The mosquito midgut-specific stages of the malaria parasite as targets for transmission blocking interventions

Die Moskitomitteldarmstadien des Malariaparasiten als Ziele für übertragungsblockierende Eingriffe

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Affidavit

I hereby confirm that my thesis entitled **"The mosquito midgut-specific stages of the malaria parasite as targets for transmission blocking interventions"** is the result of my own work. I did not receive any or support from commercial consultants. All sources and/ or materials applied are listed and specified in the thesis.
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Hiermit erkläre ich an Eides statt, die Dissertation „**Die Moskitomitteldarmstadien des Malariaparasiten als Ziele für übertragungsblockierende Eingriffe**" eigenständig, d.h. insbesondere selbständig und ohne Hilfe eines kommerziellen Promotionsberaters, angefertigt und keine anderen als die von mir angegebenen Quellen und Hilfsmittel verwendet zu haben.
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Summary

Malaria is a vector-borne disease caused by the protozoan parasite of the genus *Plasmodium* and it is transmitted from human to human by female *Anopheles* mosquitoes during a blood meal. For malaria transmission to occur, the malaria parasite must undergo a crucial developmental sexual phase inside the mosquito midgut. In this study, we sought to investigate the interplay of the malaria parasite in the mosquito midgut with regard to the identification of novel types of transmission blocking intervention strategies. These strategies are aimed at reducing the spread of malaria by blocking the development of the mosquito midgut-specific stages of *Plasmodium*. We focused on three aspects.

The first aspect was to investigate the interplay between mosquito midgut bacteria and malaria parasites in order to determine the potential influence of malaria parasites on the composition of the mosquito gut microbiota and also determine midgut bacteria which could be exploited as vehicles for the generation of paratransgenic *Anopheles* mosquitoes. We analyzed the microbial diversity of gut bacteria of the Asian malaria vector *Anopheles stephensi* during development and under different feeding regimes, including feeds on malaria parasite-infected blood, using the human pathogenic *P. falciparum* as well as the rodent malaria model *P. berghei*. 16S rRNA and DGGE analyses demonstrated a reduction in the microbial diversity during mosquito development from egg to adult and identified the gram-negative bacterium *Elizabethkingia meningoseptica* as the dominant species in the midgut of laboratory-reared male and female mosquitoes. *E. meningoseptica* is transmitted between generations and its predominance in the mosquito midgut was not altered by diet, when the gut microbiota was compared between sugar-fed and blood-fed female mosquitoes. Furthermore, feeds on blood infected with malaria parasites did not impact the presence of *E. meningoseptica* in the gut. Interestingly, extracts from *E. meningoseptica* exhibited antibacterial, antifungal and antiplasmodial activities, which may account for its dominance in the midgut of the malaria vector. Isolates of *E. meningoseptica* were cultivable, making the bacterium a potential candidate vehicle for the generation of paratransgenic *Anopheles* mosquitoes.

The second aspect of this thesis was to determine transcriptome changes that occur during the first half hour following transmission of *P. falciparum* to the mosquito vec-

tor in order to better understand gene regulation mechanisms important for the change of hosts and determine novel proteins which could be exploited in malaria transmission blocking interventions. We initially used suppression subtractive hybridization (SSH) to compare mRNA levels of *P. falciparum* gametocytes before and 30 min following activation. We identified a total of 126 genes for which transcript expression changed during gametogenesis. Among these, 17.5% had putative functions in signaling, 14.3% were assigned to cell cycle and gene expression, 8.7% were linked to the cytoskeleton or motor complex, 7.9% were involved in proteostasis and 6.4% in metabolism, 12.7% were genes encoding for cell surface-associated proteins, 11.9% were assigned to other functions, and 20.6% represented genes of unknown function. For 40% of the identified genes there has as yet not been any protein evidence. We further selected a subset of 34 genes from all the above ontology groups and analyzed the transcript changes during gametogenesis in detail by quantitative real-time RT-PCR. Of these, 29 genes were expressed in gametocytes, and for 20 genes transcript expression in gametocytes was increased compared to asexual blood stage parasites. Transcript levels of eight genes were particularly high in activated gametocytes, pointing at functions downstream of gametocyte transmission to the mosquito which could be exploited in malaria transmission blocking strategies.

The last aspect of this thesis was to determine the transmission blocking effect of a range of antimicrobial molecules as transmission blocking agents. The molecules were either isolated from insect hemolymph or recombinantly expressed in tobacco and designed to act either directly on the mosquito midgut stages or cover receptors on mosquito tissues like the midgut epithelium which the parasite would need for transit. We were able to show an antiplasmodial and transmission blocking effect of the antimicrobial molecule harmonine, a defense compound isolated from the hemolymph of the Asian ladybug *Harmonia axyridis.* Harmonine thus represents a potential lead structure for the development of novel antimalarials.

Zusammenfassung

Die Tropenkrankheit Malaria, wird durch eine Infektion mit einzelligen Parasiten der Gattung *Plasmodium* verursacht und durch den Stich der weiblichen *Anopheles*-Mücke von Mensch zu Mensch verbreitet. Dabei kann eine erfolgreiche Übertragung des Parasiten auf den Menschen nur dann stattfinden, wenn der Parasit seine sexuelle Entwicklungsphase im Mitteldarm der Mücke erfolgreich durchläuft. Ziel dieser Arbeit war es daher, die Wechselwirkungen des Malariaparasiten im Mitteldarm der Mücke in Hinblick auf die Identifizierung möglicher neuer transmissionsblockierender Strategien zu untersuchen. Der Zweck von transmissionsblockierende Strategien ist es, der Verbreitung der Malaria durch die Mücke entgegenzuwirken, indem die Entwicklung des Parasiten in der Mücke unterbunden und dadurch der Lebenszyklus des Parasiten unterbrochen wird. Der Schwerpunkt der vorliegenden Arbeit lag auf insgesamt drei Aspekten.

Der erste Aspekt der Arbeit befasste sich mit der Wechselwirkung zwischen dem Parasiten und der mikrobiellen Darmflora der Mücke. Dabei sollte der mögliche Einfluss des Parasiten auf die Darmflora untersucht werden und weiterführend die potentielle Verwendung von Darmbakterien als Vehikel für die Herstellung paratransgener Mücken erforscht werden. Vergleichende16S-rRNA- und DGGE-Analysen an der Darmflora des asiatischen Malariavektors *Anopheles stephensi* zeigten eine deutliche Reduktion der mikrobiellen Diversität während der Entwicklung vom Ei zur adulten Mücke. Zudem konnte das gram-negative Bakterium *Elizabethkingia meningoseptica*, das sich stadien- und generationsübergreifend verbreitet, als dominante Darmspezies bei im Labor aufgezogenen weiblichen und männlichen *An. stephensi* festgestellt werden. Die Dominanz von *E. meningoseptica* wurde zudem nicht durch die Aufnahme von infiziertem Blut oder einer veränderten Nahrung beeinflusst. Für die Studien wurde sowohl der humanpathogene Parasit *P. falciparum* als auch der Nagermalariaerreger *P. berghei* verwendet. Weiterführende Versuche zeigten, dass Extrakte von *E. meningoseptica* antibakterielle, antifungale und antiplasmodiale Aktivitäten aufwiesen, die ein möglicher Grund für die Dominanz dieser Spezies im Mitteldarm des Vektors waren. Isolate von *E. meningoseptica* sind im Labor kultivierbar; dadurch stellt das Bakterium einen potentiellen Kandidaten zur Generierung von paratransgenen *Anopheles*-Mücken dar.

Ein zweites Ziel dieser Arbeit war es, mögliche Unterschiede in der Genexpression von *P. falciparum* darzustellen, die in den ersten 30 Minuten nach dessen Übertragung auf die Mücke erfolgen. Dies hatte zum einen zum Zweck, die durch den Wirtswechsel hervorgerufenen Genregulationen besser zu verstehen, und bot zum anderen die Möglichkeit, neue Proteine zu identifizieren, die als potentielle transmissionsblockierende Ziele genutzt werden können. Mittels *supression substractive hybridization (SSH)* konnten insgesamt 126 Gene identifiziert werden, deren Expression sich während der Gametogenese verändert. Die identifizierten Gene konnten einer Vielzahl von putativen Funktionen wie zum Beispiel in der Signaltransduktion (17,5%), im Zellzyklus (14,3%) oder im Zytoskelett (8,7%) zugeordnet werden. Des Weiteren wurden 7,9% der Gene eine Funktion in der Proteastase und 6,4% in metabolischen Prozessen zugeordnet. 12,7% der Gene kodierten für zelloberflächenassoziierte Proteine. 11,9% der Gene hatten anderen Funktionen, während 20% der Gene keine putative Funktion zugeordnet werden konnte. Etwa 40% der identifizierten Genprodukte waren bisher nicht in Proteomstudien nachgewiesen worden. In weiterführenden Analysen wurden 34 Gene aus jeder ontologischen Gruppe ausgewählt und deren Expressionsveränderung per quantitativer *real time* RT-PCR im Detail untersucht. Für 29 Gene konnte dabei eine Transkriptexpression in Gametozyten nachgewiesen werden. Zudem wiesen 20 Gene eine erhöhte Expression in Gametozyten im Vergleich asexuellen Stadien auf. Insgesamt zeigten 8 Gene besonders hohe Transkriptlevel in aktivierten Gametozyten, was auf eine Funktion dieser Proteine während der Übertragung des Parasiten auf die Mücke hindeutet und diese somit potentielle Angriffspunkte für transmissionsblockierende Strategien darstellen könnten.

Im letzten Teil dieser Arbeit stand die Untersuchung verschiedener antimikrobieller Substanzen in Bezug auf ihre transmissionsblockierenden Eigenschaften im Vordergrund. Die Substanzen waren entweder direkt aus der Hämolymphe verschiedener Insekten isoliert oder rekombinant in transgenem Tabak exprimiert worden. Dabei wurden die rekombinanten Peptide so ausgewählt, dass sie entweder gegen die Mitteldarmstadien des Parasiten wirken oder mückenspezifische Rezeptoren blockieren, die der Parasit für seine weitere Entwicklung benötigt. Dabei konnte gezeigt werden, dass das antimikrobielle Molekül Harmonin, ein Abwehrmolekül aus der Hämolymphe des asiatischen Marienkäfers *Harmonia axyridis*, antiplasmodiale als auch transmissionsblockierende Eigenschaften besitzt. Harmonin stellt daher eine potentielle Leitstruktur für die Entwicklung neuer Malariawirkstoffe dar.

1. Introduction

1.1 Malaria

Malaria is a vector-borne devastating disease caused by protozoan parasites of the genus *Plasmodium* and transmitted by female *Anopheles* mosquitoes. The disease in humans is caused by four main species; *Plasmodium falciparum, P. vivax, P. ovale* and *P. malariae* with *P. falciparum* being the agent responsible for malaria tropica which is considered as the most deadly form of the disease (reviewed in Greenwood et al, 2008). Human infections with *P. knowlesi,* a malaria parasite that normally infects monkeys, have also been described in virtually all southeast Asian countries and the species is now considered as the fifth human pathogen (White, 2008; Cox-Singh and Singh 2008; reviewed in Singh and Daneshvar, 2013). In endemic regions, co-infection of humans with more than one *Plasmodium* species is possible (Mehlotra *et al*., 2000).

According to the WHO World Malaria Report, malaria is responsible for approximately 216 million clinical cases and 665,000 deaths annually affecting mainly children below the age of five and pregnant woman principally in Sub-Saharan Africa (WHO Malaria Report, 2011). Estimates also show that about 3.3 billion people are at risk of malaria in 99 countries with ongoing malaria transmission (Figure 1). Specific population risk groups include young children in stable transmission areas who have not yet developed protective immunity against the most severe forms of the disease, pregnant women since the disease leads to low birth weight and high rates of miscarriages which can result to maternal death, people with HIV/AIDS and international travelers who lack immunity and are visiting malaria endemic areas.

The onset of malaria symptoms takes place 10-15 days after being bitten by an infected mosquito, resulting in fever, chills, headaches, and vomiting (Ashley et al., 2003; Sherman 2005; Acharya et al., 2007). If these symptoms are not addressed with chemotherapeutic treatment, the disease may progress to severe malaria (Ashley et al., 2003; Ndam et al., 2007), which consists of severe anaemia, acute respiratory failure, hypoglycemia, renal failure, pulmonary edema, seizures, and unarousable coma (Ndam et al., 2007).

Malaria cases continue to rise in affected areas due to weak health systems, widespread poverty (Hargreaves et al., 2000) and climate change which favors the abundance and distribution of the malaria vectors (Khasnis and Nettleman 2005).

Also, efforts to completely eradicate the disease have been hampered by the lack of an effective vaccine and the rapid emergence of drug resistant parasites and insecticide-resistant mosquitoes. The WHO recommends the use of artemisinin-based combination therapies (ACTs) as the first-line treatment for *falciparum* malaria in all endemic regions. However, there have been reports of the emergence of resistance to artemisinins (Dondorp et al., 2009; Phyo et al., 2012) thereby threatening the use of the drug. Hopes of a highly efficient malaria vaccine are also being lost as the RTS, S/AS01 vaccine which was shown in a phase III clinical trial to reduced malaria by half in children 5 to 17 months of age during a 12 months period after vaccination (Agnandji et al., 2011) now shows just an efficacy of 16% over a 4 years period (Olotu et al., 2013). There is therefore the need to develop new strategies aimed at eradicating the disease. The malaria transmission blocking strategies which are aimed at disrupting the transmission of the disease from human to human by the mosquito seems to be the best alternative since the mosquito midgut malaria parasite stages undergo severe losses in parasite number.

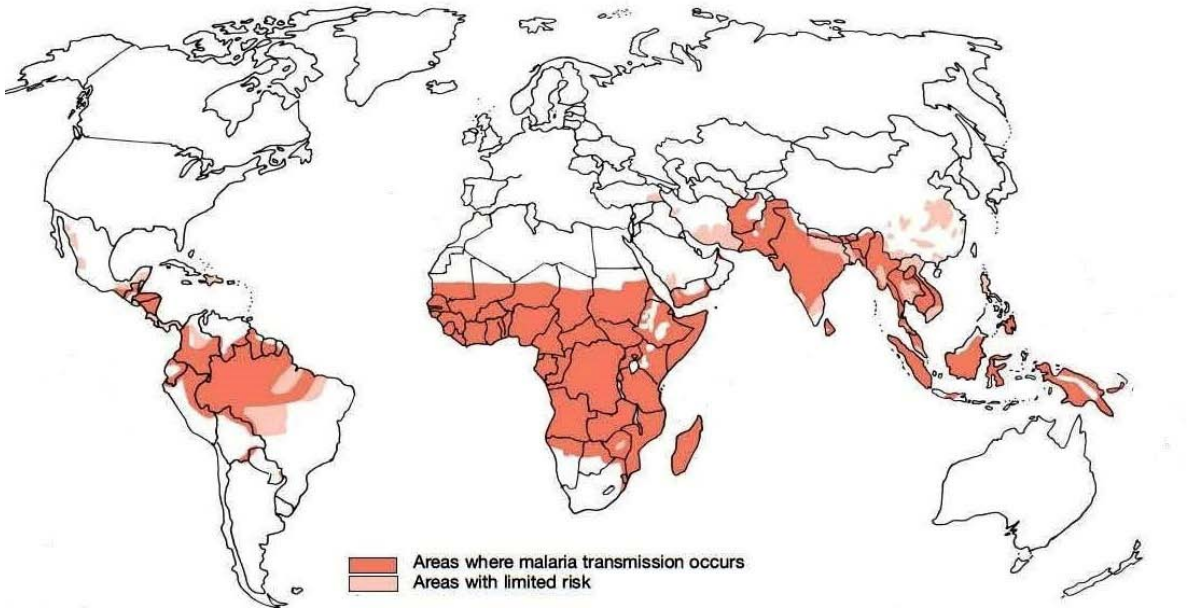

Figure 1: Countries or areas at risk of malaria transmission (Adapted from http://www.iamat.org/pdf/world_malaria_risk_chart.pdf)

1.2 The *P. falciparum* life cycle

P. falciparum exhibits a complex life cycle which alternates between the human host and the *Anopheles* mosquito (Figure 2). Infection is initiated through the bite of an infected female *Anopheles* mosquito and the release of sporoziotes from the salivary gland into the blood stream during feeding (Matuschewski 2006). The sporozoites are carried in the blood stream to the liver within half an hour where they invade hepatocytes. In the hepatocytes, the sporozoites begin to multiply asexually and within 6-15 days produce uninucleated merozoites contained inside host cell vesicles called merosomes. The merozoites are eventually released into the blood stream through budding of the merosome where they invade red blood cells. Within 48 h, the parasites begin replicating mitotically, progressing through a set of stages i.e. ring, trophozoite and schizont (Figure 3A). The mature schizonts then burst releasing merozoites, which immediately invade new uninfected red blood cells and the cycle continues leading to the clinical manifestations of the disease. Some of the schizonts are stimulated to release merozoites which develop into male and female gametocytes that mature from stage I to stage V in the blood (Figure 3B). Maturation of *P. falciparum* from stage I to stage V takes approximately 10 days. The mature gametocytes circulate in the human's blood stream, but remain dormant until they are taken up by a blood-feeding mosquito. In the midgut of the mosquito, the gametocytes become activated by the drop in temperature and the mosquito derived molecule xanthurenic acid (Billker et al., 1998; Garcia et al., 1998; reviewed in Kuehn and Pradel, 2010). Male and female gametes emerge from gametocytes and fertilization occurs to form a zygote. The zygote further develops into a motile ookinete which traverses the midgut epithelium. At the basal lamina the ookinete transforms to a sessile oocyst which matures and produce thousands of sporozoites that become released and invade the mosquito salivary gland. The life cycle is complete when the mosquito infects another human following a blood meal.

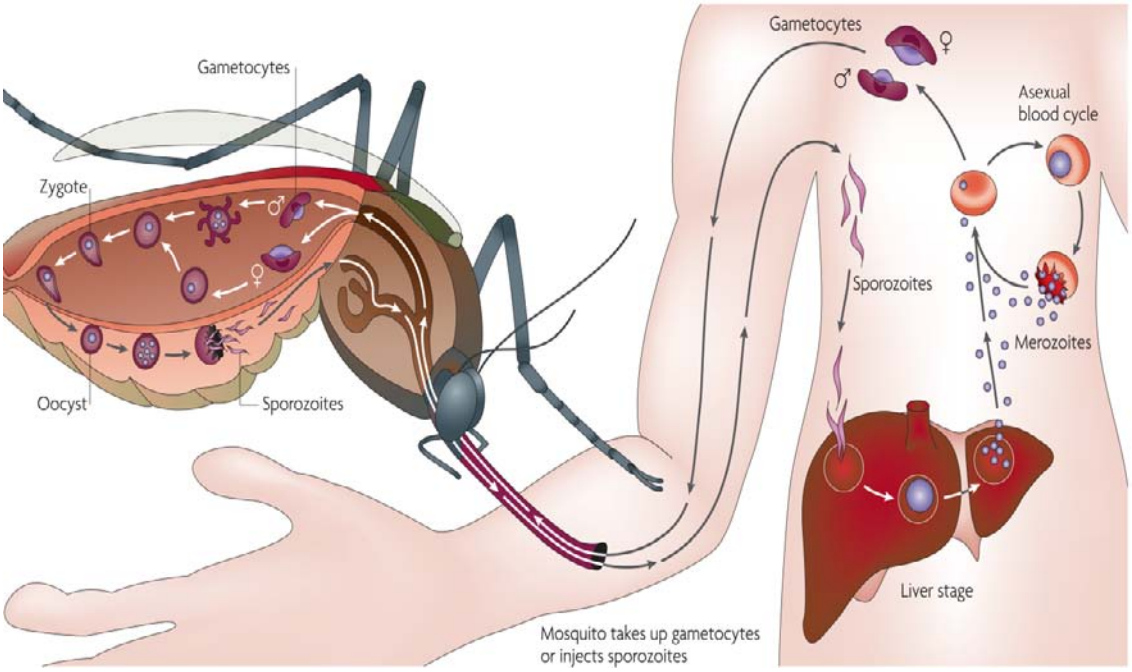

Figure 2: The malaria life cycle (Su et al., 2007).

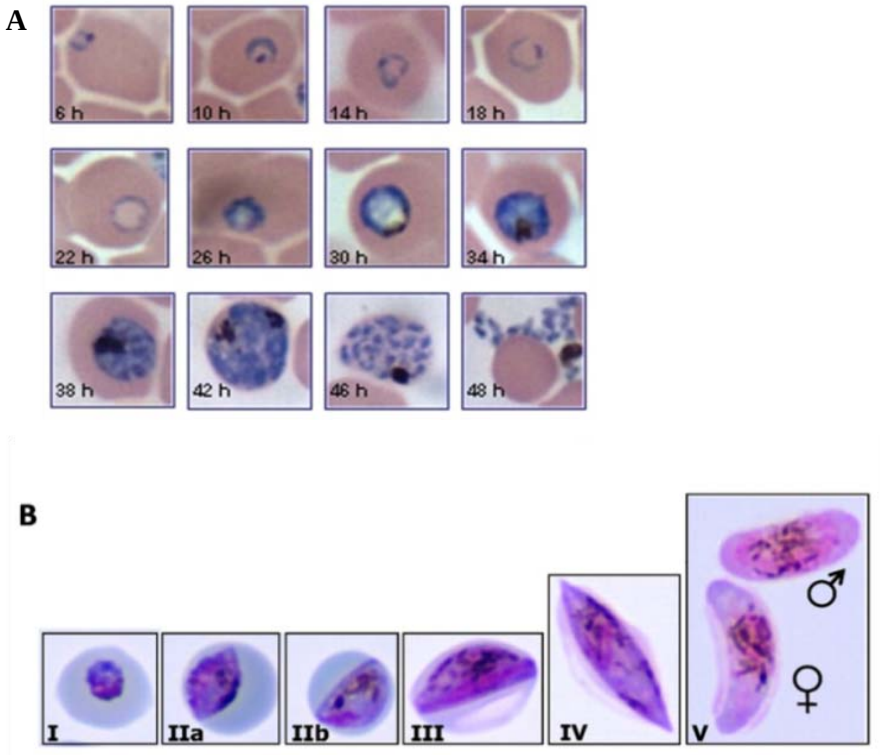

Figure 3: Blood stages of *P. falciparum*. (A) Shows the time course of development from ring stages to schizonts in vitro (Adapted from Radfar et al., 2009). The ring stage is observed between 6 and 22 h, the trophozoites between 22 and 38 h and the schizont stage is observed between 38 and 48 h and merozoites at 48 h just before invasion. (B) Shows the different gametocytes stages during development from stage I to V (modified from Sutherland 2009).

1.3 The malaria vector-*Anopheles*

Female mosquitoes of the genus *Anopheles* are vectors of the *Plasmodium* parasites that cause malaria. There are approximately 465 *Anopheles* species and about 70 of these can spread malaria parasites between humans (Sinka et al., 2012). Different *Anopheles* species transmit the disease depending on the region and the environment. *Anopheles gambiae* and *Anopheles funestus* mosquitoes are the main vectors in Africa, where 90% of malaria-related deaths occur (Lindh et al., 2005) and *Anopheles stephensi,* the main vector in Asia is responsible for the transmission of the malaria parasite *P. vivax* (Rani et al., 2009).

The *Anopheles* mosquito undergoes four main stages during its life cycle i.e. egg, larva, pupa, and adult (Figure 4). The first three stages (egg, larva and pupa) are aquatic and take about 5-14 days, depending on the species and the ambient temperature. Eggs are laid on water by females and develop into larvae within 2-3 days depending on the temperature. *Anopheles* larvae go through four stages, or instars, after which they metamorphose into pupae. At the end of each instar, the larvae molt, shedding their exoskeleton, to allow for further growth. The larvae feed on algae, bacteria, and other microorganisms in the surface microlayer (Wotton et al., 1997). The pupae then develop into adults within 2-3 days. Male *Anopheles* mosquitoes feed exclusively on sugar sources and therefore do not transmit the disease. Female mosquitoes also feed on sugar from different sources but need blood for the development of their eggs.

