX_101400	PFL2405c	PFG377 protein

Table 5.A6
Highly expressed *Plasmodium falciparum* gametocyte transcripts with corresponding proteins detected in the *Plasmodium falciparum* gametocyte proteome.
Proteins with ≥ 10 spectra detected are denoted (*).

ID		Product Description
MAL8P1.62		conserved Plasmodium protein, unknown function
PF07_0040		lysophospholipase, putative
PF07_0072	*	calcium-dependent protein kinase 4
PF08_0059	*	protein kinase c inhibitor-like protein, putative
PF10_0164		early transcribed membrane protein 10.3, etramp10.3
PF10_0210	*	deoxyribose-phosphate aldolase, putative
PF11_0114		actin-like protein homolog, ALP1 homolog
PF11_0172		folate/biopterin transporter, putative
PF11_0188	*	heat shock protein 90, putative
PF11_0465	*	dynamin-like protein
PF13_0011	*	plasmodium falciparum gamete antigen 27/25
PF13_0070	*	branched-chain alpha keto-acid dehydrogenase, putative
PF13_0121		dihydrolipamide succinyltransferase component of 2-oxoglutarate dehydrogenase complex
PF13_0144	*	oxidoreductase, putative
PF14_0039		conserved Plasmodium protein, unknown function
PF14_0124	*	actin II
PF14_0131		conserved Plasmodium protein, unknown function
PF14_0280	*	phosphotyrosyl phosphatase activator, putative
PF14_0290	*	conserved Plasmodium protein, unknown function
PF14_0352	*	ribonucleoside-diphosphate reductase, large subunit
PF14_0421	*	apicoplast 1-acyl-sn-glycerol-3-phosphate acyltransferase, putative
PF14_0493		sortilin, putative
PF14_0576		ubiquitin carboxyl-terminal hydrolase, putative
PFA0520c	*	chromatin assembly factor 1 protein WD40 domain, putative
PFB0685c		acyl-CoA synthetase, PfACS9
PFB0795w		ATP synthase F1, alpha subunit, putative
PFC0176c		conserved Plasmodium protein, unknown function
PFC0381c		conserved Plasmodium protein, unknown function
PFC0525c	*	glycogen synthase kinase 3
PFC0915w	*	ATP-dependent RNA Helicase, putative
PFD0310w	*	sexual stage-specific protein precursor
PFD1035w		steroid dehydrogenase, putative
PFD1050w	*	alpha-tubulin ii
PFE0225w		3-methyl-2-oxobutanoate dehydrogenase (lipoamide), putative
PFE0340c		rhomboid protease ROM4
PFE0395c		6-cysteine protein, putative
PFE0675c		deoxyribodipyrimidine photolyase (photoreactivating enzyme, DNA photolyase), putative
PFF0425w	*	conserved Plasmodium protein, unknown function
PFF0895w	*	malate dehydrogenase
PFI0770c		conserved Plasmodium protein, unknown function
PFL0105w		conserved Plasmodium protein, unknown function
PFL0585w		polyubiquitin
PFL0725w	*	thioredoxin peroxidase 2
PFL0795c	*	male development gene 1
PFL0960w		D-ribulose-5-phosphate 3-epimerase, putative

Table 5.A7
Highly expressed *P. falciparum* gametocyte transcripts with corresponding proteins detected in the *P. falciparum* macrogamete proteome.
Proteins with ≥ 10 spectra detected are denoted (*).