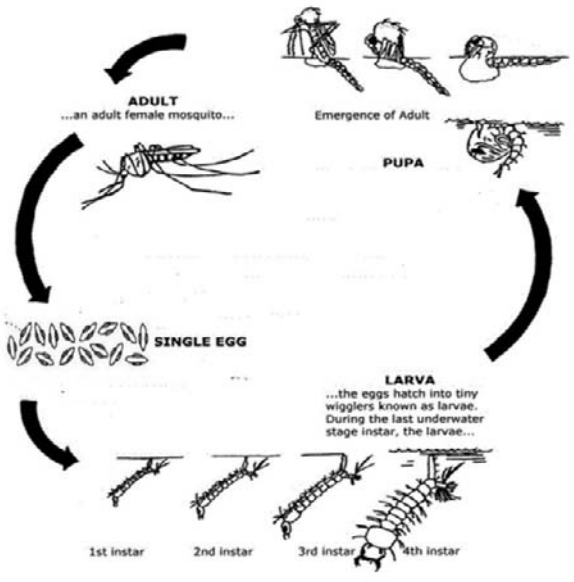

Figure 4: Life cycle of *Anopheles* species. (Modified from www.wumcd.org/mosquito/lifecycle.html)

1.4 The malaria life cycle in the mosquito midgut

For malaria transmission to occur, the malaria parasite must undergo a crucial developmental sexual phase inside the mosquito vector (Figure 5) which involves multiple developmental steps i.e. gametogenesis, fertilization, followed by zygote, ookinete and oocyst formation (reviewed in Kuehn and Pradel, 2010). This sexual phase starts when *Plasmodium* gametocytes (male and female gametocytes) are ingested by *Anopheles* mosquitoes during a blood meal. In the midgut of the mosquito, the gametocytes become activated by the drop in temperature and the mosquito derived molecule xanthurenic acid (Billker et al., 1998; Garcia et al., 1998; reviewed in Kuehn and Pradel, 2010). Gametocyte activation leads to rounding up of the cell within 5 mins, followed by parasite egress from the enveloping erythrocyte. The egress process involves the rupture of two membranes, the parasitophorous vacuole membrane (PVM) and the erythrocyte membrane (Sologub et al., 2011; reviewed in Wirth and Pradel, 2012). During gametogenesis the microgametocyte replicates its genome three times in order to produce eight motile microgametes. Following the fusion of micro- and macrogametes a zygote forms within 30 mins post blood meal and develops into an infective ookinete within the following 24 hours. The motile ookinete possesses an apical complex which enables it to traverse the midgut epithelium before settling down and forming an oocyst between epithelium and basal lamina (reviewed in Pradel, 2007).

Noteworthy, the midgut stages have to persevere outside a host cell for more than one day. During this time period, the cells are highly vulnerable to the aggressive factors of the gut, which among others include bacteria as well as human immune cells, antibodies and complement proteins present in the blood meal, and this exposure results in an approximate 1000-fold loss of parasite abundance (Vaughan et al., 1994; (reviewed in Pradel 2007; Kuehn and Pradel, 2010)). Because of these reasons, the malaria parasite midgut stages are considered bottle necks of the parasite life cycle (Figure 6) and represent prime targets for transmission blocking strategies (Abraham et al., 2004; Drexler et al., 2008).

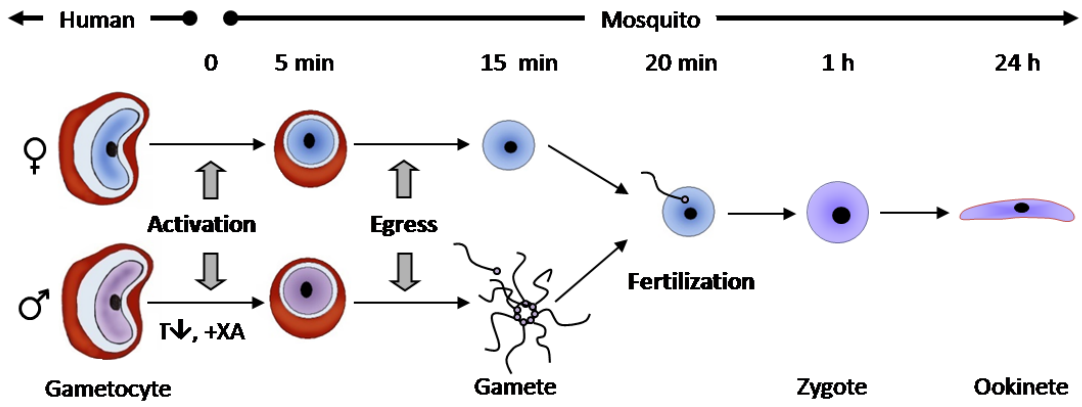

Figure 5: *P. falciparum* development in the mosquito midgut (modified from Kuehn and Pradel, 2007). Following maturation of intraerythrocytic gametocytes in the human host, they are taken up by the blood feeding female mosquito. Once they enter the midgut, they become activated and round up and emerge from the host erythrocyte. During gametogenesis, the female macrogametes transform into a macrogamete, while the activated male microgamete form eight motile microgametes within 15 min post blood meal. Within approximately 20 min post activation, the motile microgamete fertilizes the macrogamete resulting in the formation of a zygote which transforms into an infective ookinete within 24 h.

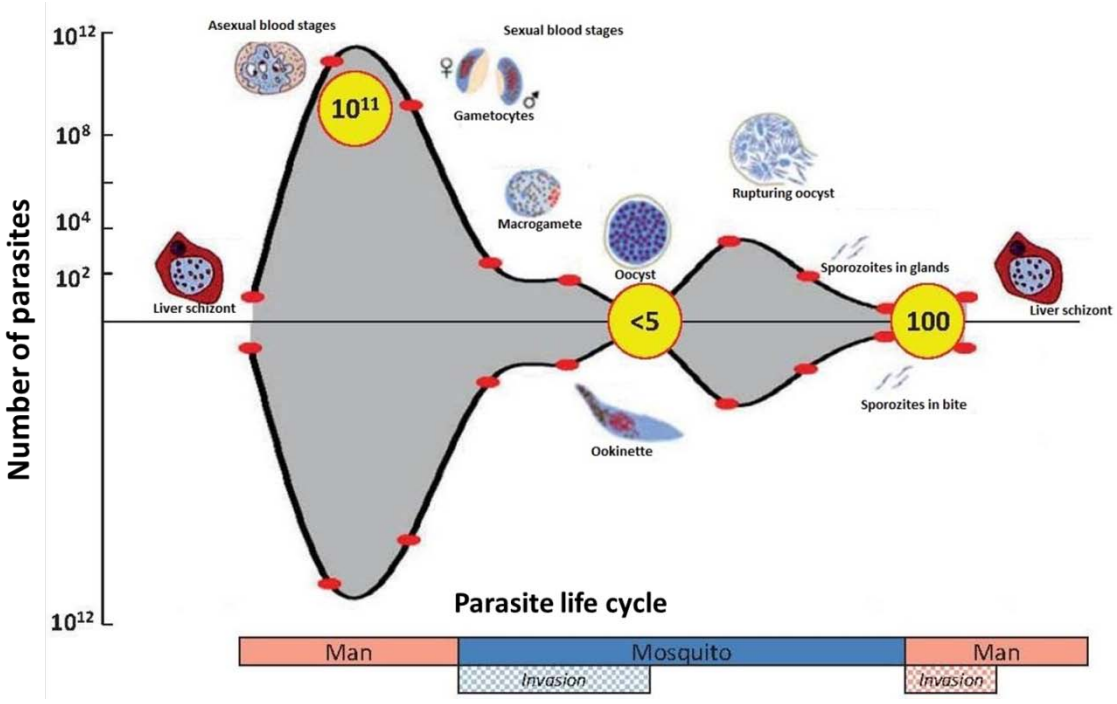

Figure 6: Bottleneck in malaria parasite life cycle (modified from http://www.malariavaccine.org/files/11122009_VectorbiologyandTBVs_ASTMH-DC_SINDEN.pdf). From the hundreds of thousands of parasites in the human blood, the number of parasites drops below five ookinetes in the mosquito midgut

1.5 *Anopheles* mosquito midgut bacteria

The midgut of *Anopheles* mosquitoes is inhabited by bacteria and several studies have investigated these midgut bacteria either from lab-reared and field collected malaria vectors (Table 1). In a recent study on *An. gambiae* in Cameroon, Boissière and colleagues reported that 90% of gut bacteria from mosquitoes collected in natural breeding sites belong to the Proteobacteria, while in lab-reared mosquitoes more than 95% of the bacteria belong to the *Elizabethkingia* sp. (Boissière et al., 2012). In a broad study whereby 454 pyrosequencing was used to investigate the bacterial diversity in the guts of eight species of mosquitoes collected from the coastal region of Kenya, four main classes of bacteria of the Gammaproteobacteria, Alphaproteobacteria, Flavobacteria and Gram-positive Bacilli classes were found to be dominant (Osei-Poku et al., 2012). Also, broader variety of midgut bacteria were identified in field-caught *An. stephensi* from Southern Iran, in which 5 genera were identified in the adult midgut, including *Pseudomonas, Alcaligenes, Bordetella, Myroides* and *Aeromonas* (Chavshin et al., 2011). Similarly, *An. stephensi* caught in the Eastern Mediterranean Region exhibited a broad variety of midgut bacteria, most of which belonged to the γ-Proteobacteria class (Dinparast et al., 2011). Rani and colleagues (Rani et al., 2009) reported the dominance of uncultured Paenibacillaceae in male *An. stephensi* caught in Haryana, India, while in field-caught females and in larvae *Serratia marcescens* dominated (Rani et al., 2009). In lab-reared mosquitoes, on the other hand, the authors identified *Serratia marcescens* and *Elizabethkingia meningoseptica* as the dominant species. Lindh and coworkers (Lindh et al., 2005) identified 16 bacterial species in the midgut of field-caught *An. gambiae* and *An. funestus* in Western Kenya, but again only two dominant bacteria species were found in lab-reared *An. gambiae*, i.e. *Pantoea stewartii* and *E. meningoseptica* (Lindh et al., 2008). The combined data show a high bacteria diversity in the gut of field-caught mosquitoes as compared to lab reared mosquitoes and the bacteria species differs independent of mosquito species and habitat indicating that microbial content of mosquito midguts is a result of the native aquatic source of the breeding grounds.

Table 1: Overview of bacteria diversity in field and lab-reared *Anopheles* mosquitoes

Mosquito species	Bacterial species	Field/lab	Reference
Anopheles gambiae	*Asaia spp., Burkholderia spp., Acinetobacter spp., Ralstonia spp., Methylobacterium spp., Sphingomonas spp., Pseudomonas spp., Stenotrophomonas spp, Stenotrophomonas spp, Streptococcus, Comamonas, Sediminibacterium spp., Escherichia-Shigella spp., Bradyrhizobium spp., Staphylococcus spp., Fusobacterium spp., Elizabethkingia spp., Gluconacetobacter spp., Neisseria spp., Schlegelella spps., Prevotella spp., Cedecea spp., Serratia spp.*	field	Boissière et al., 2012
	Acinetobacter sp., Bacillus pumilus, Bacillus sp., Enterobacter sp., Pseudomonas putida, B. cereus, Exiguobacterium mexicanum, Kocuria turfanensis, Pantoea sp., Pseudomonas rhodesiae, Staphylococcus sp.,Athrobacter sp., Comamonas sp., Knoellia sp.	field	Cirimotich et al., 2011
	Asaia sp. Burkholderia sp., Elizabethkingia sp., Bacillus spp	lab	Chouaia et al., 2010
	Enterobacter asburiae, Microbacterium sp., Sphingomonas sp., Serratia sp.,Chryseobacterium meningosepticum, Asaia bogorensis, Bacillus subtilis, Enterobacter aerogenes, Escherichia coli, Herbaspirillum sp., Pantoea agglomerans, Pseudomonas fluorescens, Pseudomonas straminea, Phytobacter diazotrophicus, Serratia marcescens	lab	Dong et al., 2009
	Sphingomonas spp., Phenilobacterium spp., Asaia spp., Burkolderia spp., Aquabacterium sp., Acinetobacter spp., Pseudomonas spp.	field	Favia et al., 2007
	Acidovorax sp., Anaplasma ovis, Anaplasma sp., Bacillus sp., Bacillales sp., Mycoplasma sp.,Paenibacillus sp., Rhodococcus cornybacteriodes,Thorsellia anopheles, Pseudomonas sp., St. maltophilia,Vibro matshnikovii, Aeromonas sp., Enterobacteriaceae sp.	field	Lindh et al., 2005
	Achromobacter xylosoiydans, Bacillus cereus, Bacillus coagulans, Bacillus mucoides, Bacillus thuringensis, Hydrogenophaga pseudoflava, Pseudomonas putida, Ps. stutzeri, Cedecea davisae, Escherichia coli,Klepsiella pneumonia, Morganella morgani, P. agglomerans, Pantoea ananas, Salmonella choleraesuis, Salmonella enteritidis.	field	Straif et al., 1998
	Flavobacterium spp., Ps. Cepacia, Ps. gladioli, Aeromonas hydrophila, Cedecea lapagei, Klyvera cryocrescens, P. agglomerans, Serratia spp.	lab	Pumpuni et al., 1996
An. stephensi	*Pseudomonas spp., Shewanella spp., Exiguobacterium spp., Klebsiella spp., Enterococcus spp., Microbacterium spp., Chryseobacterium spp., Rhodococcus spp., Kocuria spp., Aeromonas spp., Alcaligenes spp., Bordetella spp., Myroides spp., Enterobacter spp.*	field	Chavshin et al., 2012
	Pantoea agglomerans, Pantoea stewartii, Bacillus pumilus, Sphingomonas paucimobilis.,	lab	Dinparast et al., 2011
	Brevundimonas aurantiaca, Lysinibacillus sphaericus, Rahnella aquatilis	field	Dinparast et al., 2011

	Micrococcus sp., Staphylococcus hominis, S. saprophyticus, Acinetobacter, iwofii,A. radioresistens, A. johnsonii,Flexibacteriaceae, Bacillus sp., Paenibacillus alginolyticus, P. chondroitinus, Paenibacillaceae, Herbaspirillum sp., Photorhabdus luminescens, Chryseobacterium indologenes, A. hemolyticus, Citrobacter freundi, Leuconostoc citreum, Achromobacter xylosoxidans, Acinetobacter sp., Pseudomonas putida, Bacillus cereus, B. firmus, Exiguobacterium, Acinetobacter venetianus, Aeromonas sobria, Acinetobacter popoffii, Pseudomonas anquilliseptica, Calothrix sp., Brevibacterium paucivorans, Dysqonomonas sp.Staphylococcus cohnii, S. suis, Bacillus thermoamylovorans, Lactobacillus, Azoarcus sp., Leptothrix sp., Hydroxenophaga, Enterobacter aerogenes, Ignatzschineria larvae sp, *Enterobacter sp., Serratia sp.*	field	Rani et al., 2009
	Chryseobacterium meninqosepticum, Agrobacter sp., Pseudomonas mendocina, Serratia marcescens, Elizabethkingia meningoseptica, A. tumefaciens, Pseudomonas tolaasii, Klebsiella sp, Comamonas sp., uncultured bacteria clone	lab	Rani et al., 2009
	Asaia spp., Gluconobacter asaii, Acetobacter aceti, Sphingomonas rhizogenes	lab	Favia et al., 2007
	Flavobacterium spp., Ps. cepacia, A. hydophila, C.lapagei, P. agglomerans	lab	Pumpuni et al., 1996
	Staphylococcus spp., Ewingella americana, serratia marcescens,	lab	Pumpuni et al., 1993
An. arabiensis	*Acinetobacter sp., Bacillus. Pumilus, Bacillus spp., Enterobacter sp., Pseudomonas spp., B. cereus, Exiguobacterium mexicanum, Kocuria turfanensis, Pantoea sp., Staphylococcus sp., Arthrobacter sp., Comamonas sp., Enterobacter sp., Knoellia sp.*	field	Cirimotich et al., 2011
An. albimanus	*Flavobacterium spp., Acinetobacte spp., Ps. Capacia, Pantoea agglomerans, Serratia spp.*	lab	Pumpuni et al., 1996
	Enterobacter amnigenus, .E. cloacae, Enterobacter sp., S. marcescens, Serratia sp.	field	Gonzalez- Ceron et al., 2003
An. funestus	*Bacillus megaterium, Brevunzdiumonas diminuta, Comamonas testeroni, Flavobacterium resinovorum, Gluconobacter cerinus, Pseudomonas mendocina, Ps. stutzeri, St. maltophilia, C. davisa, E. coli, Erwinia chrysanthemum, K. pneumonia, Klyvera cryosceens, P. agglomerans P. ananas, S. choleraesuis.*	field	Straif et al., 1998
	Janibacter anopheles, Spiroplasma ap.	field	Lindh at al., 2005
An. sinensis	*Elizabethkingia meningoseptica, Brevibacillus centrosporus, Comomonas testosterone, Aeromonas punctata, Aeromonas hydrophila, Serratia ficaria, Pseudomonas fluorescence, Pectobacterium carotovorum, Rahnella aquatilis.*	lab	Li and Tang 2010
An. maculipennis	*Serratia spp., Asaia spp., Staphylococcus spp.*	field and lab	Favia et al., 2007
	Aeromonas bivalvium, Pseudomonas mendocina, Lysinibacillus sphaericus, Aeromonas punctata.	field	Dinparast et al., 2011

1.6 Effect of midgut bacteria on malaria infection in the mosquito

The midgut bacteria of insecst have been shown to play a vital role in preventing the development of pathogens. Several studies have reported a protective role of *Anopheles* midgut bacteria against malaria infection in the mosquito. The treatment of the mosquito midgut bacteria with antibiotics results in enhanced *Plasmodium* infection (Beier et al., 1994; Dong et al., 2009) with gram-negative bacteria highly implicated in reducing the mosquito infection with the malaria parasite (Pumpuni et al. 1993; Pumpuni et al. 1996; Gonzalez-Ceron et al. 2003; Azambuja et al., 2005). Noden et al., also showed an improved conversion of ookinetes to oocyts in *P. falciparum* using anti-bacterial antibodies generated against total midgut lysates (Noden et al., 2011). Also, the co-infection of bacteria with *Plasmodium* has been shown to reduce the number of developing oocyts in the mosquito midgut both in field and laboratory conditions (Dong et al., 2009; Gonzalez-Ceron et al. 2003; Cirimotich et al., 2011). The mechanism by which midgut bacteria inhibit *Plasmodium* infection has been studied only to a limited extend and it has been suspected that midgut bacteria play a role either directly by the production of various enzymes and toxins or physical barriers which hinder the interaction between *Plasmodium* ookinetes and midgut epithelium (Pumpuni et al. 1993; Azambuja *et al.*, 2005) or indirectly by stimulating the mosquito's innate immune system to produce antimicrobial molecules which then also act against the *Plasmodium* parasite (Dong et al., 2009). Cirimotich and colleagues reported the isolation of an *Entobacter* bacterium from wild mosquitoes in Zambia and showed that the inhibition of *Plasmodium* development by the bacterium is through the production of reactive oxygen species which target *Plasmodium* parasites in the mosquito midgut (Cirimotich et al., 2011). Other studies have implicated the activation of the mosquito's innate immune response by bacteria. Meister and others were able to show that mosquito gut bacteria drastically proliferate after a blood meal and the uptake of *Plasmodium*-infected blood triggers immune defense mechanisms in the mosquito that are directed against both the proliferated bacteria and the malaria parasites (Meister et al., 2009). Global transcription profiling of septic and aseptic mosquitoes identified a significant subset of immune genes that were mostly up-regulated by the mosquito's microbiota, and which also include several anti-*Plasmodium* factors (Dong et al., 2009). Antimicrobial factors up-regulated by bacterial infections, which have implications on malaria parasites, include members of the

fibrinogen-related protein FREP family (Dong et al., 2009) and the gram-negative bacteria-binding protein (GNBP) family (Warr et al., 2008).

1.7 Gene expression and regulation in *Plasmodium* gametocytes

The identification of genes specifically expressed in *Plasmodium* gametocytes have been facilitated by the availability of genome sequence data and the application of approaches such as proteomics, microarrays and gene knockout studies (reviewed in Moreira et al., 2004). *P. falciparum* transcriptome analyses have detected 250 to 300 genes that are specifically upregulated at the mRNA level during gametocyte development (Young et al., 2005; Sivestrini et al., 2005). The *P. falciparum* proteome on the other hand revealed more than 900 proteins in gametocytes, 315 of which are found exclusively in gametocytes (Lasonder et al., 2002). Key proteins specifically expressed in *Plasmodium* gametocytes include Pfs16, Pfg27, Pfpeg3, Pfpeg4, Pfg377, Pfs230, pfs48/45 and PfCCps (reviewed in Pradel et al., 2007).

Pfs16 a transmembrane protein expressed on the parasite parasitophorous vacuolar membrane (PVM) at the onset of gametocyte maturation has been described (Baker et al., 1994; Bruce et al., 1994; Silvestrini et al., 2005; Eksi and Williamson, 2011). Gene disruption studies of Pfs16 have shown a reduction in gametocyte production and an impaired ability of male gametocytes to exflagellate (Kongkasuriyachai et al, 2004). Pfg27 is transcribed at approximately 30 h post invasion (Carter el al., 1989; Sharma et al., 2003) and it is hypothesized to play a role in the extended period of gametocyte development in *P. falciparum* (reviewed in Baker et al., 2010). Pfg27 is also suspected to enable the formation of a multi-protein complex that mediates transduction of external signals and leads to its interaction with specific RNAs (Sharma et al., 2003).

The male development gene 1 (PfMdv-1) also known as PfPeg3 is expressed in intracellular vesicles in both male and female gametocytes but is later localized to the surface of developing ookinetes (Furuya et al., 2005; Lal et al., 2009). The disruption of the *P. falciparum* gene Pfpeg3 is linked to early arrest in male gametocytogenesis (Furuya et al., 2005).

Pfge4 renamed as *P. falciparum* gametocyte development 1 gene (Pfgdv1) has been shown by Eksi and colleagues using gametocyte-defective parasite lines and genetic complementation that the gene encoding a peri-nuclear protein, is critical for early sexual differentiation (Eksi et al., 2012). Transcriptional analysis of Pfgdv1 negative

and positive parasite lines identified a set of gametocytogenesis early genes (Pfge) that were significantly down-regulated in the absence of Pfgdv1 and expression was restored after Pfgdv1 complementation (Eksi et al., 2012).

The Pfg377 protein is localized in the osmiophilic bodies of female gametocytes (Alano et al., 1995; Severini et al., 1999) and gene-disruption of pfg377 results in the lack of osmiophilic bodies in female gametocytes and inability of the parasite to egress from host erythrocytes pointing at an important role in gametocyte emergence (de Koning-Ward et al., 2008).

Pfs230 and Pfs48/45 belong to the 6-cysteine repeat protein family (reviewed in Aly et al., 2009). Pfs230 is expressed in gametocytes and gametes plasma membrane where it forms a membrane bound complex with the GPI anchored Pfs48/45 (Kumar, 1987; Kumar and Wizel, 1992). Knockout studies of Pfs230 revealed an important unidentified function during gamete fertilization in the mosquito midgut (Eksi el al., 2006). Pfs48/45 is expressed in gametocytes and male gametes and gene deletion mutants in *P. berghei* showed an essential function during fertilization (van Dijk et al., 2001).

A highly conserved family of six secreted proteins has also been shown to be expressed in the *P. falciparum* gametocyte parasitophorous vacuolar membrane (Pradel et al., 2004). They comprise multiple adhesive domains (Lasonder et al., 2002; Pradel et al., 2004). Five of these proteins possess common Limulus coagulation factor C (LCCL) domains and are termed PfCCp1 up to PfCCp5, whereas a sixth protein, PfFNPA, lacks this domain but shares architectural features similar to the other PfCCp proteins. Gene disruption of pfCCp2 or pfCCp3 result in a complete blockage of sporozoite transition from the mosquitoes midgut to the salivary glands indicating that these proteins are essential for the parasite development in the vector (Pradel et al., 2004) while gene disruption of pfCCp4, on the other hand revealed no essential function for sporozoite transition from the midgut to the salivary glands (Scholz et al., 2008).

An important post-transcriptional mechanism to regulate gene expression in *Plasmodium* gametocytes known as translational repression has been reported (Paton et al., 1993; Vervenne et a., 1994; Abraham et al., 2004; Hall et al., 2005). In this mechanism, mRNA encoding for proteins that are required after gametocyte activation in the mosquito such as P25 and P28 are synthesized in gametocytes but translatinally repressed (Mair et al., 2006). In a study conducted by Hall and colleagues, where the *P. berghei* gametocyte transcriptome was compared with the proteomes of gametocytes

and ookinetes, 9 genes were identified for which transcripts accumulated in gametocytes but were not translated until the ookinete stage (Hall et al., 2005). In a recent study conducted by Saeed and coworkers, it was shown that in *P. berghei*, the mRNA transcript of some members of the *Plasmodium* LCCL protein family (PbLAP4, PbLAP5 and PbLAP5) accumulates in gametocytes but their proteins are not expressed indicating that they are translationally repressed (Saeed et al., 2013).

The mechanism of translational repression in *Plasmodium* had been proposed (Figure 7) (Mair et al., 2006). Using the rodent malaria parasite *P. berghei*, Khan and colleagues identified in the gametocyte specific proteome (Khan et al., 2005) an RNA helicase DOZI (development of zygote inhibited), a member of the DEAD-Box RNA helicase as part of a ribonucleoprotein complex involved in translational repression of female gametocytes (Mair et al., 2006). DOZI is highly expressed in the cytoplasmic bodies of female gametocytes where it interacts with some *Plasmodium* mRNAs. Mair and others were able to show that gene targeted disruption of DOZI resulted in a down regulation of approximately 370 transcripts targeted for degradation rather than translation and also preventing zygote development (Mair et al., 2006). In 2010, Mair and others identified an interaction partner of DOZI called CITH (homolog of worm CAR-I and fly trailer hitch) as a component of messenger ribonucleoprotein complex (mRNP) involved in the maternal mRNA translational repression machinery (Mair et al., 2010). CITH mutants revealed that the expression of 232 transcripts were significantly changed with 183 mRNAs more than 2 fold down regulated and 127 mRNAs were found to be destabilized in both data sets (Mair et al., 2010). Also gene deletion mutant parasites of the two core mRNP proteins (DOZI and CITH) result in fertilization but the zygotes fail to develop into ookinetes in a female gametocyte mutant fashion.

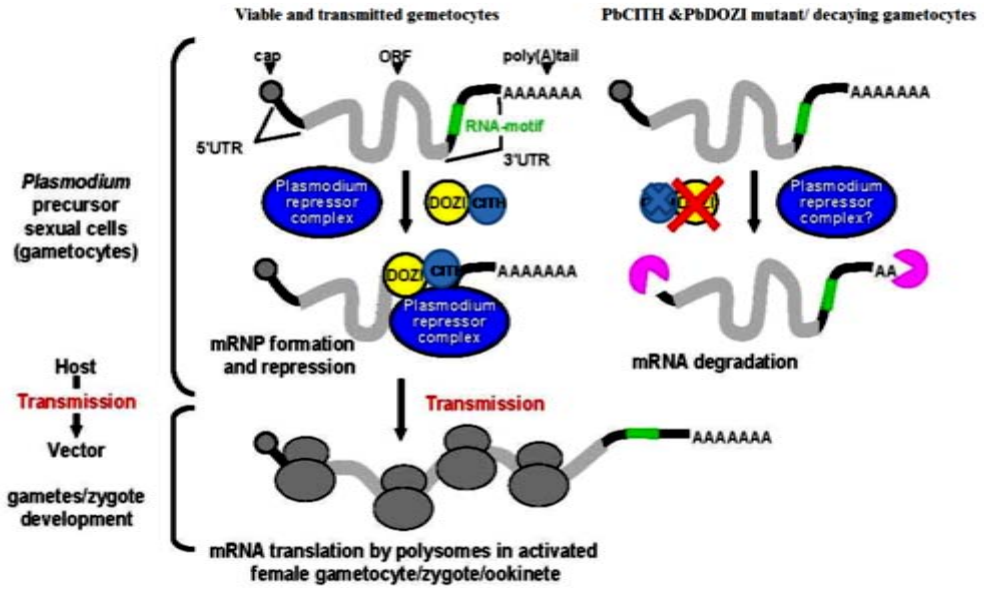

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Figure 7: Proposed mechanism of translational repression (Modified from Mair et al., 2006). In the presence of the RNA helicase DOZI and its interaction partner CITH specific transcripts are assembled into translationally quiescent mRNPs in the female gametocytes that are the precursors of the female gametes. These mRNAs are stored for later translation after gamete formation and fertilisation, which occurs in the mosquito. In the absence of DOZI and CITH the transcripts are neither stored in silent mRNPs nor transported to translating polysomes but instead are specifically degraded

1.8 Transmission blocking strategies in the mosquito

Malaria transmission blocking strategies are aimed at reducing the prevalence of malaria infection in endemic communities by targeting *Plasmodium* within the insect host. These strategies thereby reduce exposure of the human population to infectious mosquito. This section gives a brief overview of the current malaria transmission blocking strategies in the mosquito.

1.8.1 Malaria transmission blocking vaccines (TBVs)

Malaria transmission blocking vaccines (TBVs) are designed to prevent the transmission of malaria from an infected to an uninfected individual by the *Anopheles* vector. Such vaccines do not protect an individual from infection but rather reduce the transmission of the disease. TBVs target sexual phase surface antigens of the malaria parasite by relying on human antibodies to inhibit the parasites from developing in the mosquito host together with the involvement of complement or cell-mediated destruc-

tion of parasites (Figure 8A). Studies have also shown that it is also possible to raise antibodies by inoculating the host with recombinant DNA plasmids containing the gene encoding for the surface antigens which can produce antibodies with transmission blocking activities (Lobo et al., 1999; Coban et al., 2004; LeBlanc et al., 2008).

The leading malaria TBV candidates include Pfs25, Pfs28, Pfs48/45 and Pfs230 and their orthologs in *P. vivax* (reviewed in (Pradel 2007; Coutinho-Abreu and Ramalho-Ortigao, 2010; Arévalo-Herrera et al., 2011)). The TBV potential of Pfs25 has been demonstrated using different expression system. Goodman and others were able to express the antigen in recombinant chimpanzee adenovirus 63 (ChAd63), human adenovirus serotype 5 (AdHu5) and modified vaccinia virus Ankara (MVA) viral vectored vaccines and demonstrated that sera from Ad-MVA Pfs25 immunized mice inhibited the transmission of *P. falciparum* to the mosquito both ex vivo and in vivo (Goodman et al., 2011). Also, multiple versions of the Pfs25 antigens in a plant virus-based transient expression system have also been generated and sera from immunized mice showed strong transmission blocking activity in standard membrane feeding assay (Farrance et al., 2011). Antibodies against algae-produced Pfs25 have also been showed to exhibit transmission blocking activity (Gregory et al., 2012). In others studies, the TBA potential of Pfs25 was demonstrated using the vaccinia virus as delivery system of this antigen to the mammalian hosts (Kaslow et al., 1991), or using recombinant Pfs25 expressed in yeast (Barr et al., 1991; Kaslow et al., 1991). However, in phase 1 trial aimed at assessing the safety and immunogenicity of recombinant Pfs25 formulated with Montanide ISA 51, a water-in-oil emulsion, it was reported that the candidate vaccine induce transmission blocking immunity in humans but reported unexpected side effects (Wu et al., 2008).

Pfs28 is a 28 KDa protein encoded by a paralogue of the gene encoding Pfs25 and it is adjacent to Pfs25 in the genome (Duffy and Kaslow, 1997). The antigen has also been tested in TBA and it has been shown that antibodies produced by the injection of yeast Pfs28 in the presence of alum, significantly reduced the infection of *An. freeborni* mosquitoes with *P. falciparum* (Duffy and Kaslow, 1997). The authors further showed a lower infectivity when vaccination was carried out with yeast Pfs28 and Pfs25 antigens injected at the same time. Gozar and colleagues also showed that Pfs25/Pfs28 fusion proteins were significantly more potent than either Pfs25 or Pfs28 alone in eliciting antibodies in mice that blocked oocyst development in *Anopheles freeborni* mosquitoes (Gozar et al., 1998). Therefore there may be an advantage in combining

Pfs25 and Pfs28 in the same vaccine since immunization with both may be synergistic in inducing transmission blocking antibodies.

Other TBV candidates to prevent the spread of *P. falciparum* include Pfs230 and Pfs48/48 (reviewed in Pradel 2007). Pfs230 and Pfs48/45 contain 14 and 3 copies, respectively; of a motif that has a characteristic pattern of conserved cysteines (Williamson et al. 1993; Carter et al. 1995; Templeton and Kaslow, 1999; Gerloff et al. 2005) and are classified to belong to the 6-cysteine repeat protein family (reviewed in Aly et al., 2009). These proteins are expressed on the surface of gametocytes and gametes (reviewed in Williamson, 2003). A disruption of the gene encoding for Pfs230 leads to reduced fertilization and oocyst formation (Eksi et al., 2006). Also, because Pfs230 is a very large molecule, it has been difficult to determine which regions would be effective in a vaccine. However, fragments have been expressed as *E.coli* MBP-fusion proteins and shown to induce some transmission blocking activity (Williamson et al., 1995). Although antibodies against Pf230 have been shown to block the transmission of *P. falciparum* in the mosquito, the transmission blocking activity of Pfs230 monoclonal antibodies was completely lost when complement was inactivated (Quakyi et al., 1987) indicating that the activity is mainly mediated by complements. In this regard, other studies have confirmed a complement mediated transmission blocking effect of antibodies against Pfs230 (Healer et al., 1997; Tachibana et al., 2011). Farrance et al. produced a region of the Pfs230 antigen in a plant-based transient-expression system and showed that the antigen induces a strong immune response in rabbits that is able to reduce oocyst count in the presence of complements by greater than 99% as determined by standard membrane feeding assays. (Farrance et al., 2011) Pfs48/45 is mainly expressed in *P. falciparum* gametocytes and gametes and has been shown to play a central role in male gamete fertility (van Dijk et al., 2001). Immunization of mice with this recombinant protein led to production of antibody titers that were capable of significantly reducing *P. falciparum* intensity in *An. stephensi* (Outchkourov et al., 2008). In another study, a harmonized codons approach was used to successfully express recombinant full length Pfs48/45 in *E. coli* and antibodies generated showed greater than 93% transmission blocking activity in membrane feeding assays (Chowdhury et al., 2009).

1.8.2 Transgenesis

Transgenesis in *Anopheles* mosquitoes is the genetic modification of the vectors to express midgut effector genes whose products can inhibit the transmission of malaria (Figure 8B). Several studies have shown that transgenesis targeting *Plasmodium* development is feasible (reviewed in (Coutinho-Abreu et al., 2010; Wang and Jacobs-Lorena, 2013)). Genetically engineered *An. stephensi* mosquitoes expressing a 12 amino acid salivary gland and midgut peptide 1 (SM1) substantially impaired in their ability to transmit malaria (Ito et al., 2002). In another study, the genetic manipulation of *An. stephensi* to express phospholipase-2 (PLA2) led to an 87% reduction in *P. berghei* oocyst intensity as compared to non-PLA2 expressing controls (Moreira et al., 2002). C-type lectin (CELIII) was also expressed in *An. stephensi* mosquitoes under the control of a midgut-specific *An. gambiae* derived carboxypeptidase promoter and shown to be cytotoxic to *P. berghei* ookinetes (Yoshida et al., 2007). Kim and colleagues also transformed *An. gambiae* mosquitoes with cecropin A driven by a carboxylpeptidase promoter from *Aedes aegypti* and were able to show that the transformed mosquitoes were able to inhibit *P. berghei* by 61% on average as compared to controls (Kim et al., 2004). *Anopheles* mosquitoes have also been transformed to produce antimicrobial peptides (Kim et al., 2004) and single chain antibodies targeting *Plasmodium* proteins (Isaacs et al., 2011).

Despite the advances in the development of transgenic mosquitoes to limit malaria transmission, many challenges exist to the application of transgenetic mosquitoes in the field, for example, there is the lack of an effective drive mechanism to introduce the transgene into a population. An important additional challenge faced by genetic drive approaches, in general, is that anopheline vectors frequently occur in the field as reproductively isolated populations, thus posing a barrier for gene flow from one population to another (Wang et al., 2012). Also, a serious problem is the fitness of transgenic mosquitoes in natural habitats.

1.8.3 Paratransgenesis

Paratransgenesis is an alternative approach to interfere with malaria transmission based on the elimination of mosquito-specific parasite stages using genetically modified symbionts (Figure 8C). Bacteria mainly isolated from mosquito midgut have been exploited in this approach (see section 1.5) but, viruses capable of infecting mosquitoes like the densonucleosis viruses (DNVs) have also been tested (Ren et al., 2008).

For a bacterium to be used as a paratransgenic weapon, it must possess some basic requirements which include; stability and ability to compete with other bacteria in the mosquito midgut, in vitro culturability and possibility for easy genetical alteration, possession of an efficient means of introduction into the mosquito (vertical or transstadial transmission), effector gene product should not impair symbiont and vector fitness, effector gene product should be secreted to assure interaction with the malaria parasite (reviewed in Wang and Jacobs-Lorena 2013). Additional advantages would be that the bacterium is not eliminated by the diet of the mosquito (sugar, blood or plasmodia) or the bacterium is able to inhibit the malaria parasite on its own by producing inhibitory substances or activating mosquito immune response.

The paratransgenic approach was first demonstrated by Durvasula and his colleagues whereby they genetically modified a bacterium, *Rhodococcus rhodnii,* within the midgut of the kissing bug *Rhodnius prolixus* to express cecropin A and showed that it could break the transmission of trypanosomiasis (Durvasula et al., 1997). Several studies have also shown that paratransgenesis is feasible in malaria-transmitting *Anopheles* mosquitoes. *E. coli* was transformed with a plasmid expressing cecropin A fusion protein and fed to lab-reared *An. stephensi*, and was shown to inhibited the development of *P. berghei* (Yoshida et al., 2001). Furthermore, Riehle and others genetically engineered *E. coli* to display two anti-*Plasmodium* effector molecules, SM1 and phospholipase A2, on their outer membrane surfaces and showed that the bacteria significantly inhibited *P. berghei* development in *An. stephensi* mosquitoes (Riehle at al., 2007). However, their studies showed that the transgenic *E. coli* survived poorly in the mosquito midgut, excluding this bacterial species as a paratransgenic tool. Therefore there is the need to search for new bacteria to be exploited in this strategy.

The paratransgenic approach is advantageous over transgenesis in that; genetically modified bacteria can be easily produced in large quanties for testing since they can be easily grown in culture as compared to mass production of transgenic mosquitoes, bacteria can be easily freeze dried for both storage and transportation making introduction in remote areas possible as compared to mosquitoes. Lastly, the fitness of mosquitoes transformed with genetically modified bacteria in natural habitat is not affected as compared to the case with transgenic mosquitoes (reviewed in Cirimotich et al., 2011). The disadvantage of this approach is that proper characterisation of the bacterium is needed to avoid the release of a genetically harmful bacterium into the environment.

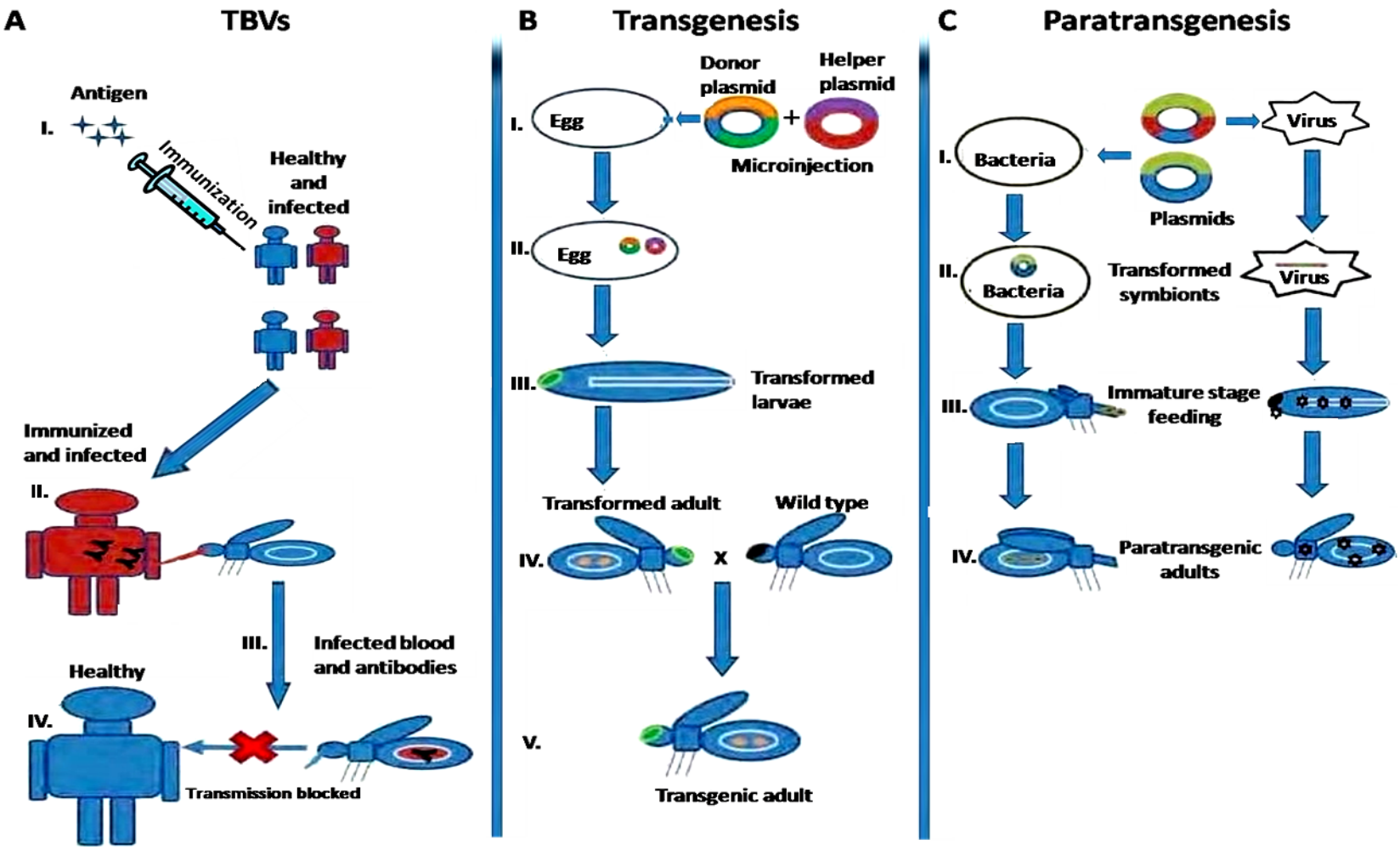


Figure 8: Schematic depiction of transmission blocking strategies (Modified from Coutinho-Abreu & Ramalho-Ortigao 2010). (A), strategy for transmission blocking vaccines (TBVs). (B), strategy for generation of transgenesis mosquitoes. (C), strategy for paratransgenesis using bacteria and viruses

1.8.4 Transmission blocking drugs (TBDs)

TBDs are drugs which can be used to kill or inhibit the sexual stages of *Plasmodium* thereby reducing the transmission of malaria from human to human by the mosquito (reviewed in (Pradel, 2007, Kiszewski, 2011)). Like TBVs, a TBD would only kill gametocytes or other mosquito stages of the malaria parasite and will confer no direct benefit to an infected person with malaria. However, the drug will simply reduce the chance of that infected person to pass on infections to mosquitoes and thus, to other people thereby reducing the transmission of the disease. This could in turn benefit individuals by reducing the chances of re-infection. These drugs are intended to be used in combination with current anti-malarial drugs so as to treat infected individuals and at the same time preventing these individuals from transmitting the disease to mosquitoes. Till date, none of the clinically used anti-malarial drugs has been shown to completely eliminate matured gametocytes. Primaquine is the only drug that has been shown to be highly effective against matured gametocytes (Grewal, 1981; Vale et al., 2009; White 2013) and a single dose has been used alongside first line therapies and shown to be effective in reducing *P. falciparum* gametocytes in blood and therefore reducing transmission of malaria (Burgess and Bray, 1961; Gunder, 1961; Shah et al., 2013; Sutano et al., 2013,). Although primaquine has been shown to be highly effective as a TBD, it is being avoided due to its adverse effect on glucose-6-phosphate dehydrogenase (G6PD) deficient individuals. Thus the drug cannot be used in areas where glucose-6-phosphate dehydrogenase (G6PD) deficiency is prevalent thereby requiring that individuals be tested before being administered the drug. Screening of individuals before drug administration will be very expensive especially as many infected people are from very poor countries.

Artemisinin combination therapies (ACTs) which is the current drug of choice for treating malaria in endemic regions contains artemisinin derivatives that have been shown to reduce gametocytes in *P. falciparum* infections (Chen et al., 1994; Price et al., 1996). However, ACTs do not result in complete clearance of gametocytes in the blood and treated individuals are still able to infect mosquitoes (Targett et al., 2001) thereby limiting their transmission blocking potentials. Other compounds which have been shown to exhibit transmission blocking activity include Tafenoquine, an 8 aminoquinoline relative of primaquine shown to inhibit sporogony of *P. vivax* (Coleman et al., 2001; Ponsa et al., 2003). Epoxomicin, a naturally derived compound from Acti-

nomycetes bacteria which acts as a protease inhibitor has also been shown to reduce *P. falciparum* gametocytes in vitro (Aminake et al., 2011) and a dose of 10 nM completely kills gametocytes after 72 h (Czesny et al., 2009). Methylene blue, the first synthetic drug ever used against malaria has also been shown to exhibit strong gametocytocidal activity against *P. falciparum* in vivo when it was administered in combination with amodiaquine and artesunate as compared to standard artesunate-amodiaquine controls (Coulibaly et al., 2009). Neem, a product derived from *Azadirachta indica,* a tree from India, exhibits significant activity against the sexual stages of *P. falciparum* in vitro (Udeinya et al., 2006; Udeinya et al., 2008).

Proteases of *Plasmodium* have also been considered as potential candidates for transmission blocking drug targets against malaria. Using *P. berghei*, it was shown that exflagellation of gametocytes can be blocked by the cysteine/serine inhibitors TPCK and TLCK as well as the metalloprotease inhibitor 1, 10-phenanthroline (Torres et al., 2005). This study was confirmed by Rupp and colleagues (Rupp et al., 2008) using *P. falciparum* therefore linking a major involvement of serine proteases and metalloproteases in the ability of microgametes to exflagellate. Also, the membrane permeable cysteine protease inhibitor E64d has been shown to block oocysts formation by 80 to 100% (Eksi et al., 2007).

Most TBDs are designed to kill gametocytes within the human blood since it is difficultto have the drugs at reasonable concentrations in human sera so as to have a transmission blocking effect in the mosquito once taken up. A novel strategy will be to modify plants in malaria endemic areas to express these TBDs (peptides) in stable form in their nectar. Since *Anopheles* female mosquitoes also take sugar meals between blood meals, they can take up the transmission blocking agent through the nectar of the transmission blocking plants. The nectar containing the transmission blocking agent stays in the mosquito midgut and is able to reduce the infection of the mosquito when it takes up blood infected with *Plasmodium.*

1.9 Overview of antimicrobial molecules tested in study

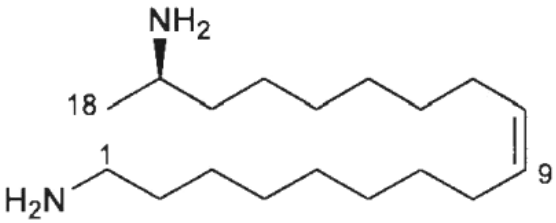
Harmonine ((17R,9Z)-1,17-diaminooctadec-9-ene) is an antimicrobial molecules isolated from the hemolymph of the Asian harlequine ladybird *Harmonia axyridis* (Röhrich et al., 2012). *Harmonia axyridis* also known as the Asian lady beetle (the harlequin ladybird) originated from Asia and the species was introduced as a biologi-

cal control agent against aphid and/or coccid pests into North America, Europe and the Soviet Union. Since its introduction, the species has become an invasive species especially in Europe outpowering and dominating the most abundant native European ladybirds, *Coccinella septempunctata* and *Adalia bipunctata* (Roy et al., 2008). Its invasive success has been attributed to its enduring resistance against diverse pathogens and its ability to exude droplets of haemolymph containing deterrent alkaloids with harmonine as principal antimicrobial compound through its leg joints when threatened or attacked.