ID		Product Description
MAL13P1.103		conserved Plasmodium protein, unknown function
MAL13P1.347		conserved Plasmodium protein, unknown function
MAL7P1.99		conserved Plasmodium protein, unknown function
MAL8P1.72	*	high mobility group protein
MAL8P1.79	*	conserved Plasmodium protein, unknown function
PF10_0061		conserved Plasmodium protein, unknown function
PF10_0066		thioredoxin, putative
PF10_0196		cytoplasmic dynein intermediate chain, putative
PF10_0303	*	25 kDa ookinete surface antigen precursor (pfs25)
PF11_0279		conserved Plasmodium protein, unknown function
PF13_0134		conserved Plasmodium protein, unknown function
PF13_0168		CPW-WPC family protein
PF13_0269	*	glycerol kinase, putative
PF13_0329		conserved Plasmodium protein, unknown function
PF14_0244	*	ABC transporter, (EPP family)
PF14_0275		conserved Plasmodium protein, unknown function
PF14_0523		protein phosphatase, putative
PF14_0636		conserved Plasmodium protein, unknown function
PF14_0672		cyclic nucleotide phosphodiesterase, putative
PFA0190c		actin-related protein, ARP1
PFA0225w		LytB protein
PFA0490w		conserved Plasmodium protein, unknown function
PFB0730w		DEAD/DEAH box helicase, putative
PFC0262c		conserved Plasmodium protein, unknown function
PFC0570c		conserved Plasmodium protein, unknown function
PFC0895w		CPW-WPC family protein
PFE0645w	*	conserved Plasmodium protein, unknown function
PFF0380w		conserved Plasmodium protein, unknown function
PFI0705w	*	conserved Plasmodium protein, unknown function
PFI1210w	*	conserved Plasmodium protein, unknown function
PFI1330c		conserved Plasmodium protein, unknown function
PFI1380c		conserved Plasmodium protein, unknown function
PFI1550c		adenylate kinase, putative
PFL0550w		HSP20-like chaperone
PFL0630w		iron-sulfur subunit of succinate dehydrogenase
PFL0655w		conserved Plasmodium membrane protein, unknown function
PFL0660w	*	dynein light chain 1, putative
PFL0915c	*	conserved Plasmodium protein, unknown function
PFL1030w		membrane skeletal protein, putative
PFL1685w		conserved Plasmodium protein, unknown function
PFL2320w		conserved Plasmodium protein, unknown function
PF14_0039	*	conserved Plasmodium protein, unknown function
PF14_0576	*	ubiquitin carboxyl-terminal hydrolase, putative
PFB0795w	*	ATP synthase F1, alpha subunit, putative
PFC0176c	*	conserved Plasmodium protein, unknown function
PFC0381c	*	conserved Plasmodium protein, unknown function
PFE0225w	*	3-methyl-2-oxobutanoate dehydrogenase (lipoamide), putative
PFL0105w	*	conserved Plasmodium protein, unknown function
PFL0960w	*	D-ribulose-5-phosphate 3-epimerase, putative

Table 5.A8
Highly expressed *Plasmodium falciparum* gametocyte transcripts with corresponding proteins detected in the *Plasmodium falciparum* zygote and ookinete proteome.
Proteins with ≥ 10 spectra detected are denoted (*).

PF08_0100	*	ruvB-like DNA helicase, putative
PF10_0039		membrane skeletal protein IMC1-related
PFF0095c		conserved Plasmodium protein, unknown function
PF10_0062		NOT family protein, putative
PF10_0101		conserved Plasmodium protein, unknown function
PF10_0195		kinesin, putative
PF10_0228		conserved Plasmodium protein, unknown function
PF10_0330		ubiquitin conjugating enzyme, putative
PFB0161c	*	conserved Plasmodium protein, unknown function
PFC0185w		membrane skeletal protein IMC1-related
PFC0250c		AP endonuclease (DNA-[apurinic or apyrimidinic site] lyase), putative
PFF0305c		ubiquitin conjugating enzyme E2, putative
PFF0750w		cyclin-dependent protein kinase, predicted
PFL0800c		CelTOS, putative
PFL1295w		conserved Plasmodium protein, unknown function
PFL1770c		conserved Plasmodium protein, unknown function
PFI1380c	*	conserved Plasmodium protein, unknown function
PFE0225w	*	3-methyl-2-oxobutanoate dehydrogenase (lipoamide), putative
PFL0105w	*	conserved Plasmodium protein, unknown function
PF08_0100	*	ruvB-like DNA helicase, putative

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