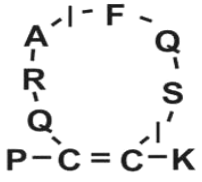
SM1 is a small synthetic dodecapeptide that is thought to inhibit the development of *Plasmodium* parasites in the mosquito midgut by binding to proteins on the lumen of the mosquito midgut required for parasite invasion (Ghosh et al., 2001). Different recombinant variants of the pure peptide expressed in tobacco were produced.The small SM1 peptide was transferred to a stabilising scafford, a so called cystine-knot mini protein (McoTi) or an agouti related protein (AGRP) which is expected to provide an ideal framework for anti-plasmodial peptides to be delivered to insect guts as the peptides gain stability for an extended period of time in the mosquito midgut.

Psalmopoeus cambrigei falciparium killers (PcFk1 and PcFK2) are peptides of 33 and 28 amino acid residues respectively which were isolated from the venom of Trinidad chevron tarantula *Psalmopoeus cambridgei* and reported to have antiplasmodial activity against the intra-erythrocytic stage of *Plasmodium falciparum in vitro* (Choi et al., 2004).

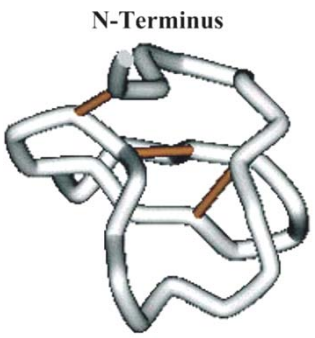
Harmonine was provided by Prof. Dr. Andreas Vilcinskas of the Fraunhofer Institute for Molecular Biology and Applied Ecology IME, Gießen, Germanywhile the recombinant peptiede were produced by Prof. Dr. Heribert Warzecha of University of Darmstadt, Germany.

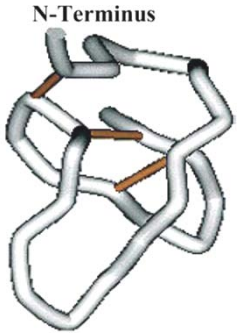


Harmonine ((17R,9Z)-1,17-diaminooctadec-9-ene)



SM1





Three-dimensional (3D) structure of PcFK1 3D-structure of PcFK2

Figure 9: Structures of antimicrobial molecules tested in study. Structure of harmonine was obtained from Röhrich et al., 2012, SM1 (Ghosh et al., 2009) and PcFK1& PcFK2 (Choi et al., 2004).

1.10 Objective of this study

Efforts to completely eradicate malaria have been hampered by the lack of an effective vaccine and the rapid emergence of drug-resistant parasites and insecticide-resistant mosquitoes. Therefore alternative strategies of combating the disease are urgently needed. For malaria transmission to occur the malaria parasite must undergo a crucial developmental sexual phase inside the mosquito midgut and because these sexual stages are highly vulnerable resulting in an approximate 1000-fold loss in parasite abundance in the moquito midgut, they represent prime targets for transmission blocking strategies. It was the main aim of this PhD thesis to investigate the interplay of malaria parasites with the content of the mosquito midgut with regard to the identification of novel types of transmission blocking interventions. Such interventions are aimed at reducing the spread of malaria by blocking the development of the mosquito midgut-specific stages of *Plasmodium*. The thesis focused on three specific objectives

Specific objective 1

The first objective of this study was to investigate the interplay between mosquito midgut bacteria and malaria parasites. Here, we aimed at investigating the bacterial diversity in the gut of lab-reared *An. stephensi* mosquitoes during development from egg to adult via 16S rRNA analysis and to determine potential changes in the gut microbiota of the female mosquito under different feeding regimes. We were particularly interested in investigating the tripartite interactions between gut bacteria, *Plasmodium* parasites and the mosquito host following a blood meal in order to determine the potential influence of malaria parasites on the composition of the mosquito gut

microbiota and also determine gut bacteria which could be exploited as vehicles for the generation of paratransgenic *Anopheles* mosquitoes.

Specific objective 2

To identify transcriptome changes in the malaria parasite during the initial stage of parasite transmission from human to mosquito. To this end, we wanted to determine the changes in the *P. falciparum* gametocyte transcriptome following activation in order to gain in-depth knowledge on the molecular switch-over that takes place in the parasite during transmission from human to mosquito. By analysing the upregulation of transcript following parasite transmission to the mosquito we hoped to identify new plasmodial proteins which could be exploited as targets for malaria transmission blocking interventions, particularly for transmission blocking vaccines or transmission blocking drugs.

Specific objective 3

The last objective was to evaluate the effect of antimicrobial molecules as transmission blocking agents preventing the spread of malaria. Here, we tested the effect of a range of antimicrobial molecules as transmission blocking agents. The molecules were either isolated from insect hemolymph or recombinantly expressed in tobacco and designed to act directly on the midgut stages of the malaria parasite or cover receptors on mosquito tissues like the midgut epithelium which the parasite would need for transit.
The general idea for the production and testing of the transmission blocking activities of these recombinant peptides was to be able to develop a modified plant which is present in malaria endemic areas that expresses a transmission blocking agent on its nectar in a stable form. Since *Anopheles* female mosquitoes also take sugar meals between blood meals, they can take up the transmission blocking agent through the nectar of the transmission blocking plants. The nectar containing the transmission blocking agent stays in the mosquito midgut and is able to reduce the infection of the mosquito when it takes up blood infected with *Plasmodium*.

2. Materials and Methods

2.1 Material

2.1.1 Labwares

Table 2: List of labwares used in this study and their suppliers

Labwares	Suppliers
96 well plates Nunc	Hartenstein, Germany
Amicon ultra 10K 15 ml	Millipore, Germany
Cell culture flasks, 25 cm^3, 75 cm^3	Schubert&Weiss
Coverslips	Hartenstein, Germany
Cryotubes 2 ml	Greiner, Flacht
Eppendolf tubes 1.5 ml, 2.0 ml	Sarstedt, Nürnbrecht
Falcon tubes 15, 50 ml	Sarstedt, Nürnbrecht
Filters 250 ml ,500 ml, 0,2 µm	Biochrom, Germany
Gloves	Laborhaus Scheller, Roth, Germany
Hybond-ECL, 20 cm x 3 m	GE Healthcare/Amersham Biosci-ence, München
Membrane feeder	Lenz Laborglas, Germany
Microscope slides	Hartenstein, Germany
Pap Pen for Immuno staining	Sigma, Germany
Pasteur pipettes glass, 230 mm	Hartenstein, Germany
Pasteur pipettes glass, 230mm, with filter	Hartenstein, Germany
PCR tubes	Sarstedt, Nürnbrecht
Petridishes	Laborhaus Scheller, Germany
Pipette tips 20 µl, 200, 1000µl	Sarstedt, Nürnbrecht
SafeSeal Tips Professional 300µL	Biozym, Germany
Serological Pipettes, 2 ml, 5 ml, 10 ml, 25ml	Sarstedt, Nürnbrecht
Sterile filters (0.2 µm pore size)	Laborhaus Scheller, Germany
Measuring cylinders	Roth, Germany

2.1.2 Instruments

Table 3: List of instruments used in this study and their suppliers

Instruments	Manufacturer
AccuJet® pro	Brand, Germany
Bunsen burner	Schütt, Germany
Centrifuge	Heraeus, Germany
Centrifuge , Biofuge fresco	Heraeus, Germany
Centrifuge Beckmann J2-HC	Beckmann, München
CFX 96 Real-Time Detection System	Bio-Rad, Germany
Dry block heating thermostat	Lab-4you, Berlin
Electrophoresis chamber	Bio-Rad, Germany
Electrophoresis chamber MIDI 1, MAXI	Roth, Germany
Electrophoresis power supply	Bio-Rad, Germany
Gel documentation system, Gel Doc 2000	Bio-Rad, Germany
Light microscope laborlux 11	Leitz, Wetzlar
Light microscope Leica DMLS	Leica, Solms
LSM Zeiss confocal laser scanning microscope	Zeiss, Germany
Rotar-mixer- picofuge	Stratagene, Germany
Mosquito incubator model 2015	VWR, West Chester, USA
pH-Meter InoLab	WTW, Weilheim
Micropipettes	Eppendorf, Hamburg
Shaker SM 30 control	Edmund Bühler GmbH, Tübingen
Spectrophotometer NanoDrop ND-1000	Peq-Lab, Erlangen
Sterile bench HERA Safe	Heraeus, Germany
TCS SP5 confocal laser scanning microscope	Leica, Germany
Vacuum Pump Laboport	KNF, Freiburg
Vortex Genie 2	Scientific industries
Water bath	Memmert, Germany
Weighing balance	Kern, Germany
Western blot apparatus Mini-Trans-Blot	Bio-Rad, München
Spectrophotometer multiskan Ascent Thermo Elect	Thermo Electron Corporation, Finland

Sonication device Sonoplus HD70	Bandelin, Germany
Transmission Electron microscope	Zeiss EM10 Zeiss,Germany
Power supply	Bio-Rad, Germany
PCR thermocycler primus 25 advanced	Peq-Lab, Germany
Chromatography column PolyPrep®	Bio-Rad, München

2.1.3 Chemicals

Chemicals used in this study were purchased from AppliChem (Darmstadt), ATCC (Manassas, USA), Dianova (Hamburg), GE Healthcare (Uppsala, Sweden), Amersham Bioscience (München), Invitrogen/Gibco/Molecular Probes (Karlsruhe), Merck/Novagen/Calbiochem (Darmstadt), Roth (Karlsruhe), Sigma/Fluka (Taufkirchen), WAK Chemie (Steinbach) and Santa Cruz Biotechnology (Heidelberg).

2.1.4 Antimicrobial molecules tested in this study

Antimicrobial molecules tested in this study i.e. harmonine and recombinantly produced peptides in tobacco (Salivary gland and midgut peptide 1 (SM1) and *Psalmopoeus cambrigei falciparium* killers) were provided by Prof. Dr. Andreas Vilcinskas of the Fraunhofer Institute for Molecular Biology and Applied Ecology IME, Gießen, Germany and Prof. Dr. Heribert Warzecha of University of Darmstadt, Germany respectively.

2.1.5 Commercially available systems and enzymes

Table 4: List of commercial kits and enzymes used in this study

Kits and enzymes	Suppliers
Fast Plasmid Mini kit	Eppendorf, Hamburg, Germany
MasterPure DNA purification kit	Epicentre Biotechnologies, Germany
Maxima SYBR green qPCR master mix	Thermo Scientific, Bonn, Germany
NucleoSpin Plasmid QuickPure	Macherey-Nagel, Düren
NucleoSpin® Extract II	Macherey-Nagel, Düren

Oligotex mRNA Mini Kit	Qiagen, Hilden
PCR-Select cDNA Subtraction Kit	Clontech, Mountain View, CA, USA
pGEM®-T Easy vector	Promega,Mannheim, Germany
QIAamp Blood Mini kit	Qiagen, Hilden
RNAse-free DNAse I	Qiagen, Hilden
SuperScript III First-Strand Synthesis	Invitrogen, Karlsruhe
Restriction endonucleases and buffers	New England Biolabs Frankfurt, Germany
PHUSION polymerase and buffer	New England Biolabs Frankfurt, Germany
GOTaq polymerase and buffer	New England Biolabs Frankfurt, Germany
T4-DNA-ligase and buffer	New England Biolabs Frankfurt, Germany

2.1.6 Antibodies and antisera

Table 5: List of antibodies and antisera used in this study

Antibodies and antisera	Antibody type	Dilution WB or IFA	Provider
Goat anti-mouse IgG Alexa 594	Secondary	1:1000 (IFA)	Molecular Probes
Goat anti-mouse IgG, Alexa 488	Secondary	1:1000 (IFA)	Molecular Probes
Goat anti-rabbit IgG Alexa 594	Secondary	1:1000 (IFA)	Molecular Probes
Goat anti-rabbit IgG, Alexa 488	Secondary	1:1000 (IFA)	Molecular Probes
Mouse anti Pfactin 1	Primary	1:50 (IFA)	AG Pradel
Mouse anti Pfactin 2	Primary	1:200 (IFA)	AG Pradel
Mouse anti- PfPK2	Primary	1:20 (IFA)	Brügl-AG Pradel
Mouse anti- PfPPLP6	Primary	1:50 (IFA)	Wirth-AG Pradel
Mouse anti- Pfs230	Primary	1:500 (IFA)	Kim Williamson, Chicago
Mouse anti-PfCLK4	Primary	1:75 (IFA)	Kern-AG Pradel
Mouse anti-Pfs16	Primary	1:500 (IFA)	Kim Williamson, Chicago
Mouse anti-Pf 14-3-3	Primary	1:100 (IFA&WB)	Produced in this study

Mouse anti-α5-SU	Primary	1:20 (IFA)	Aminake- AG Pradel
Neutral goat serum	Blocking	1% (IFA)	AG Pradel
Neutral mouse serum	Control	Same as Ab tested	AG Pradel
Rabbit anti-PfGAP50	Primary	1:50 (IFA)	Julian Rayner, England
Rabbit anti-Pfs25	Primary	1:1000 (IFA)	ATCC
Rabbit anti-MSP-1	Primary	1:1000 (IFA)	ATCC

2.1.7 Microorganisms and parasites

- ***E.coli* BL21 (DE3) RIL:** *E. coli* protein expression cell line.
- ***E.Coli* Nova Blue and *E.Coli* Top 10:** *E.coli* cell lines for cloning.
- ***E.coli* 536:** Cell lines for bioactivity testing.
- ***Staphyloccocus aureus* 8325:** Cell lines for bioactivity testing.
- ***Candida albicans* S314:** Cell lines for bioactivity testing.
- **HeLa cells:** Immortal cell line used in scientific research, it is derived from cervical cancer cells taken from Henrietta Lacks, who died from her disease in 1951. HeLa cell lines were used for cytotoxicity assays.
- ***P. falciparum* 3D7 (MRA 102):** Originally cloned from NF54 isolate (MRA-1000) by limiting dilution. Applications: Genome sequencing strain, CQ sensitive (www.mr4.org). Cell line was used for malstat assay.
- ***P. falciparum* Dd2 (MRA 150):** Derived from W2-Mef, which was selected from clone W2 for resistance to mefloquine. Applications: CQ-resistant (intermediate), pyrimethamine-resistant, mefloquineresistant. (www.mr4.org). This cell line was used for the malstat assay.
- ***P. falciparum* WT NF54 strain:** A gametocyte-producing strain isolated in 1982 from West Africa (Ponnudurai et al., 1982). This cell line was used for the production of gametocytes.
- ***P. falciparum* F12 strain:** Parasite subclones derived from gametocyte producing clone 3D7, which completely lost ability to produce gametocytes (Alano et al., 1995).
- ***P. berghei* ANKA Strain**: Two strains were used, a wild type strain and a strain constitutively expressing GFP through out the parasite life cycle under the control of the eef1a promoter (http://www.mr4.org/MR4ReagentsSearch/parasites/MRA-865.aspx).

2.1.8 Buffers, solutions and media

Table 6: List of buffers, solutions and media

Buffers, Solutions and Media	Components
10 % Triton-X100	10 ml of 100% Triton X-100, dissolve in 100 ml dH_2O, store at 4°C
10 x Giemsa buffer	0.7 g KH_2PO4 1.0 g Na_2HPO4 dissolve in 800 ml dH_2O, adjust pH to 7.2 and make up volume to 1l.
10 x Incomplete medium	10.43 g RPMI 1640 powder 5.94 g HEPES 0.05 g Hypoxanthine dissolve in 100 ml ddH_2O sterile filter using a 0.22 µm bottle top filter (Millipore)
10 x Phosphate-buffered-saline (PBS)	80 g NaCl 2 g KCl 14.4 g of Na_2HPO_4 2.4 g of KH_2PO_4 dissolve in 800 ml dH_2O, adjust pH to 7.4, make up volume to 1l and autoclave.
10 x SDS-PAGE running buffer	29 g Tris 144 g Glycerol 10 g SDS dissolve in 1l dH_2O
10 x TBS	12.1 g Tris 87.3 g NaCl dissolve in 800 ml dH_2O, adjust pH to 7.5 and make up volume to 1l.
10 x PAGE running buffer	29 g Tris 144 g Glycerin 10 g SDS Add ddH_2O to 1000 ml.
10% AlbuMax II™ stock	10 g AlbuMax II™, dissolve in 100 ml ddH_2O Sterile filter using a 0.22µm syringe filter.
10% Ammonium per sulfate (APS)	0.1g APS in 1ml dH_2O, aliquot in tubes, store -20°C
1000 x Hypoxanthin stock (0.4M)	0.5 g hypoxanthin, dissolve in 100 ml ddH_2O Sterile filter using a 0.22µm syringe filter
1mM Xanthurenic acid	0.05 g Xanthurenic acid 1 ml 0.5 M NH_4OH 243 ml of ddH_2O.
2 x Sample bufferis	2.5 ml 500 mM Tris-HCL. pH 6.8 2.0 ml Glycerin 4.0 ml 10 % SDS 0.5 ml 0.1 % Bromophenol blue Add ddH_2O to 10 ml.

40% Acrylamide :Bisacrylamide (37.5:1)	38.93g Acrylamide 1.07g Bis acrylamide 100 ml TAE Sterile filter (0.45µm) and store at 4°C till use
50 x TAE buffer	242 g Trizma Base (2M) 57.1 ml Acetic Acid (1M) 100 ml 0.5M EDTA (pH8.0) Dissolve in 800 ml dH_2O, adjust pH to 7.5 and make up volume to 1l.
A^+Medium	500 ml 1x RPMI 1640 50 ml humanes Serum A^+ 550 µl 1000x Hypoxanthin 550 µl 1000x Gentamicin
Detergent buffer for inclusion bodies	20 mM Tris/HCl pH 7.5 2 mM EDTA pH 8.0 200 mM NaCl 1 % Deoxycholate 1 % NP-40 in ddH_2O
DGGE denaturing solution 0% (10% AA)	25 ml of 40% acrylamis/bisacrylamide 2 ml of 50x TAE 73 ml of distilled H_2O
DGGE denaturing solution 100% (10% AA)	25 ml of 40% acrylamis/bisacrylamide 2 ml of 50x TAE 33 ml of distilled H_2O 40 ml of Formamide 42 g Urea Heat at 50°C to dissolve urea
Elution buffer for GST purification	50 mM Tris pH 8.0 10 mM reduced glutathione in ddH_2O
Equilibration buffer	12.1 g Tris 5.8 g Nacl 10.2 g $MgCl_2$ Add ddH_2O to 800 ml, adjust pH to 9.5 and make volume to 1000 ml
Gel green staining solution	1.17g of NaCL 60µl of gel green add 200ml of dH_2O.
GlcNac-Medium	550 ml A^+-Medium 6,08 g N-Acetyl-Glucosamine Sterile filter
Glycerolytes 57	26.66 g Sodium lactate 570 g Glycerol 300 mg KCl 517 mg Na_2PO_4,H2O (Monobasic sodium phosphate) 1242 mg (dibasic Sodium Phosphate) add ddH_2O to nearly 800 ml, adjust pH to 6.8 with HPO_4 make up volume to 1 l. Store at

	4°C
Ketamin/Xylazin (Anesthetizing solution)	Add 150 µl of 2% Xylazin (dissolved in methanol) to 1 ml 10% Ketamin (dissolved in H_2O).
LB (Luria-Bertani) medium	10 g Tryptone 5 g Yeast extract 5 g NaCl dissolve in 1l dH_2O and autoclave
LB (Luria-Bertani) agar	10 g Tryptone 5 g Yeast extract 5 g NaCl 12 g Agar dissolve in 1l dH_2O and autoclave
Lysis buffer for GST purification	50 mM Tris pH 8 350 mM NaCl 10 % Glycerin 1 m ß- Mercaptoethanol 10 mM Imidazol
Lysis buffer for inclusion bodies	50 mM Tris ph 8.0 0.25 % Sucrose (w/v). 1 mM EDTA in ddH_2O
Lysis buffer Western-Blot (NETT)	150 µl of 5M NaCl 50 µl of Triton-X 100 250 µl of 1M Tris pH 8 50 µlof 0,5M EDTA add 5 ml dH2O
Malstat reagent	1 ml 10% Triton X-100 1 g L (+) Lactate 0.33 g Tris 0.033 g 3-Acetylpyrimidin-adenine dinucleo-tide (3-APAD) dissolve in 100 ml dH_2O and adjust pH to 9
SAX (gametogenesis activation solution)	100 µM Xanthurenic acid 1.67 mg/ml Glucose 8 mg/ ml NaCl 1 mg/ml Tris pH 8.2 in ddH_2O
SDS (sodium dodecyl sulfate) solution (10% w/v)	10g SDS dissolve in 100 ml dH_2O
SOC medium	20 g Tryptone 5 g Yeast Extract 2 ml of 5 M NaCl. 2.5 ml of 1M KCl. 10 ml of 1M MgCl2 10 ml of 1M MgSO4 Dissolve in 980 ml dH_2O and autoclave then add 20 ml of 1M sterile filtered glucose.
Stop buffer	1.2 g Tris

	0.4 g EDTA Add ddH_2O to 800 ml, adjust pH to 8.0 and make volume to 1000 ml.
Transfer buffer	3.03g Tris 14.4 g Glycerin 200 ml Methanol Add ddH_2O to 1000 ml.
Washing buffer inclusion bodies	0.5 %riton X-100 1 mM EDTA pH 8.0 in ddH_2O
X-Gal stock (25mg/ml)	250 mg of X-Gal dissolved in 10 ml N,N -dimethylformamide, and protect from light with aluminium foil. Store at -20°C.

2.1.9 Plasmids

pGEM T-Easy

The pGEM®-T Easy cloning vector is a high copy number linearized vector with a T added to both 3´-ends. Selection of recombinants is by Ampicillin resistance and Blue/White Selection (Figure 10).

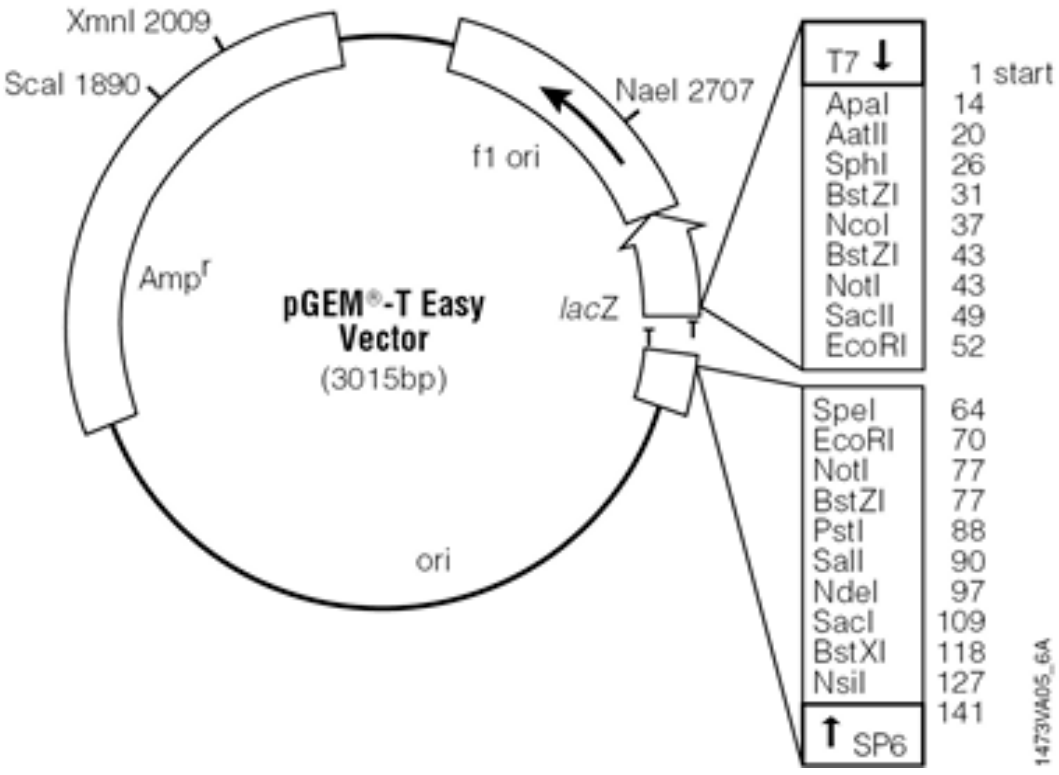


Figure 10: pGEM T-Easy vector map showing Ampicillin resistant gene and restriction sites (http://www.promega.com).

pGEX 4T-1

This is a Glutathione-S-transferase (GST) tag high copy expression vector with an Ampicillin resistant gene for selection (Figure 11).

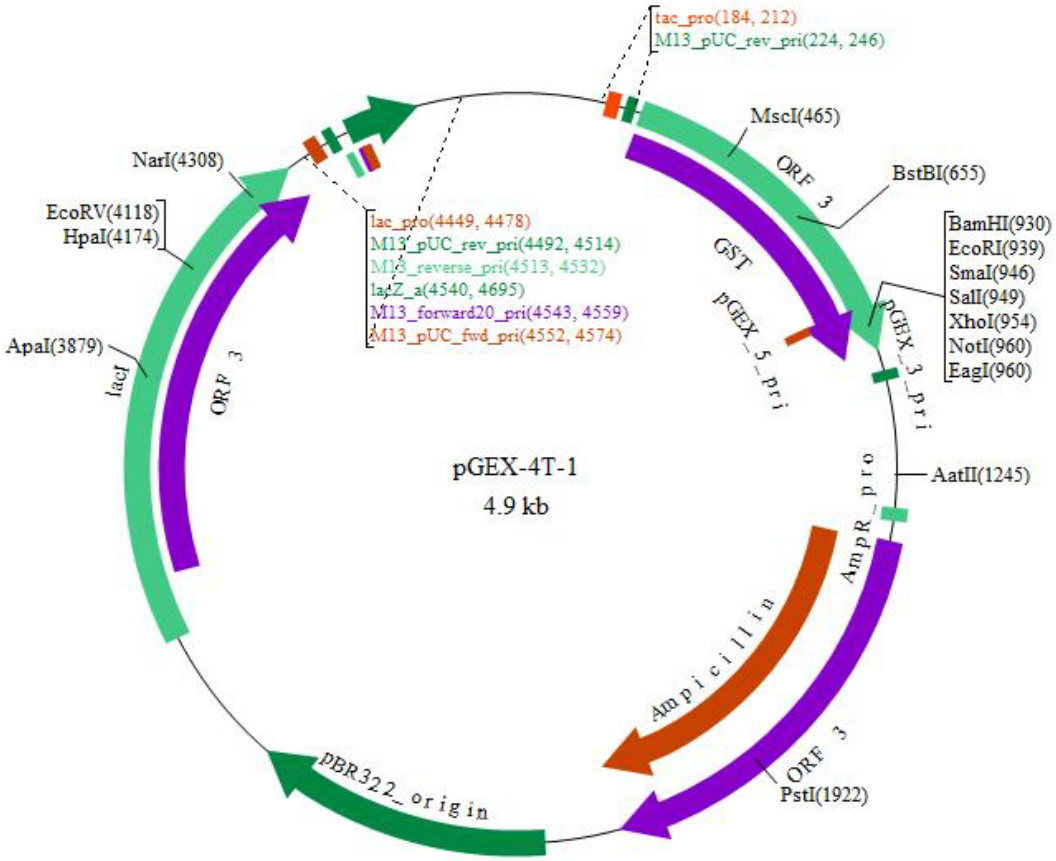


Figure 11: pGEX-4T-1 expression vector represented with restrictions sites and ampicillin resistant gene (http://www.biovisualtech.com/bvplasmid/pGEX-4T-1.htm).

2.1.10 Genes investigated and oligonucleotides

Table 7: List of genes and oligonucleotides used in this study

PlasmoDB ID	Name/feature	Sense and antisense primer (5‘---3‘)	Annealing temp. (°C)
RT-PCR primers			
PF3D7_0302100	CLK-4	GCCATACGATCCGTCTGTTT	60
		CATGCATGCAAAGGACCATA	60
PF3D7_1238900	PK2	ATCCGAATGATCCAAGCTCA	60
		TTACCTCGGAAAGGTGGGTA	60
PF3D7_0827800	SET3	TGGTGAGAAGTAGCGACGAG	60

		TTACGACACCTCTCCCTTCG	60
PF3D7_1115200	SET7	TGGGTTCCAGAACCTGATAAA	60
		ATGATGGCCGAAAGCATTAT	60
PF3D7_0815800	VPS9	TGTTACCTTGTGCTGATGAA	60
		CAGCATTCGCTACTTTTTCT	60
PF3D7_1136400	TPR	TGTTGATCAAAATATCGTTCATGT	60
		AAAAAGTTCTTGAACTAGTTGCTCTTT	60
PF3D7_0215400	WD40	GCCAAATCACCAGCAAAAAT	60
		GCTGATGGGGACATATGGTT	60
PF3D7_0818900	HSP70-1	GAATCGGTTTGTGCTCCAAT	60
		CAACTGTTGGTCCACTTCCA	60
PF3D7_0727400	Proteasome SU α5	GTGCGATGAGTGGTTTGATG	60
		AAGCAACTCCGAATGGTCTG	60
PF3D7_0807500	Proteasome SU α6	GTTTTTGTGCGGGATATCGT	60
		GCTTCAATTGCCAAAATGGT	60
PF3D7_0422300	Alpha - tubulinII	GGCCATCTCCTCAAGTATCG	60
		TCAACATTCAAAGCACCATCA	60
PF3D7_1246200	actinI	AAAGAAGCAGCAGGAATCCA	60
		TTGATGGTGCAAGGGTTGTA	60
PF3D7_1412500	actinII	GGAATGTCCGGGTTTACACA	60
		TCTTTCAGGTGGAGCAATGA	60
PF3D7_0918000	GAP50	TCTTTGGGTGATTGGGGTAA	60
		TCCATGCTGGATCATTTAAGC	60
PF3D7_1351700	ALV6	TCAAAACATCTACTCGCACCA	60
		CCCGAATGAAAATTCGTACC	60
PF3D7_1103500	CPW-WPC	ATTCAGTGAAGGCACAAATC	60
		CGTCTGGAATATCATCTGCT	60
PF3D7_0406200	Pfs16	CAAGGTGGACTATCTCAAGG	60
		TTTATCATCATCTGCGTTCTT	60
PF3D7_1218800	PSOP17	ATTACCATCGGCAAAACCAT	60
		CCAATTCCTCCAAATCCAGA	60

PF3D7_1033200	ETRAMP10.2	CTGCCCTTGTCGTTACAGGT	60
		CCGAATTTACGGTACGTGCT	60
PF3D7_1031000	Pfs25	AATGCGAAAGTTACCGTGGA	60
		CAAGCGTATGAAACGGGATT	60
PF3D7_1444800	FBPA	TGTACCACCAGCCTTACCAG	60
		TTCCTTGCCATGTGTTCAAT	60
PF3D7_1457000	SPP	CAGTATGCTTGGTTTAGGAGA	60
		TAAAGAAGAGCTGGTTGAGG	60
PF3D7_0207700	SERA4	ATACTGGTTCCCAAGGAGAT	60
		TTTCTGGTGGTGCTAATTCT	60
PF3D7_0817600	PPLP6	GGGAGTACCACCACCCTTTT	60
		GTCGTTTTATGGGCAGCACT	60
PF3D7_1239400	SP	TGTTCAAAAACAACAGTTCCAA	60
		TCCATTGGTTTGCTAGGTGA	60
PF3D7_0704100	6 TMs	TCAGACGATCACGCTATTGG	60
		TCCCGTCTGACTATTTCCTTG	60
PF3D7_1021700	4 TMs	AAACGAAAGGATGCCACAAG	60
		CGCTTGGGCTGCTACTACAT	60
PF3D7_0417400	2 TMs	TGCAAAGACGAAACATTCCA	60
		TGTCCGTATGCTTCAAGGAA	60
PF3D7_1225600	-	TGATGAAAAGTCCAAAGGGAAT	60
		TTTTCCGGTCCTCATCACTT	60
PF3D7_1321000	-	AGAAGATCATGTACATCTTCACAAA	60
		TCATCATTTGATGCAAGTACGAG	60
PF3D7_0925700	HDAC1	TGGTCTTTTTCAATTTCAGCAA	60
		GCTTCCTCAACACCATCTCC	60
PF3D7_1133400	AMA1	GGATTATGGGTCGATGGA	60
		GATCATACTAGCGTTCTT	60
PF3D7_1455800	PfCCp2	TCGGATGGAGAATCCGTT	60
		GTATCCCATGTCTTGTGA	60
PF3D7_0708000	-	GGTTGGAGAAGATACACTATGAGG	60

		CAAAGAGGATTATTCTCGTCGGT	60
PF3D7_1023000	-	TGTCTACCTTCATACTCATGTTCG	60
		AAGATGCCTCCTGCTAATAACC	60
PF3D7_1026600	-	GATGATGACGCAGGACATACTC	60
		TGGCTGGGTAATTTAATATCTCCC	60
PF3D7_1251200	Coronin	TATCCTGATCCGTCAAATAACCTG	60
		GATAACTCCGATCATTCCTCCC	60
PF3D7_1438800	-	TGCCTGTTATGGAAGATGAGC	60
		GCAAATAGGATATACAACGTTCCC	60
PF3D7_0818200	14-3-3	CCGAAAGATATGATGAAATGGCAG	60
		TGCTCATCTCTTTCTGTTCAACAC	60
PF3D7_1316700	-	GTGGACATAAAGCCCTTATTTGAC	60
		GTTTCGAGACAATCTTGGTTCC	60
PF3D7_0717700	Seryl-tRNA synthetase	AAGTAGCAGGTCATCGTGGTT	60
		TTCGGCACATTCTTCCATAA	60
Recombinant protein primers			
PF3D7_0818200	14-3-3	TAGGATCCGCAACATCTGAAGAATTAAA	45
		TAGCGGCCGCTTATTCTAATCCTTCGTCTTTTGA	45
Other primers			
T7 and SP6	pGEM-T Easy	GTAATACGACTCACTATAGGG	45
		ATTTAGGTGACACTATAG	45
27f and 1492r	16s rRNA gene	AGAGTTTGATCATGGCTCAG	54
		TACGGCTACCTTGTTACGACTT	54
341f and 907r	16s rRNA gene	CCTACGGGAGGCAGCAG	54
		CCGTCAATTCMTTTGAGTTT	54
341f +GC and 907r	16s rRNA gene	CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCA CGGGGGGCCTACGGGAGGCAGCAG	54
		CCGTCAATTCMTTTGAGTTT	54

Underlined sequences indicate restriction site

2.1.11 Miscellaneous materials

- Human serum and blood from A^+ group used for cell culture was purchased from Bayerisches Rotes Kreuz (BRK), Würzburg and the University Hospital Aachen.
- NMRI female mice and Balb/ c mice were obtained from Charles River laboratories, Sulzfeld.
- Gas bottles containing a mixture of 5% O_2, 5% CO_2 in 90% N_2 used for in vitro *Plasmodium* culture was purchased from Westfalen AG, Münster.

2.2 Methods

2.2.1 Entomology methods

2.2.1.1 Mosquito rearing and maintenance

An. stephensi colonies were maintained under standard insectary conditions at 26 ± 0.5°C, 80 ± 2% humidity and a 12/12 h light/dark cycle in gauze-covered, wire-framed cages. Eggs production was induced by allowing adult female mosquitoes to feed on blood from an anesthetized non-infected Balb/c mouse. Four days later eggs were collected on filter paper in a beaker containing 0.1% sea salt solution which was placed in the cage a day before. The eggs were carefully transferred into a clean plastic tray containing 0.1% sea salt solution for hatching and emergence of larvae over 2 days. Following emergence of larvae, they were reared at a fixed density of approximately 300 larvae per 3 litres of 0.1% sea salt solution in tray. Larvae were fed on floating dry fodder pellets (cat food) daily and any surplus “old” food was remove and discarded. The larvae develop to pupae 6 to 8 d post emergence from eggs and were collected daily, washed with distilled water and placed in small beakers containing 0.1% sea salt solution then transferred to cages for adult mosquito emergence. Adult mosquitoes were fed via a pad soaked with sterile filtered 5% saccharose solution supplemented with 0.05% para-aminobenzoic acid (PABA).

Figure 12: Mosquito rearing and maintenance. The white plastic tray (below) contain eggs which subsequently hatch to larvae then to pupae and the pupae are then collected and transferred to the wire-framed cages (above) where adult mosquitoes emerge and are maintained.

2.2.1.2 Mosquito preparation and feed for bacteria diversity determination

An. stephensi eggs were surface-sterilized by washing in 70% ethanol and rinsed twice with PBS and then homogenized in 100 µl of PBS for DNA extraction. Instar stage IV larvae and pupae were collected, surface-sterilized for 5 min in 70% ethanol, then rinsed twice in PBS and the midguts were dissected under sterile conditions (see section 2.2.1.5 for midgut dissection). Adult mosquitoes were collected and the females separated from the males. Adult male and female mosquitoes were fed on sterile sugar solution while a subset of female mosquitoes were fed on human and mouse blood in the absence and presence of *Plasmodium* gametocytes containing fresh blood. Mosquitoes were either fed on *P. berghei*-infected mice or on mature *P. falciparum* gametocyte cultures *ex vivo*, using a sterile membrane-feeding apparatus consisting of an artificial membrane stretched across the base of a water-jacketed glass cylinder at 37°C (see section 2.2.1.3, Figure 14). At 24 h post-feeding, the mosquito midguts were dissected. Twenty-five midguts of each group were pooled and homogenized in 100 µl PBS for DNA extraction. One liter each of water prior to be used for larvae-rearing and water used for rearing of larvae and pupae were collected and filtered through a 0.2 µm filter. Total DNA extracted from the filters was used for controls.

2.2.1.3 Transmission blocking assays using *P. falciparum*

This was used to test the transmission blocking effect of harmonine and it was conducted using the standard membrane feeding assay (SMFA) with mosquitoes which had been fed with 5% sterile saccharose solution supplemented with 0.05% PABA and 0.04 mg/ml gentamycin. Three to five day old mosquitoes were starved on the day before feeding. The next day female mosquitoes were attracted to a bottle containing hot water (approximately 40°C) which served as bait. The mosquitoes were collected using a gun aspirator and were then placed on ice for about 2 mins and male mosquitoes were removed. A maximum of 60 female mosquitoes were transferred into a pre-made small carton pot (Figure 13). *P. falciparum* mature gametocytes were tested for their infectivity by in vitro exflagellation of male gametocytes. Cultures containing gametocytes with greater than 5 exflagellation centres per field were used without purification while gametocytes were purified from cultures with 2-5 exflagellation centres per field using Percoll. The feeding system was then prepared by connecting glass feeders together with a tubing and to a circulating water bath to generate a continuous flow of hot water at 37°C through the jacket of the membrane feeders **(**Figure 14). A parafilm membrane was then stretched in both directions and place on the glass feeders maintained at 37°C. For the membrane feeding, the procedure was performed entirely at 37°C and as quick as possible to prevent the in vitro exflagellation of male gametocytes. The gametocytes were pelleted at 37°C by centrifugation at 1800 g for 5 min and the pellet mixed with an equal volume of freshly collected washed RBCs (A^+). Then inactivated A^+ human serum was added to the mixture in a ratio of 1:2 and the desired concentration of harmonine to be tested added, then mixed and immediately transferred to the feeders. The feeders were then placed on top of the mosquito pots for the mosquitoes to feed on the blood in the dark for 20 mins. After feeding, they were transferred to a sealed safety level three insectary where they were maintained at 26°C and fed with 5% sterile saccharose supplemented with 0.04 mg/ml gentamycin. The transmission blocking effect of the compound was determined by dissecting and counting the number of oocysts in the mosquito midgut 7-12 days after infection which represent the time in which oocysts become visible in the mosquito midgut.

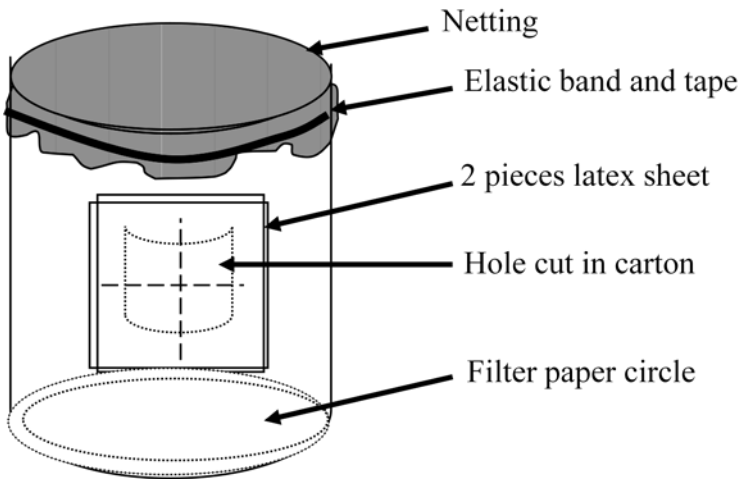


Figure 13: Preparation of pot for maintenance of infected mosquitoes (Adapted from Carter al., 2008). A small hole of approximately 2 cm square was cut in the side of a paper carton and 2 squares of latex sheeting was used to cover the hole with a slit cut into each one to allow for entry of the mosquito aspirator tube. Then a tape was used to secure over the hole. A circle filter paper was then fitted to the base of the pot and stretched netting was placed over the top of the pot and secured with an elastic band and tape.

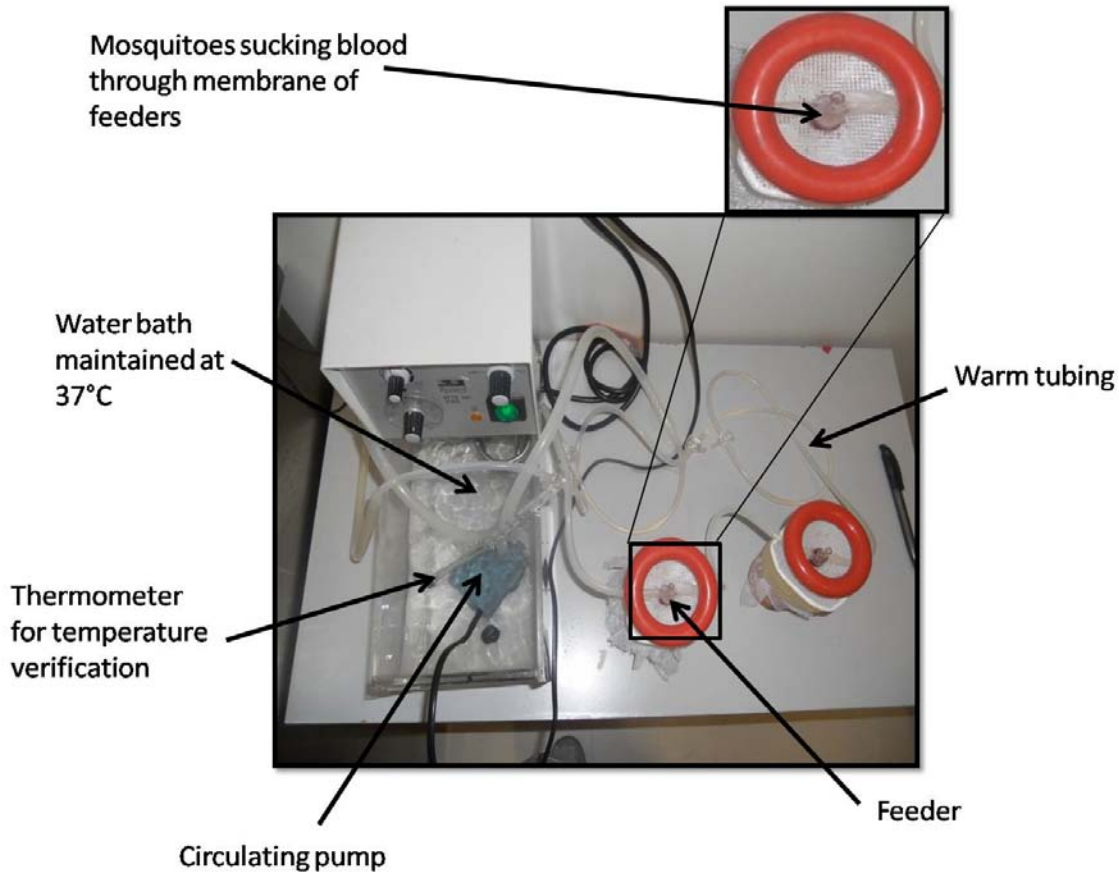


Figure 14: *P. falciparium* Standard membrane feeding assay. Adult mosquitoes were transferred into small pots and fed with blood containing mature gametocytes or blood alone through a membrane feeder.

2.2.1.4 Transmission blocking assays with *P. berghei*

P. berghei constitutively expressing GFP throughout the life cycle was used to determine the transmission blocking effect of recombinantly produced peptides tested in this study. Three to 5 d old mosquitoes were collected as described in section 2.2.1.3 and starved for one day prior to peptide feed. The mosquitoes were then fed with peptides (5 ml of 0.5 mg/ml) containing 10% saccharose and food color (to determine peptide-fed mosquitoes) using cotton pads for 2 days after which they were allowed to feed on an anesthetized *Plasmodium berghei* infected mouse (1-10% parasitaemia) for 15 min. Mouse were anesthetized by peritoneal injection of 100 µl of Ketamin/Xylazine solution (150 µl of 2% Xylazin in methanol and 1 ml 10% Ketamin in H_2O) per 10 g body weight of mouse. The mosquitoes were transferred to a safety level 3 insectary and maintained at 19-20°C with 5% sterile saccharose solution supplemented with 0.05% PABA. The transmission blocking effect of the peptides were determined by dissecting and counting the number of oocysts in the mosquito midgut 7-12 days after infection and or the number of sporozoites in the salivary glands greater than 14 days after infection.

2.2.1.5 Mosquito midgut dissection for oocyst determination

The numbers of oocysts in the mosquito midgut were counted 7-12 days post infection to determine the transmission blocking effect of substances tested in this study. To obtain the midguts, mosquitoes were removed from the pots using a gun aspirator and anesthetized by placing the gun aspirator on ice. The anesthetized mosquitoes were transferred onto a petri dish embedded on ice and examined visually for the presence of eggs in the abdomen since this is proof of a previous blood meal. Mosquitoes with eggs in the abdomen were then dissected under a binocular light microscope using a 40 x objective. Midguts were extricated by holding the anterior of the abdomen with tweezers whilst at the same time a second pair of tweezers was used to gently pull on the apex of the abdomen until the gut came out. The tweezers were used to cut through the alimentary canal at the junction of the midgut and the hindgut, simultaneously severing the malpighian tubules. The hindgut and malpighian tubules were discarded leaving only the midgut on the slide. For *P. falciparum* gametocyte-fed mosquitoes, oocysts in midgut were visualized following staining with of 0.2% mercurochrome and oocysts were counted under the light microscope at 40 x objective (Figure 15). For visualization of oocysts in *P. berghei*-fed mosquitoes, the midguts were immersed in 1

x PBS and the GFP expressing oocysts counted under a fluorescent microscope at 40 x objective.

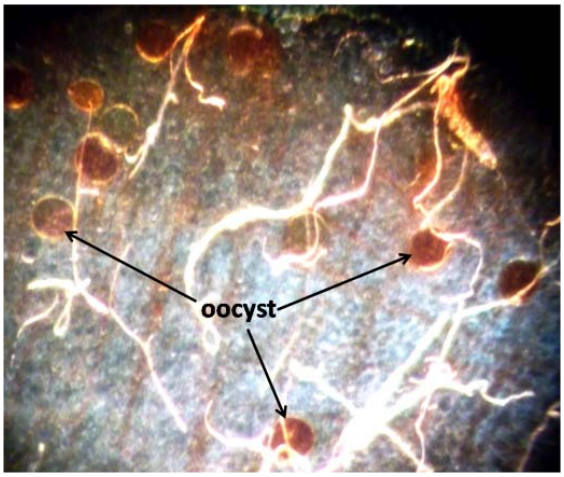


Figure 15: *P. falciparum* oocysts in mosquito midgut stained with mercurochrome.

2.2.1.6 Mosquito salivary gland dissection for sporozoites determination

In order to determine sporozoites in mosquito salivary glands, mosquitoes between 14-17d post-feeds were dissected which represent the time in which the sporozoites become visible in the salivary gland. The mosquitoes were aspirated from the pots, anesthetized as described in section 2.2.1.5, and placed on a glass slide mounted under a binocular light microscope containing a drop of 1 x PBS. A dissecting needle was placed gently on the thorax of the mosquito below the region where the salivary glands lie and a second needle on the 'neck' of the mosquito (apex of head and thorax) and the head was gently detached without cutting. Then another needle was used to gently push down on the thorax to expose the salivary glands. Intact salivary glands are comprised of a pair of three lobes each, two lateral lobes and a medial lobe. The number of lobes obtained from each dissected mosquito was counted and salivary glands obtained for each group were pooled together in one tube containing 50 µl PBS on ice. To liberate the sporozoites from the glands, the glands were macerated using an eppendorf pestle and 10 µl of the sporozoite suspension was loaded onto a Neubauer counting chamber and a phase contrast microscope was used to count the number of sporozoites present in the 4 x 4 grid under the 40 x magnification. The number of sporozoites per salivary gland lobe was determined as:

$$No\ of\ sporozoites\ per\ salivary\ gland\ lobe = \frac{No\ of\ sporozoites\ per\ \mu l\ \times \text{total volume (}50\mu l\text{)}}{Total\ number\ of\ salivary\ gland\ lobes\ obtained}$$

2.2.2 Cell biology methods

2.2.2.1 Thawing of *P. falciparum* parasites

To start a new *P. falciparum* culture, cryotubes containing frozen cultures in glycerolyte were allowed to thaw at RT. 1 ml of the culture was transferred into a 15 ml falcon tube, 200 µl of sterile 12 % NaCl was added, and the tube was swirled gently and incubated for 2 min at RT. Afterwards, 10 ml of 1.6% NaCl was added drop wise and the culture centrifuged at 1000 g for 5 min. The supernatant was discarded and 10 ml of 0.2% Glucose/0.9% NaCl solution was added drop by drop and the mixture centrifuged at 1000 g for 5 min. Then the supernatant was removed and the pellet resuspended in 5 ml of complete medium containing 500 µl of RBC and transferred to a 25 cm^3 culture flask for cultivation.

2.2.2.2 Freezing of *P. falciparum* parasites

In order to store *P. falciparum* cultures, high parasitaemia cultures with greater than 5%, mainly ring forms, were transferred into a 15 ml falcon tube and centrifuged at 1000 g for 5 min. The supernatant was removed and 5 pellet volumes of glycerolyte was added and 1 ml aliquots were transferred into 2 ml cryotubes and allowed to stand at RT for 5 min then stored at -80°C till needed.

2.2.2.3 Preparation of blood smears

To prepare blood smears, 100 µl of cultures were transferred into 1.5 ml eppendorf tubes and the tubes were centrifuged at 3000 g for 1 min. Thereafter, 85 µl of the supernatant was removed and the pellet resuspended with the remaining 15 µl of medium. Ten microlitres of the suspension was then placed on a glass slide towards the left side and the cells were smeared across the slide with the help of another slide placed at an angle of 45° to get a thin film (Figure 16). The slides were then allowed to air dry and the specimens on the dried slides fixed with methanol for 30s, allowed to dry and stained with Giemsa for 10-20 min. After staining, the slides were washed with distilled water to remove excess stain, dried and observed using 100 x objectives under oil immersion with a light microscope.

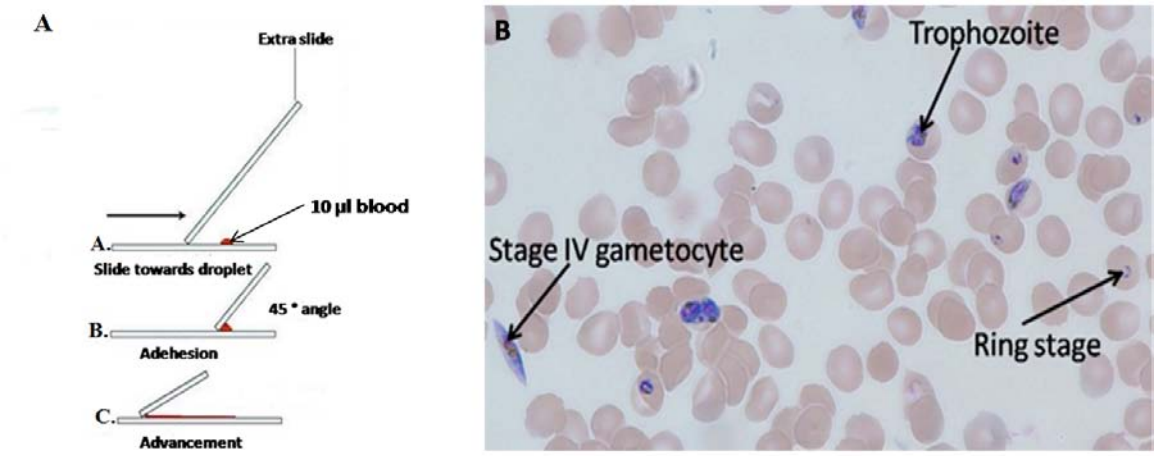


Figure 16: Parasite blood smear preparation. (A), Procedure for preparation of blood smears. (B), Giemsa stained blood smear from a *P. falciparum* mixed culture.

2.2.2.4 Estimation of parasitaemia

Percentage of infected erythrocytes (% parasitaemia) was estimated from Giemsa-stained slides (section 2.2.2.3). To this end, an area of thin blood film where the erythrocytes were uniformly distributed was observed using a 1000 fold magnification under oil immersion and the number of erythrocytes in the field was counted. Without changing the field, the number of infected erythrocytes was also counted. The same process was repeated for about 4-5 fields and the percentage parasitaemia calculated as follows:

A = mean infected erythrocytes from field counted

B = mean total erythrocytes (infected and non infected) from field counted

$$Percentage\ parasitaemia\ (\%) = \frac{A}{B} \times 100$$

2.2.2.5 Cultivation of *P. falciparum*

Chloroquine (CQ)-sensitive strain 3D7 and CQ-resistant strain Dd2 were cultivated in human erythrocytes with Albumax II medium as previously described (Trager and Jensen, 1976; Cranmer et al., 1997). RPMI medium 1640 was supplemented with hypoxanthine, 0.5% Albumax II and gentamycin. *P. falciparum* F12 asexual strain was cultivated in RPMI medium 1640 *in vitro* in the presence of 10% inactivated A^+ human serum. To generate gametocytes, *P. falciparum* isolate strain NF54 was cultivated in RPMI medium 1640 *in vitro* in the presence of 10% inactivated human serum as described (Ifediba and Vanderberg, 1981). For gametocytes used for qRT PCR the culture medium was supplemented with 50 mM N-acetyl glucosamine for approximately 5 days to kill the asexual blood stages as soon as stage I gametocytes started to

emerge in the NF54 culture. The gametocyte culture was then maintained in normal culture medium without GlcNac until immature (stage III and IV) or mature stage V gametocytes were harvested and enriched by Percoll gradient purification. All cultures were maintained at 37°C in an atmosphere of 5% O_2, 5% CO_2, 90% N_2 (Figure 17). Cultures were also synchronized by repeated sorbitol treatment (section 2.2.2.6). Continuous cultures were maintained in 25 cm^3 flasks and every 48 h, a blood smear was prepared and the parasitaemia estimated. The parasitaemia was then maintained at less than 3% to avoid stressing the parasites by removing an aliquot of the culture with high parasitaemia into a new flask and adding fresh medium to 5ml and fresh erythrocyte to final haematocrit of 5%. Gametocytes were cultured in 75 cm^3 flasks and the cultures were fed daily by removing spent medium and adding fresh medium.

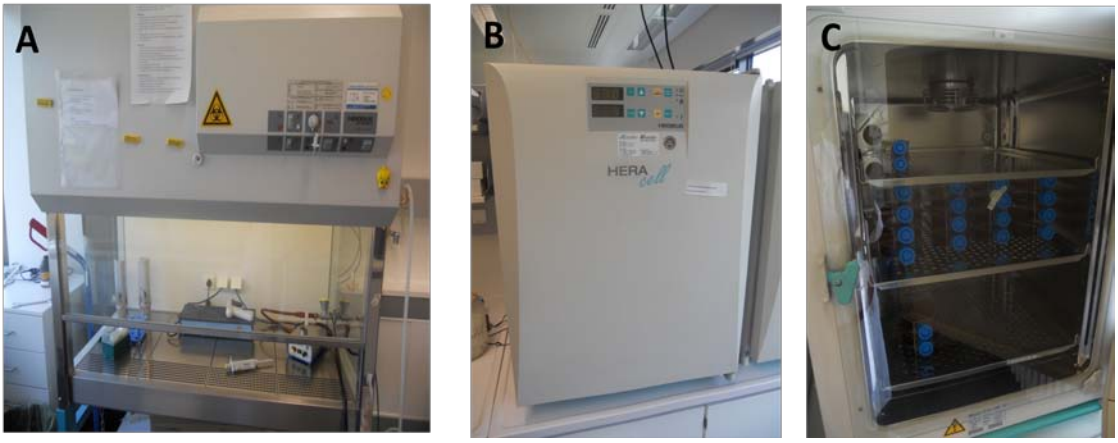


Figure 17: *P. falciparum* parasite culture. (A), clean working bench for manipulation of parasite cultures. Bench contains a hot plate which maintains the cultures at 37°C. (B), and (C), 37°C incubator for maintaining cultures in culture flasks.

2.2.2.6 Synchronization of parasite cultures

Parasite cultures which were mainly ring stages were transferred into a 15-ml falcon tube and the tube centrifuged at 1000 g for 2 min. The supernatant was removed and the pellet resuspended in 10 volumes of 5% sorbitol and kept at room temperature for 10 min. Thereafter, the supernatant was removed following centrifugation at 1000 g for 2 min and the pellet was resuspended in 10 volumes of complete medium to wash. The supernatant was again removed after centrifugation at 1000 g for 2 min and the pellet resuspended in 5 ml of warm complete medium and transferred into a 25 cm^3 flasks for in vitro cultivation.

2.2.2.7 *P. berghei* maintenance

Two *P. berghei* ANKA strains were used in this study, one wild type strain and the other constitutively expressing GFP throughout the life cycle. The parasites were maintained in Balb/c mice. For initial infection of mice, approximately 10^7 *P. berghei*-parasitized red blood cells were injected intraperitoneally to a recipient mouse. Mice positive for mature gametocytes were used to infect *An. stephensi* mosquitoes. Following 14-21 days post-infection, some mosquitoes were dissected and assessed for sporozoites. Infective mosquitoes were used to infect other mice by mosquito feeds. These mice were monitored as above and once the parasitaemia reached 10%, blood was collected via cardiac puncture and used for syringe blood-to-blood passages. Housing and handling of animals followed the guidelines of the animal welfare committee of Lower Franconia.

2.2.2.8 Counting cells using a Neubauer counting chamber

The Neubauer counting chamber was used to count HeLa cells required for seeding in the MTT assay and sporozoites from mosquito salivary glands. For the determination of cell number for the MTT assay, 50 µl of HeLa cell suspension was mixed with 450 µl of 0.1% Trypan blue (prepared with PBS). A coverslip was placed over the grid of the chamber and 10 µl of mixture was loaded on one side of the grid by placing the tip of a pipette at the edge of the coverslip so that the mixture fills the chamber by capillary action. The number of viable cells was counted (non viable cells were stained blue while viable cells were not) in the 4 corner squares which are subdivided into 16 tertiary squares omitting cells lying on the cancelled lines (Figure 18). The mean count of viable cells per the four corner squares was calculated and the viable cell concentration per ml was determined using the following formula:

$$Number\ of\ cell/ml = M \times df \times 10^4$$

M = mean count of 4 corner quares

df = dilution factor(10 ie 50 µl of HeLa cell suspension + 450 µl of 0.1% Trypan blue).

10^4 = conversion factor for the counting chamber.

For counting sporozoites, 10 µl of salivary gland suspension was loaded in the chamber and the number of sporozoites per µl determined as above.

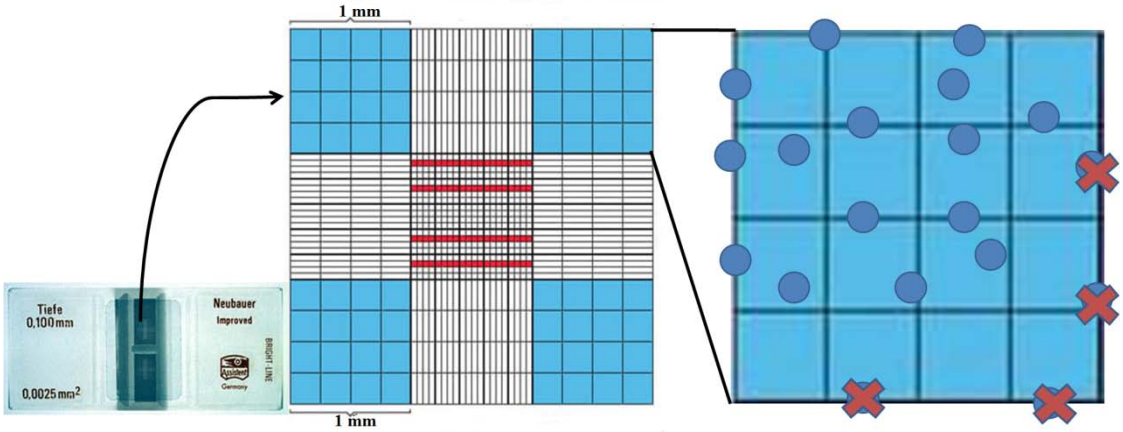


Figure 18: Neubauer counting chamber indicating counting grid and how cells were counted (Modified from http://elearning.studmed.unibe.ch).

2.2.2.9 Purification of asexual blood stages

Asexual blood stages were purified in order to isolate genomic DNA or to obtain parasite lysates. To this end, cultures were transferred into falcon tubes and centrifuged at 1000 g for 5 min. Afterwards the pellet was washed with 10 ml of ice cold PBS and the supernatant was discarded after another centrifugation at 1000 g for 5 min. To lyse red blood cells, pellets were resuspended in 0.15% saponin in PBS by gently pipetting up and down until the blood turned dark red and incubated on ice for 10 min. When lysis was completed, the tubes were centrifuged at 3000 g for 10 min and the supernatant was discarded. The pellet was then washed once with PBS and centrifuged at 3000 g for 10 min. After discarding the supernatant, the parasite pellet was then stored at -20°C or used immediately.

2.2.2.10 Purification of gametocytes by Percoll

Gametocytes were purified as previously described by Kariuki and coworkers (Kariuki et al., 1998). Spent medium was removed from culture flask containing gametocytes and 5 ml of 1 x ICM was added and transferred into a prewarmed 50 ml falcon tube. The tube was then centrifuged at 1800 g for 5 min and the pellet washed once with 1 x ICM medium and centrifuged at 1800 g for 5 min and the pellet resuspended to a final volume of 2 ml with 1 x ICM medium. Ninety percent Percoll was prepared by addition of one volume of 10 x ICM to nine volumes of stock Percoll solution (100%). The solution was then diluted with 1 x ICM to make 80%, 65%, 50% and 35% Percoll solutions. Two millilitres (2ml) of each of these diluted solutions was run slowly down the side of a tilted 15-ml test tube, starting with the heaviest (80%), to form the layered gradient and the tube prewarmed at 37°C. The gametocyte suspension was carefully

layered over the discontinuous Percoll gradient and centrifuged at 1300 g for 10 min. The interface between the first and the second layer (Figure 19A) was collected and washed 2 times with 5-10 ml incomplete medium followed by centrifugation at 1300 g for 5 min. Purified gametocytes were checked for purity and infectivity by examining thin Giemsa-stained smears (Figure 19B) and checking for exflagellation respectively.

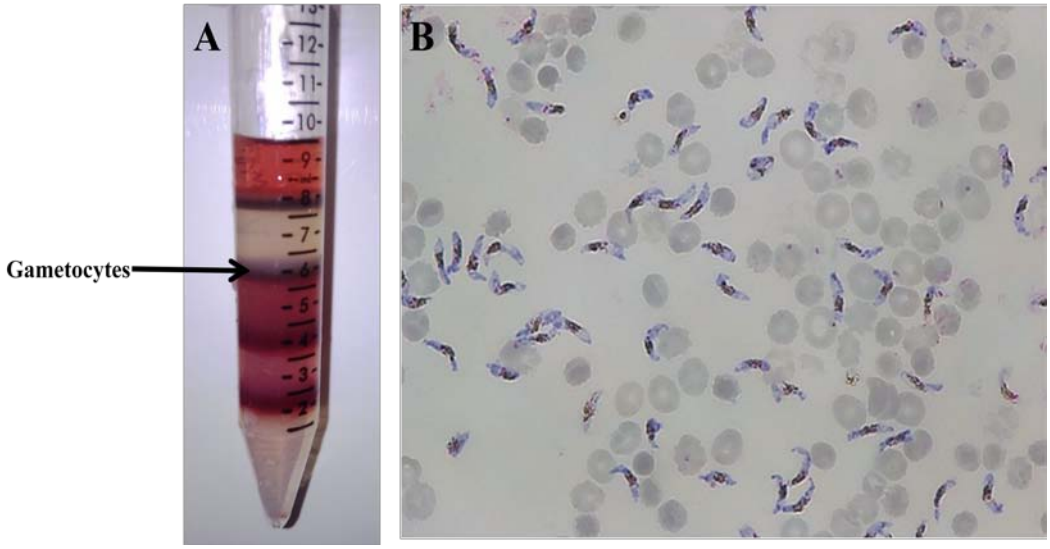


Figure 19: *P. falciparum* gametocyte purification. (A), gametocyte layer following percoll gradient separation. (B), Giemsa stained smear of gametocytes following purification with percoll**.**

2.2.2.11 Malstat assay

Harmonine and *E. meningoseptica* extracts were screened for growth inhibition activity against erythrocytic stages of *P. falciparum* at concentrations between 0.39 to 50 µM and 2.5 to 0.02 mg/ml respectively using the Malstat assay which measures the activity of the *Plasmodium*-specific enzyme lactate dehydrogenase as earlier described (Makler and Hinrichs; 1993, Makler et al., 1993). Synchronized ring stages of *P. falciparum* strains 3D7 and Dd2 were plated in triplicates in 96-well plates (200 µl/well) at a parasitaemia of 1% in the presence of the compounds. Harmonine was dissolved in dimethyl sulfoxide (DMSO) while *E. meningoseptica* extracts were dissolved in methanol. Chloroquine, disolved in double-distilled water, served as a positive control in the experiments. Incubation of parasites with DMSO or methanol alone at a concentration of 0.5% vol. served as negative. Parasites were cultivated *in vitro* for 72 h. Aliquots of 20 µl were removed and added to 100 µl of the Malstat reagent in a 96-well microtiter plate. The assessment of pLDH activity was obtained by adding a 20 µl

of a mixture of NBT (Nitro Blue Tetrazolium)/Diaphorase (1:1; 1mg/ml stock each) to the Malstat reaction, and optical densities were measured at 630 nm. Each compound was tested 2 to 4 times and the IC_{50} (50 % inhibitory concentration) values were calculated from variable-slope sigmoidal dose-response curves using the GraphPad Prism program version 4.

2.2.2.12 MTT cytotoxicity test

HeLa cells (1.3 $x10^3$ cells per well) were plated in 96-well microtiter plates and cultured at 37°C overnight. Harmonine was added to the cultures in concentrations of 0.2-100 µM in triplicate, and the cells were incubated for 48 h. DMSO at a final concentration of 0.5 % vol. was used as negative control and a toxic concentration of 15% vol. DMSO as positive control. After 48 h of drug treatment, Twenty microlitres of MTT (3-(4, 5-Dimethylthiazol-2-yl)-2,5 Diphenyltetrazoliumbromid; 5 mg/ml) was added to each well and incubated for 3 h at 37°C. The medium was subsequently replaced by 100 µl solubilization solution (5% SDS, 0.1M HCl in 100 %vol. DMSO) incubated for 30 min under rotation at RT to solubilize the formazan crystals and the optical density of the solution was measured at 550 nm (Multiscan Ascent). The OD550 values of compound-treated cultures were normalized to the OD550 values of DMSO control (set to 100%).

2.2.2.13 Gametocyte toxicity test

P. falciparum NF54 parasites were grown at high parasitaemia to favor gametocyte formation. Upon appearance of gametocytes stage II, 1 ml of culture was aliquoted in triplicate in a 24-well plate in the presence of harmonine at IC_{50} (4.8 µM) and IC_{90} (50 µM) concentrations and *E. meningoseptica* extract at IC_{50} concentrations (0.25 mg/ml, as determined by Malstat assay). The gametocytes were cultivated for 7 d and the medium was replaced daily. For the first 48 h of cultivation, the gametocytes were treated with test compound; subsequently the medium was compound free. After 7 d, Giemsa-stained blood smears were prepared and the gametocytemia was evaluated by counting the number of gametocytes stages IV and V in a total number of 1000 erythrocytes.

2.2.2.14 Erythrocyte lysis test

To investigate the effect of harmonine on erythrocyte integrity, a volume of 1 ml of washed uninfected erythrocytes was resuspended in regular medium at a final haematocrit of 5 % and plated in triplicates in a 96-well plate (200 µl/well) in the presence of

harmonine at different concentrations. Medium supplemented with 0.15% saponin was use for lysis control and 0.5%vol. DMSO was used for negative control. After 48 h of incubation at 37°C, the plate was centrifuged at 500 g for 2 min. A volume of 100 µl of the resulting supernatant was carefully transfered to another plate and the optical density was measured at 550 nm with a spectrophotometer.

2.2.2.15 Exflagellation inhibition assay

Inhibition of microgametogenesis was investigated by the exflagellation inhibition assay. A volume of 100 µl of mature NF54 gametocyte cultures was pre-incubated with harmonine in concentrations ranging between 0.39 µM and 100 µM for 15 min at 37°C. The sample was then transferred to RT and xanthurenic acid (XA) was added at a concentration of 100 µM for activation. After another 15 min, the numbers of exflagellation centers (Figure 20) were counted in 30 optical fields using a Leica DMLS microscope by 400-fold magnification. Three independent experiments were performed and the inhibition of exflagellation was calculated as a percentage of the number of exflagellation centers in compound-treated cultures in relation to the number of exflagellation centers in untreated controls. The IC_{50} values were calculated from variable-slope sigmoidal dose-response curves using the GraphPad Prism program version 4.

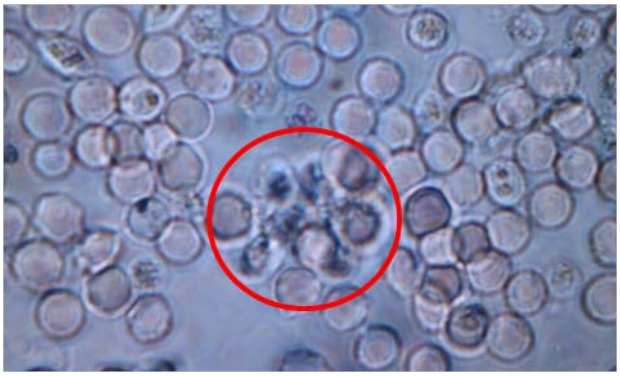


Figure 20: Exflagellation centre following gametocyte activation (red circle)

2.2.2.16 Zygote development assay

Inhibition of zygote formation was investigated using the Zygote development assay. Mature gametocyte cultures were pre-incubated for 15 min at 37°C in the presence of Harmonine at IC_{50} (4.8 µM) and IC_{90} (50 µM) concentrations. The cultures were then activated as described above and incubated for another 12 h at RT. Unfixed specimens were fluorescently labeled with antibodies against Pfs25 in combination with a fluorochrome-conjugated secondary antibody, and the number of zygotes was counted in 90

optical fields using a Leica DMLS microscope by 400-fold magnification. Two independent experiments were performed.

2.2.2.17 Indirect immunofluorescence assay

Asexual F12 blood stage parasites, non-activated NF54 gametocytes and gametocytes at 30 min post-activation were air dried on glass slides and fixed for 10 min in −80°C methanol. For membrane permeabilization and blocking of non-specific binding, fixed cells were incubated for 30 min in 0.01% saponin/0.5% BSA/PBS and 1% neutral goat serum in PBS. Preparations were then successively incubated for 1.5 h each at 37°C with the primary antibody diluted in 0.01% saponin/0.5% BSA/PBS. Binding of primary antibody was visualized using fluorescence-conjugated goat anti-mouse or anti-rabbit secondary antibodies (Alexa Fluor 488 or Fluor 596) diluted in 0.01% saponin/0.5% BSA/PBS. Nuclei were highlighted by incubating the specimens with Hoechst nuclear stain for 1 min and cells were mounted with anti-fading solution AF2 and sealed with nail varnish. Digital images were taken using a Leica TCS SP5 confocal laser scanning microscope and processed using Adobe Photoshop CS software.

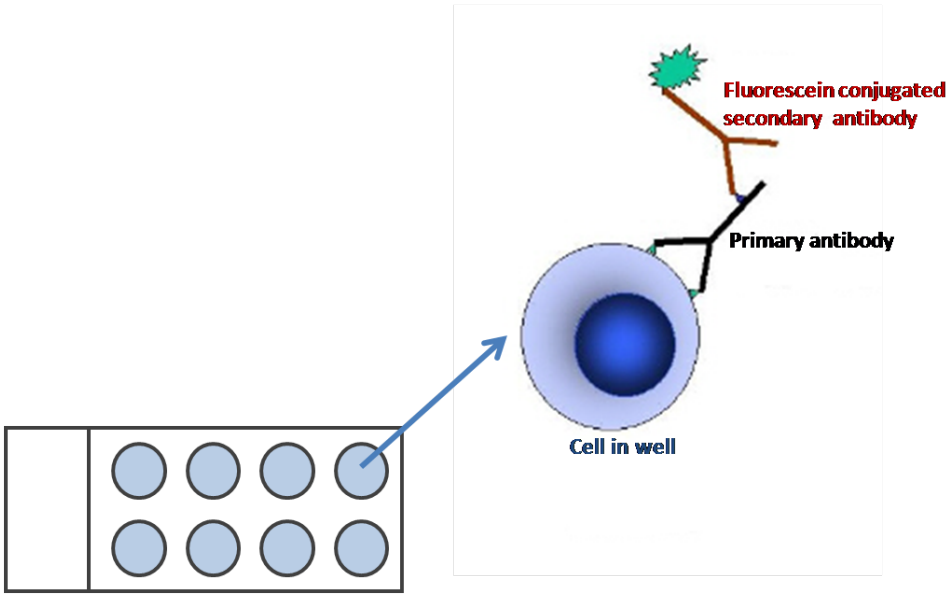


Figure 21: Indirect immunofluorescent assay (modified from http://www.mgormerod.com**).** The secondary detection antibody (fluorescein conjugated) binds to the primary antibody which is specific to the protein of interest on the parasite.

2.2.2.18 Fluorescent signal quantification

For quantitative evaluation of signal strength, digital images of 15-20 randomly selected mature and activated gametocytes were taken using a LSM Zeiss confocal laser scanning microscope under the same optimal laser scanning microscopy settings for

each antibody. The average fluorescence signal intensity of each cell, as indicated by a line edging the respective cell was measured using the LSM 510 image software and recorded.

2.2.2.19 *An. stephensi* midgut histology

Whole larvae and dissected midguts of female adult *An. stephensi* mosquitoes were fixed in 2.5% glutaraldehyde and 2% paraformaldehyde in cacodylate buffer for one day. Specimens were embedded in Epon as previously described (Sologub et al., 2011). Semi-thin sections were obtained using an ultramicrotome RMC MT-7000. Semi-thin sections were dried on slides at 60°C, stained with a mixture of 0.5% methylene blue and 0.5% AzurII for 1 min, and investigated using an Olympus BX41 fluorescence microscope at 100-fold and 1000-fold magnifications in combination with a Jenoptik ProgRes Speed XT5 camera. Digital images were processed using Adobe Photoshop CS software.

2.2.2.20 Electron microscopy of *E. meningoseptica* isolate

E. meningoseptica was cultured for 48 h. A volume of 2 ml of the culture was pelleted, fixed in 2.5% glutaraldehyde in PBS overnight. The next day, the pellet was washed 3 times for 20 min with PBS and incubated overnight in 2% osmium tetroxide / PBS. The cells were then dehydrated with increasing concentrations of ethanol (50%, 70%, 90% and 100%) and incubated two times for 30 min in propylene oxide then embedded in Epon for two days at 60°C. Sections were obtained using an ultramicrotome RMC MT-7000. Post-staining of sections was performed with 1% of uranyl acetate for 30 min. Photographs were taken with a Zeiss EM10 transmission electron microscope.

2.2.2.21 Organic extract preparation from *E. meningoseptica* isolate

The *E. meningoseptica* isolate was cultured in 100-ml Erlenmeyer flasks containing 50 ml of LB medium. The liquid cultures were grown for 2 days at 37°C, while shaking at 150 rpm. A volume of 250 µl of each liquid culture was used to inoculate 10 LB agar plates and the plates were grown at 37°C for 48 h. LB agar plates without bacteria were used as negative control. Colonies on agar plates were controlled for purity, then cut into small pieces and macerated overnight with a double volume of ethyl acetate with continuous shaking for 2 days. The ethyl acetate macerate was filtered and the filtrate was collected. A double volume of acetone was added to the agar macerate, and this mixture was shaken for 3 h for extraction of metabolites and then filtered. The

ethyl acetate and acetone filtrates were dried under vacuum at 40°C and the dry weight was determined. The extracts (agar ethyl acetate and agar acetone) were dissolved in methanol, and then stored at 4°C for the bioassays. For extraction of metabolites from broth cultures, 1 ml of the 48 h *E. meningoseptica* isolate culture was added to 350 ml LB medium and grown for 48 h at 37°C. The culture was filtered and metabolites from the cell pellet were extracted in a double volume of methanol. The mixture was shaken for 2 h and then filtered. The aqueous layer (spend medium) was extracted two times with the triple volume of ethyl acetate. The extracts (broth ethyl acetate and broth methanol) were concentrated under vacuum, their dry weights determined, dissolved in methanol and stored at 4°C for the bioassays.

2.2.2.22 Standard disk diffusion assay

The *in vitro* antimicrobial activity testing of *E. meningoseptica* extracts was carried out using the standard disk diffusion assay (Inderlied et al., 1995) against gram-positive bacteria (*Staphylococcus aureus* strain 8325), gram-negative bacteria (*Escherichia coli* strain 536) and yeast (*Candida albicans* strain S314). Sterile filter disks (6 mm) each impregnated with 25 µl of 250 mg/ml extract 3 times and allow to dry were placed on agar plates that had been inoculated with the test bacterium. After 24 h incubation at 37°C for bacteria and 30°C for fungus, the antimicrobial and antifungal potential was quantitatively assessed as diameter of the inhibition zone. Gentamicin as antibacterial and nystatin as antifungal agents served as positive controls while 50 vol% methanol served as negative control. The antibiotic susceptibility test on the *E. meningoseptica* midgut isolate was performed using the standard disk diffusion assay as described above, using 1:2 serial dilutions of the respective antibiotics (ampicillin, kanamycin, streptomycin and gentamicin) at concentrations of 7.8, 15.6, 31.25, 62.5, 125.0 and 250.0 µg/ml. Water used for dissolving the antibiotics served as negative control while gentamicin at a concentration of 10 mg/ml was used as positive control.

2.2.3 Molecular biology methods

2.2.3.1 DNA extraction

For bacterial diversity determination in midgut tissues, total DNA was extracted using the MasterPure DNA purification kit in accordance with the manufacturer's instructions. Genomic DNA (gDNA) from *P. falciparum* was isolated from purified asexual

blood stages following lysis with 0.15% saponin using the QIAamp Blood Mini Kit as recommended by the manufacturer. The isolated DNAs were eluted with distilled water and their concentration determined using a NanoDrop spectrophotometer.

DNA fragments from agarose gels were purified using the NucleoSpin® Extract II using the protocol as recommended by the manufacturer. The kit is based on the principle that DNA binds in the presence of chaotropic salt to a silica membrane. The binding mixture is then loaded directly onto NucleoSpin® Extract II columns and contaminations are removed by a simple washing step. DNA was eluted with ddH_2O.

2.2.3.2 Polymerase chain reaction

Polymerase chain reaction (PCR) was used to amplify total 16S rRNA gene from midgut tissue samples for bacteria diversity determination. PCR was also used in Real-time RT-PCR (qPCR), the amplification of genomic DNA sequences for checking qPCR primers and also for the amplification of coding sequences for recombinant protein production. Two distinct polymerases were used during this study: Go-Taq polymerase for 16S rRNA gene amplification and primer check and PHUSION polymerase for recombinant protein production because of the proof reading function.

a) 16S rRNA gene amplification

Bacterial 16S rRNA gene amplification for library construction and DGGE were performed using a PCR mix of 50 µl using the following ingredients:

Reaction Mix

10 µl Go-Taq buffer (5x)
2.5 µl $MgCl_2$ (25 mM)
1 µl dNTPs (10 mM)
1 µl Forward primer (100 pmol/ µl)
1 µl Reverse primer (100 pmol/ µl)
1.25 U Go-Taq polymerase
50-100 ng DNA
Add dd H_2O to 50 µl

For 16S rRNA gene library construction, the universal 27f and 1492r primers (Lane, 1991) were used and PCR reactions were performed under the following conditions:

PCR Condition

Initial denaturation:	94°C for 5 mins	
Denaturation:	94°C for 30 s	30 cycles
Annealing:	54°C for 30 s	
Extension:	72°C for 1 min 30 s	
Final extension:	72°C for 10 min.	

16S rRNA gene amplification for DGGE and excised DGGE bands were performed using the universal 16S rRNA gene primers 341f with GC-clamp and 907r (Muyzer et al., 1998) and the 341f without GC-clamp and 907r primers respectively. The conditions were as follows:

PCR Condition

Initial denaturation:	95°C for 5 mins	
Denaturation:	95°C for 1 min	30 cycles
Annealing:	54°C for 1 min	
Extension:	72°C for 45 s	
Final extension:	72°C for 10 min.	

Plasmid DNA containing 16S rRNA gene of correct insert size was verified by PCR amplification using T7 and SP6 pGEM-T Easy primers flanking the insert using the following conditions:

PCR Condition

Initial denaturation:	95°C for 2 mins	
Denaturation:	95°C for 30 s	30 cycles
Annealing:	45°C for 30 s	
Extension:	72°C for 2 min	
Final extension:	72°C for 3 min.	

PCRs without DNA templates were used as negative controls and the PCR products were determined on a 1% agarose gel using electrophoresis.

b) Genomic and plasmid DNA amplification

For the amplification of genomic DNA for qPCR primer check, the following master mix and PCR condition was used:

Reaction Mix

4 µl Go-Taq buffer (5x)
2.5 µl $MgCl_2$ (25 mM)
0.6 µl dNTPs (10 mM)
0.6 µl Forward primer (10 pmol/ µl)
0.6 µl Reverse primer (10 pmol/ µl)
1.25 U GoTaq polymerase
50 ng DNA
Add dd H_2O to 20 µl

PCR Condition

Initial denaturation:	95°C for 10 mins	
Denaturation:	95°C for 15 s	35 cycles
Annealing:	60°C for 1min	
Extension:	60°C for 1 min	

Final extension: 95°C for 3 min.

Others PCR reactions using Go-Taq polymerase and PHUSION polymerase were conducted using the following mix and conditions.

Go-Taq reaction

5 µl Go-Taq buffer (5x)
1.25 µl $MgCl_2$ (25 mM)
0.5 µl dNTPs (10 mM)
0.5 µl Forward primer (100 pmol/ µl)
0.5 µl Reverse primer (100 pmol/ µl)
1.25 U GoTaq polymerase
50 -100 ng DNA (or 2 µl from a colony diluted in 20 µl of dH_2O)
Add dH_2O to 25µl

PCR Condition

Initial denaturation:	94°C for 2 mins	
Denaturation:	94°C for 30 s	35 cycles
Annealing:	45°C for 30 s	
Extension:	72°C for 60 s/1Kbp	
Final extension:	72°C for 2 min.	

PHUSION reaction

10 µl Phusion buffer (10x) + $MgCl_2$
1 µl dNTPs (10mM)
1 µl Forward primer (100pmol/ µl)
1 µl Reverse primer (100pmol/ µl)
5 U Phusion polymerase
50 -100 ng DNA or cDNA
Add ddH_2O to 25µl

PCR Condition

Initial denaturation:	98°C for 30 s	
Denaturation:	98°C for 20 s	35 cycles
Annealing:	45°C for 30 s	
Extension:	60°C for 60 s/1Kbp	
Final extension:	60°C for 2 min.	

2.2.3.3 Agarose gel electrophoresis

Agarose gel electrophoresis was used to separate and analyze DNA fragments. For gel preparation, agarose was dissolved in concentrations of 1 to 3 % (w/v) in 1xTAE buffer. The mixture was boiled in a microwave until the agarose was completely dissolved and was allowed to cool at RT to about 50-60°C. The gel mixture was then

poured on a gel tray and the combs inserted and allowed to solidify. After gel solidification, the combs were removed and the tray was placed in a clean gel tank filled with 1 x TAE buffer. Samples were mixed with loading dye in the ratio of 5:1 and loaded in appropriate wells together with markers and the appropriate controls. DNA on gels was separated at 110V for about 30-60 min and then stained in Ethidium bromide and visualized using a UV transilluminator.

2.2.3.4 Ligation of PCR products into cloning vectors

For subcloning and construction of 16s rRNA gene libraries, the PCR products were ligated into the pGEM®-T Easy vector as recommended by the manufacturer. The plasmid is a linearized vector with a single 3´-terminal thymidine at both ends which greatly improve the efficiency of ligation of PCR products by preventing recircularization of the vector and a compatible overhang for PCR products generated by certain thermostable polymerases. When Phusion polymerase was used, a 3´-A overhang was added to the PCR product since the enzyme does not generate 3´-A-tailed amplicon. Adding for the 3´A overhang was done as follows:

Component	**Volume (µl)**
Purified PCR product	2-6
5x Go-Tag polymerase buffer	2
25 mM $MgCl_2$	1
Go Taq polymerase	1
Add ddH_2O to	10

The mixture was incubated at 70°C for 15- 20 min and the A-overhang product was then ligated into the pGEM®-T Easy vector as follows:

Component	**Volume (µl)**
2 x rapid ligation buffer	5
pGEM-T Easy vector (50 ng/ µl)	1
A-tailed product	2
T4 DNA ligase (3 U/ µl)	1
ddH_2O	1

The mixture was then incubated for 2-3h at RT or at 4°C overnight and 2 µl of the ligated product was used for transformation.

In order to clone into the target vector the amount of insert required was calculated using the following formula:

$$Amount\ of\ insert\ (ng) = \frac{5 \times amount\ of\ vector(ng) \times length\ of\ insert(bp)}{Length\ of\ vector\ (bp)}$$

The ligation reaction was as follows:

Component	Volume (µl)
10 x ligation buffer	5
Ligation vector (100 ng/ µl)	1
Digested product	X
T4 DNA ligase	1
ddH_2O to	20

The mixture was then incubated for 2-3h at RT or at 16°C overnight and 2 µl of the ligated product was used for transformation.

2.2.3.5 Transformation of chemically competent *E. coli* cells

To transform chemically competent *E. coli* cells, 2 µl of a ligation reaction or 2µl plasmid (50-100 ng) was mixed with 30-50 µl of chemically competent *E. coli* (TOP10 , Nova Blue or BL21). After pre-incubation for 30 min on ice, cells were subjected to a heat shock for 40-50 seconds at 42°C. Cells were chilled for 2 min on ice and resuspended in 500 µl SOC medium. Recovery was carried out for 60 min at 37°C, 220 rpm before selection on L-broth agar plates with the appropriate antibiotics.

2.2.3.6 Plasmid DNA purification

For purification of plasmid mini-preps the NucleoSpin Plasmid Quick Pure kit was used as recommended by the manufacturer. One bacterium colony was incubated with 3-5 ml of LB medium supplemented with ampicillin overnight at 37°C, 220 rpm. The next day the bacteria were pelleted at 16000 g for 1 min and plasmid DNA purified as recommended by the manufacturer. DNA was eluted with 50 µl of double distilled water and concentration determined using a NanoDrop machine.

2.2.3.7 Restriction digestion and 16s rRNA gene restriction fragment length polymorphism (RFLP)

Restriction digestion otherwise stated was carried out as follows:

Component	Volume (µl)
pDNA (up to 2 µg)	X
10 x NEB 4 buffer	5
10 x BSA (if required)	5
Restriction enzymes (20000 U/ml)	1
ddH_2O to	50

For 16s rRNA gene RFLP, the 16s rRNA gene was amplified from the pGEM-T vector using the T7 and SP6 vector primers flanking the insert. The PCR product was then digested with Msp I in a 10 µl reaction mixture as follows:

Component	**Volume (µl)**
PCR product	5
10 x NEB 4 buffer	1
Msp I (20000 U/ml	0.25
ddH_2O to	10

The mixture was then incubated for 1 h at 37°C. Afterwards 0.5 µl of Hae III (10000 U/ml) was added and incubated again for 2 h at 37°C. Fragments were separated by electrophoresis using a 3% gel at 110V for 45 mins and the restriction patterns analysed.

2.2.3.8 DNA Sequencing

Sequencing was done using cloning vector primers by the Seqlab – sequence laboratories Göttingen, Germany (http://www.seqlab.de) and the Fraunhofer Institute for Molecular Biology and Applied Ecology (Fraunhofer IME) sequencing facility. The sequencing mixture was made up of 600-700 ng of DNA plus 20 pmol of primer (forward or reverse) in a total volume of 7 µl. Suppresive subtraction hybridization inserts were sequenced using GATC Biotech, Konstanz, Germany.

2.2.3.9 Denaturing gradient gel electrophoresis (DGGE)

Denaturing gradient gel electrophoresis (DGGE) is a molecular fingerprinting method that separates PCR-generated DNA products based on the denaturing characteristics of their DNA. Therefore, different sequences of DNA (from different bacteria) will denature at different denaturant concentrations resulting in a pattern of bands with each band theoretically representing a distinct bacterial population present in the community. If PCR products from a given reaction are of similar size (bp), conventional separation by agarose gel electrophoresis results only in a single DNA band that is largely non-descriptive. DGGE can overcome this limitation by separating PCR products based on sequence differences that results in differential denaturing characteristics of their DNA. DGGE was used to get a fingerprint of the bacteria diversity in *An. stephensi* mosquitoes during development and under different diet (Figure 22). The universal 16S rRNA gene primers 341f with GC-clamp and 907r (Muyzer et al., 1998) were used for PCR amplification of bacterial 16S rRNA genes with the PCR mix and

conditions shown in section 2.2.3.2. Denaturing gradient gel electrophoresis (DGGE) was performed as described in Schmitt et al. (Schmitt et al, 2007). Lint-free tissues were used to wash glass plates, spacers and combs with 1% SDS and then 70% ethanol. The gel sandwich was then assembled by placing the small glass plate on top of the large one and a 0.75 mm spacer was placed along each edge of the plate assembly (Figure 23). The plate clamps were then attached and tightened and the entire assembly was placed into the rear slot of a pouring stand. The clamps were let loose slightly and a spacing card was used to assure the proper spacer alignment. If properly aligned, the clamps were tighten and plate assembly was removed from the pouring stand and inspected to make sure that the two plates and the spacer form a flush surface across the bottom of the assembly. The plate assembly was then clamped into place on a foam gasket placed into one of the two front slots of the pouring stand. A 20% and 80% denaturing solution using 10% acrylamide/bisacrylamide was then prepared from a 100% denaturant containing 7 M urea and 40% (vol/vol) formamide and a 0% denaturing solution lacking the denaturant urea and formamide. A gradient wheel maker was then used to produce the 10% (wt/vol) polyacrylamide gel using a 20 - 80% denaturing gradient. Fourty microlitres of samples with loading dye were then loaded on the gel and DGGE electrophoresis was performed with a Bio-Rad DCode universal mutation detection system for 6 h at 150V or 16 h at 70V and 60°C. The gels were stained for 30 min in 3x GelGreen before taking an image with a GelDoc EQ Station. Selected bands were excised with an ethanol-sterilized scalpel and incubated in 25 µl ddH_2O overnight at 4°C. A total volume of 4 µl of eluted DNA was subsequently used for reamplification with primers 341f and 907r using the PCR conditions described in section 2.2.3.2. The DNA obtained from the excised DGGE bands were ligated into the pGEM-T Easy vector and transformed into competent *E.coli* nova blue cells.

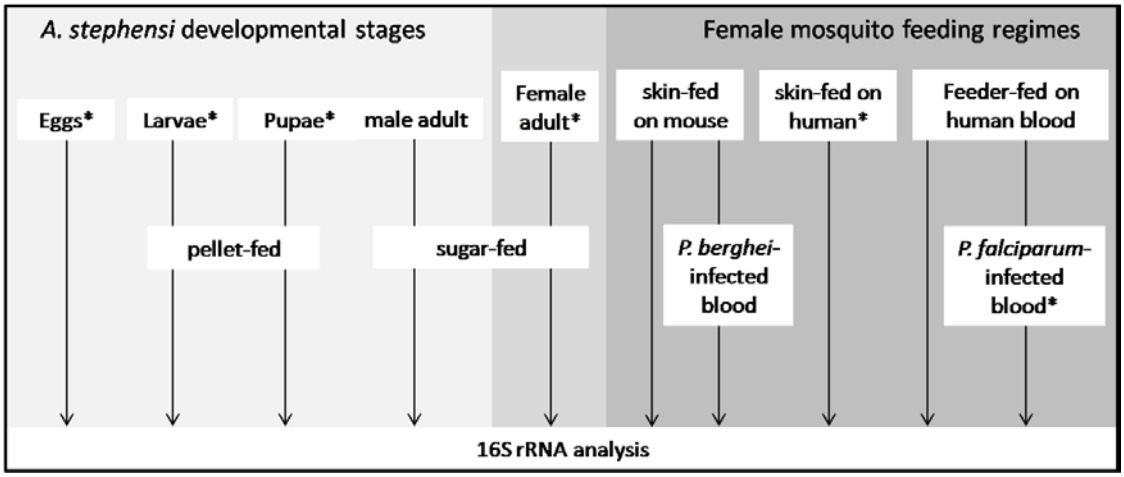


Figure 22: Schematic depicting the experimental set-up of the 16S rRNA fingerprinting analysis by DGGE. The * indicate samples from which 16s rRNA libraries were constructed.

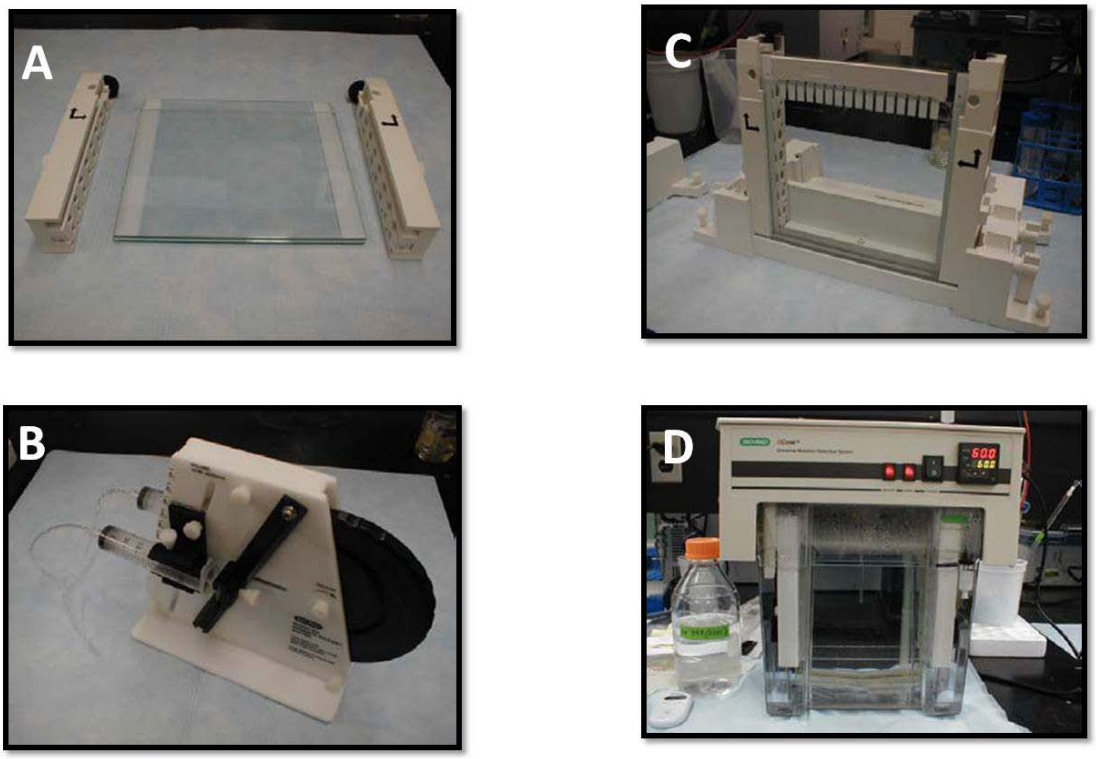


Figure 23: Setting up the DGGE apparatus
(Adapted from www.zjpostdoctor.com/userfiles/file/13093318829705.pdf)**.** (A). Components of the gel sandwich; gel clamps and spacers between two glass plates. (B), gradient wheel, the two syringes hold the high denaturant-concentration solution (back syringe) and low denaturant-concentration solution (front syringe). (C), gel assembly with comb inserted. (D), the DGGE apparatus in action.

2.2.3.10 Construction of 16S rRNA gene clone library

16S rRNA gene libraries were constructed from DNA obtained from *An. stephensi* eggs, larvae midguts, pupae midguts, as well as from midguts of female *An. stephensi* mosquitoes that were sugar-fed, skin-fed on human blood, and feeder-fed on *P. falciparum* infected blood (Figure 22 marked in asterisks). Additionally, 16S rRNA gene libraries were created from the water in which the developmental stages were raised as well as from the water tray prior to mosquito addition. The PCR products from the amplified 16S rRNA genes were ligated into the pGEM-T Easy vector and transformed into competent *E. coli* nova blue cells. Plasmid DNA was isolated using the Nucleospin plasmid extraction kit and the correct insert size was verified by PCR amplification using T7 and SP6 pGEM-T Easy primers flanking the insert followed by agarose gel electrophoresis. Fifty clones from each of the eight 16S rRNA gene libraries were characterized by double digestions with the restriction endonucleases MspI and HaeIII (RFLP; see section 2.2.3.7). The clones with identical restriction patterns were grouped, and one randomly chosen clone per group was sequenced. Sequencing was performed in both directions using the T7 and SP6 primers flanking the insert. The sequences were assembled using the program BioEdit (v7.1.3) (http://www.mbio.ncsu.edu/bioedit/bioedit.html) and controlled for chimeras and for other amplification and sequencing artifacts using the program Pintail (Ashelford et al., 2005). The sequences were compared with 16S rRNA gene sequences available in the GenBank databases by BLASTn search (http://blast.ncbi.nlm.nih.gov/). Sequences with identities above 99.0% were considered to belong to the same operational taxonomic unit (OTU). All 16S rRNA gene sequences were deposited in GenBank under the accession numbers (JX067925 - JX067934, JX110443).

2.2.3.11 Phylogenetic tree construction

The phylogentic tree was constructed with the help of Volker Glöckner. It was done in order to determine the phylogentic relationship between the *Elisabethkingia* sequences obtained from the different mosquito developmental stages and feeding regimes. The 16S rRNA gene sequences were automatically aligned using the web-based SINA aligner (v.1.2.9) (Pruesse et al., 2012) and afterwards imported into the 16S rRNA ARB-Silva database (SSURef_108). Manual corrections of the alignment and phylogenetic analysis were performed using the ARB software package (Ludwig et al., 2004). Tree construction was conducted using neighbour-joining algorithm (Jukes-

Cantor correction) with bootstrap values based on 1000 replications. DGGE derived sequences were added to the tree without altering the trees topology using the parsimony tool.

2.2.3.12 Construction of a subtracted cDNA library using the SSH method

The SSH experiment was conducted by our collaborators in the University of Giessen. The total RNA used here was isolated by Matthias Scheuermayer from Percoll-enriched mature stage V gametocytes before and at 30 min p.a., using Trizol reagent as described by the manufacturer. Subsequently, mRNA was isolated from total RNA using the Oligotex mRNA Mini Kit according to manufacturer's instructions, and the SSH method was conducted as previously described (Altincicek and Vilcinskas, 2007, 2008) using the PCR-Select cDNA Subtraction Kit from Clontech according to the manufacturer´s protocol. Two hundred and twenty nanograms (220) ng of purified mRNA were reverse-transcribed into cDNA using a cDNA synthesis primer; subsequently double-stranded cDNA was generated and digested with RsaI. The double-stranded cDNA from activated gametocytes was ligated in separate aliquots to adaptor 1 or adaptor 2R and were denaturated at 98°C for 90 s and then hybridized at 68°C for 8 h with a 30-fold excess of double-stranded cDNA of non-activated gametocytes. Subsequently, both samples were mixed together with a 10-fold excess of freshly denatured double-stranded cDNA from non-activated gametocytes and hybridized at 68°C for 16 h. The sample was then subjected to two rounds of suppression PCR with PCR-primer 1 and nested primers supplied with the kit. PCR amplifications were performed in a total volume of 25 µl using a PCR cycler with a heated lid and the 5´Prime PCR Extender System. An initial adapter extension at 75°C for 5 min was followed by a denaturation step at 93°C for 30 s and by 27 cycles of denaturation at 93°C for 15 s, annealing at 66°C for 30 s, and extension at 72°C for 90 s. A final 7 min 72°C step was added to allow complete extension of the products. The secondary PCR was performed with nested primer 1 and 2R on 1 µl of the primary PCR products for 15 cycles with an initial denaturation step at 93°C for 1 min, followed by denaturation at 93°C for 15s, annealing at 68°C for 30s and extension at 72°C for 90 s, plus a final extension step at 72°C for 7 min. Resulting PCR products of the secondary subtractive PCR were purified using the NucleoSpin Extract II kit, ligated into the pGEM-T easy vector and transformed into NEB 5-alpha competent *E. coli*). The library was plated on 2×YT agar plates containing 100 µg/ml of ampicillin and incubated at 37°C for 16 h.

2.2.3.13 Colony PCR and analysis of cDNA sequence data from SSH

A preliminary colony PCR screen on 20 colonies was performed with vector-specific primers, i.e. T7 and SP6, using the following conditions: denaturation at 95°C for 5 min followed by 30 cycles of denaturation at 95°C for 15 s, annealing at 43°C for 20 s, and extension at 72°C for 60 s. A final 7 min 72°C step was added to allow complete extension of the products. The colony PCR showed that 75% of clones contained an insert in the vector. Subsequently, 288 randomly picked clones were screened for the presence of the vector. Plasmid isolation of 134 positively screened colonies was performed with the Fast Plasmid Mini kit and the inserts were sequenced by GATC Biotech, Sequences were used to identify similar sequences of the National Center for Biotechnology Information databases using BLASTX program (BLASTX 2.2.13; http://www.ncbi.nlm.nih.gov/BLAST/) and to predict signal sequences, transmembrane regions and features, using the PlasmoDB program (www.plasmodb.org) (Aurrecoechea et al., 2009).

2.2.3.14 *E. meningoseptica* isolation and species identification

Adult female mosquitoes were dissected and 25 midguts pooled and homogenized, then diluted 1:10 in LB medium. A volume of 100 µl of the diluted homogenate was plated on an LB agar plate and incubated at 37°C for 48 h. Four random colonies were picked and bacterial genomic DNA was amplified by colony PCR using 16S rRNA gene universal primers as described above. The PCR product was separated a 1% agarose gel by electrophoresis and the purified product was cloned in the pGEM T-easy vector, sequenced and preliminarily identified by BLASTn. The corresponding *E. meningoseptica* isolate from the midgut homogenate was cultured in 3 ml of LB supplemented with 100 µg/ml ampicillin and stored in glycerol at -80°C. The corresponding 16S rRNA gene sequences were deposited in GenBank under the accession number JX110443.

2.2.3.15 Total RNA isolation

Total RNA was isolated from the mixed asexual F12 cultures, enriched immature NF54 gametocytes (stage III and IV), non-activated mature gametocytes and gametocytes at 30 min p.a. using the Trizol reagent. The samples were dissolved in 20 pellet volumes of prewarmed trizol and incubated shaking at 37°C for 5 min then stored at -80°C till use. The stored samples were thawed at 37°C and transferred to 15 ml falcon

tubes and 0.2 Trizol vol of chloroform was added, mixed well by shaking and allow to stand for 2-3 min at RT then centrifuged (30 min, 4 °C at 1400 g). The aqueous phase was then removed carefully and transferred to a fresh reaction tube and the RNA was precipitated by addition of 0.5 volumes of isopropanol. The samples were transferred to 1.5 ml eppendorf tubes and precipitation was carried out for at least 2 h at 4°C. RNA was pelleted by centrifugation for 30 min at 15,000 g at 4°C. After a wash step with 500 µl of 75% ethanol and additional centrifugation for 5 min, the supernatant was discarded, the pellets air-dried, and subsequently dissolved in 50 µl of DEPC H_2O, heated at 60°C for 30 min and placed on ice for 2 min then stored at -80°C.

2.2.3.16 Removal of genomic DNA and RNA clean up

Up to 5 µg of RNA preparations were treated with RNAse-free DNAse I to remove gDNA contamination. The reaction mixture was composed of up to 5µg of RNA, 10 µl of buffer RDD, 2.5 µl of DNAse I stock solution and 100 µl of RNAse free water. The samples were then incubated on the benchtop for 10 min. To clean up the RNA, 250 µl of diethylpyrocarbonate (DEPC) water mixed with 150 µl phenol and 150 µl chloroform was added, well shaken and incubated for 3 min at RT. The mixtures was then centrifuged at 12000 g for 10 min at 4°C, then the aqueous phase transferred to a new tube and the RNA precipitated at -20°C for at least 30 min by adding 0.1 volumes of cold 3M NaOAc (pH 4.8-5.2) and 2.5 volumes cold 100 % ethanol. After centrifugation for 30 min at 12000 g at 4°C, the pellet was washed with ice cold RNase free 75% ethanol and centrifuged at 12000 g for 10 min at 4°C. The pellet was air dried and subsequently dissolved in 25 µl of DEPC H_2O, heated at 60°C for 30 min and placed on ice for 2 min. Aliquots were then taken and the RNA concentration determined using a NanoDrop spectrophotomer. All RNA samples had A260/A280 ratios greater than 2.1.

2.2.3.17 cDNA synthesis and reverse transcriptase PCR

Two µg of a pure total RNA sample from mixed asexual F12 cultures, enriched immature NF54 gametocytes (stage III and IV), non- activated mature gametocytes and gametocytes at 30 min p.a. were used for cDNA synthesis using the SuperScript III First-Strand Synthesis System, following the manufacturer's instructions. The reaction mixture was composed of the following: 2 µg of RNA, 1 µl of Random hexamers (50 ng), 1 µl of 10 mM dNTP mix and DEPC water was added to 10 µl. Two reaction mixtures for each sample were prepared one for “sample” and the other as “no RT”control.

The samples were incubated at 65 °C for 5 min and incubated on ice for at least 1 min. The RNA-primer mix was then prepared while the samples were incubating and 9 µl of the primer mix made up of 2 µl of 10X RT buffer, 4 µl of 25 mM $MgCl_2$, 2 µl of 0.1 M DTT and 1 µl of RNAse OUT inhibitor was added to each tube. The tubes were incubated for 2 min at 25°C and 1 µl of superscript III reverse transcriptase (50U) was added to the samples except "no RT" control samples. Thereafter, the samples were incubated at 50°C for 50 min followed by inactivation of the enzyme with 15 min incubation at 70°C. The samples were then treated with 1 µl of RNAse H and incubated for 20 min at 37°C.

Controls without reverse transcriptase were used to investigate potential gDNA contamination by diagnostic RT-PCR using hdac (histone deacetylase 1; PF3D7_0925700)-specific primers. RNA quality was further verified for contamination by monitoring transcripts of stage-specific genes, i.e. ama1 (Apical membrane antigen 1) and pfccp2 by diagnostic RT-PCR.

2.2.3.18 Quantitative RT-PCR (qPCR)

Primers for real-time RT-PCR were designed using the Primer 3 software (http://frodo.wi.mit.edu/primer3/) and were initially tested on gDNA in conventional PCR with the same conditions subsequently used for real-time RT-PCR (for primer sequences see table 7). Primers for the reference gene encoding for seryl- tRNA synthetase were obtained from Salanti et al., 2003. The PCR products were subsequently separated by agarose gel electrophoresis. Primers with high specificity were further validated by testing amplification efficiency on 10-fold dilutions of gDNA using real-time RT-PCR followed by analysis of their melt curves since specific primers will produce a melt curve with a single peak. Primers with low specificity, efficiency and poor melt curves were redesigned.

Real-time RT-PCR measurements were performed using the Bio-Rad CFX96 Real-Time Detection System. The test reactions were prepared in triplicates in a total volume of 20 µl using the maxima SyBR green qPCR master mix with 20 ng of cDNA and primer concentrations of 0.3 µM. The following PCR cycling conditions were used: an initial denaturation step at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 15 s and annealing/extension at 60°C for 60 s, followed by a final extension at 95°C for 10 s and a final melt-curve analysis of 55-95°C. Controls without template and without reverse transcriptase were included in all real-time RT-PCR

experiments. Transcript expression levels were calculated by the $2^{-\Delta Ct}$ method (Livak et al., 2001, Sugden et al., 2009).

2.2.4 Protein biochemistry methods

2.2.4.1 Recombinant protein expression

Recombinant proteins were expressed as fusion proteins with a GST-tag using the pGEX 4T-1 vector. The *E. coli* BL21 (DE3) RIL cells were transformed using the vector construct (section 2.2.3.5) and recombinant proteins expression was initially tested by carrying out a mini-expression. To this end, four colonies were picked randomly and grown in LB medium containing 100 µg/ml ampicillin at 37°C. After 2 h, temperature was lowered to 30°C and the expression was induced by adding isopropyl-β-thiogalactopyranoside (IPTG) with a final concentration of 0.75 mM. Five hours later, cells were collected and mixed with 2 × SDS buffer (1:2), boiled for 10 min at 95°C and loaded on a 12% SDS gel (see section 2.2.4.2)

Bacterial colonies with induced protein expression were then used for maxi-recombinant protein expression. To this end, the bacterial colony was grown overnight in 100 ml of LB medium containing 100 µg/ml ampicillin at 37°C with shaking to obtain a starter culture. The next day, fresh medium was added to a final volume of 1500 ml and incubated with shaking at 37°C for 2 h. The temperature was then lowered to 25°C and 1500 µl of 0.75M IPTG was added to the culture to induce protein expression and incubated for 3-5 h at 25°C. After incubation the culture was centrifuged at 5000 xg at 4°C for 10 minutes, after which the supernatant was discarded and bacterial pellet stored at -20°C till use or lysed immediately for protein purification.

2.2.4.2 Purification of recombinant proteins

GST purification of soluble protein

Bacteria were lysed by resuspending pellet in 60 ml of lysis buffer followed by incubation with shaking for 1 h at 4°C. Afterwards the cells were sonicated (50 % intensity, 50 % duty cycle) on ice for 2 min to shear DNA and the lysate was centrifuged at 30000 x g for 1.5 h. The supernatant was collected and filtered through a 0.25 µm syringe filter and 1.5 ml of washed glutathione-sepharose beads added and allowed to bind to the recombinant protein while rotating overnight at 4°C. The next day, the solution was loaded onto a PolyPrep® column whereby unbound proteins passed through the matrix and the recombinant protein is bound to the beads. After

washing the column 3 times with cold PBS, the protein was eluted from the column using the elution buffer (50 mM Tris pH 8/10 mM reduced glutathione) and 8 fractions of 1ml each was collected. The fractions were then analysed on a 12% SDS-PAGE and fraction with high amount of proteins were combined and filtered through an Amicon filter tube 30000 MWCO to exchange the buffer and concentrate the protein.

Inclusion bodies purification

The bacterial pellet was resuspended in 80 ml of inclusion bodies lysis buffer and placed on ice. Twenty ml of lysis buffer containing 200 mg of lysozyme was added and the suspension was incubated on ice for 10 min. The mixture was then sonicated (50 % intensity, 50 % duty cycle) on ice for 10 min and the lysate resuspended in 200 ml detergent buffer followed by centrifugation at 5,000 x g for 10 min at 4°C. Thereafter, the pellet was resuspended in 250 ml of washing buffer and centrifuged at 5,000 x g for 10 min. This step was repeated several times, until a tight pellet was obtained. The tight pellet of inclusion bodies was then washed in 250 ml of 70 % ethanol and resuspended in 2-5 ml of sterile PBS. The pellet was sonicated until the protein solution was able to pass through a 23 G needle for subsequent immunization of mice. Protein concentration was estimated by comparison of stained SDS-gels showing a dilution series of BSA proteins of known concentration. Inclusion body proteins were stored at -20°C.

SDS polyacrylamide gel electrophoresis (PAGE)

SDS-PAGE was used in this study to separate proteins and usually a 10 % or 12 % gel was prepared as follows:

Table 8: Preparation of SDS-PAGE gel

Components	Resolving gel		Stacking gel
	10 %	12 %	5 %
H_2O	1.9 ml	1.6 ml	1.4 ml
30 % acrylamide	1.7 ml	2.0 ml	0.33 ml
1.5 M Tris pH 8.8	1.3 ml	1.3 ml	-
0.5 M Tris pH 6.8	-	-	0.25 ml
10 % SDS	0.05 ml	0.05 ml	0.02 ml
10 % ammonium persulfate (APS)	0.05 ml	0.05 ml	0.02 ml
TEMED	0.002 ml	0.002 ml	0.002 ml

The resolving gel was casted on a pre-made gel cassette up to the level of about 1 cm below the comb height and allowed to polymerise. After polymerization of the resolving gel, the stacking gel was immediately prepared and poured and then a comb carefully fitted to avoid air bubbles. The gel was then allowed to polymerise. Samples were prepared and mixed in a ratio of 1:2 with sample buffer and boiled at 95°C for 10 min then cooled down on ice for 2 min and loaded on the gel allowing a lane for loading a protein marker. The proteins were separated in 1 x SDS-PAGE running buffer for approximately 20 min at 85 V until the front dye had passed through the stacking gel and entered the resolving gel. The voltage was then switched to 125 V. When electrophoresis was complete, the gels were washed 2 times with distilled water and used for western blot or stained directly with either Coomassie staining solution or the Stain Reagent for visualization. After staining, gels were destained with Coomassie destaining solution in case of staining with Coomassie or distilled water in case of GelCode® Blue staining, and then transferred to a solution of 20% ethanol/10 % Glycerol for 1 d at RT. Thereafter the gel was sealed in a plastic foil and allowed to dry at RT. Estimation of protein sizes was done by comparison to the size of the protein marker.

Immunization of mice and generation of polyclonal antisera

Specific immune sera were generated by immunizing 6-week-old female NMRI or Balb/c mice with 100 µg recombinant protein emulsified in Freund's incomplete adjuvant, followed by a boost after 4 weeks with same amount of protein in Freund's incomplete adjuvant. Sera were collected 10 days after the boost. The housing and handling of the animals followed the guidelines of the animal welfare committee of Lower Franconia.

Western blot analysis

Parasite lysates from different parasite stages were prepared following lyses of the pasites using the NETT buffer and the lysates were separated by SDS-PAGE and transferred to a Hybond ECL nitrocellulose membrane (Amersham Biosciences, München) using a sandwich. The sandwich was built on the black side of the cassette holder with all its components put in place in the following order; a fiber pad, followed by 2 Whatman papers on top, the gel on top of the whatman papers, then the nitrocellulose membrane, afterwards 2 Whatman papers and finally a fiber pad (Figure 24). The holder cassette was then placed in a Mini-Trans-Blot-Apparatus from Bio-

Rad containing transfer buffer and ice Proteins were transferred onto the nitrocellulose membrane at 25 V for 2 h or 15 V overnight. When transfer was complete, the membranes were briefly rinsed in 1 x TBS and non specific binding sites were blocked by incubating the membrane with a solution containing 0.5 % BSA/5 % milk in TBS for 1 h at RT or overnight at 4°C on a rocker. After blocking, membranes were washed twice with 1 x TBS and incubated for 2 h at RT or overnight at 4°C with primary antibody diluted in 3 % milk/TBS. After washing for 10 min at RT, one time with 3 % milk /TBS, twice with 3 % milk/0.1 %Tween/ TBS and once again with 3 % milk /TBS, the membranes were incubated with the corresponding alkaline phosphatase-conjugated secondary antibody in 3 % TBS/milk for 1 h at RT. To remove unbound antibody, further washes were performed with 1 x 10 min with 1 x TBS, 2 x 10 min with 1 x TBS/0.1 % Tween and 1 x 10 min with 1 x TBS followed by addition of equilibration buffer for 3 min. Nitrocellulose membrane was developed in a solution of NBT/BCIP (nitroblue tetrazolium chloride and 4-chlor-3-indoxylphosphate) for 1-20 min and the reaction was stopped with stop buffer.

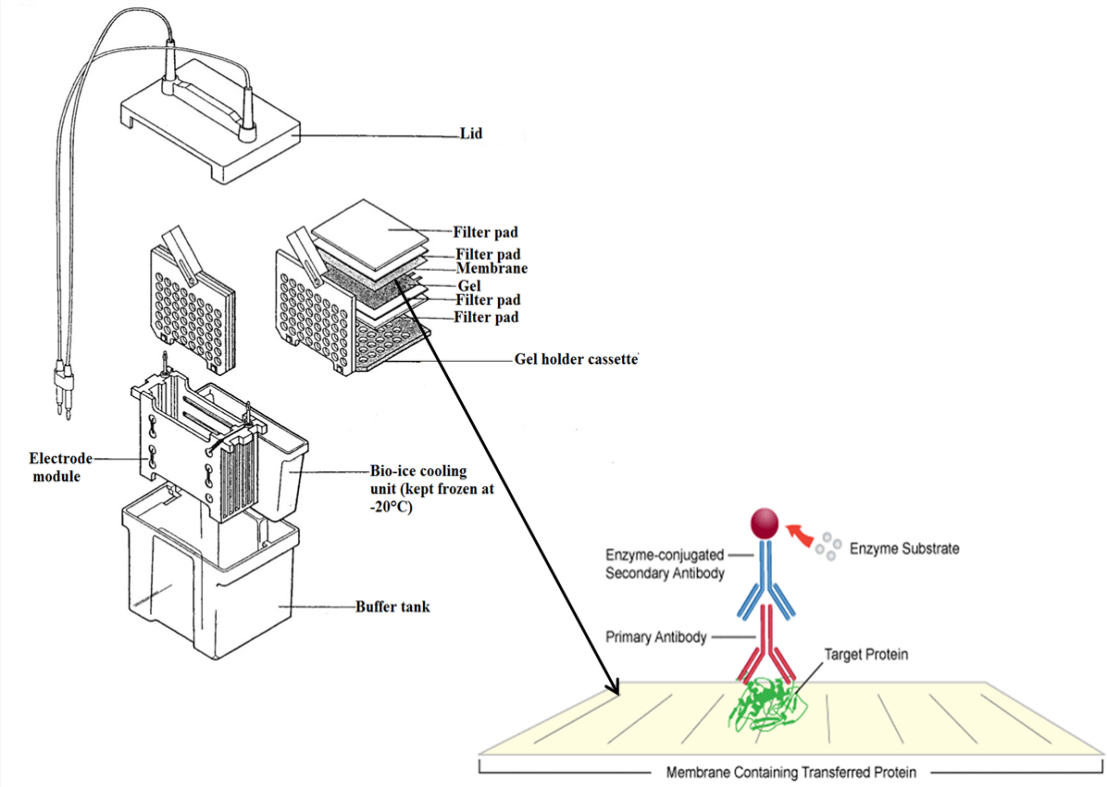


Figure 24: The western blot apparatus, the cassette sandwich preparation and the protein detection on nitrocellulose membrane (adapted from http://www.leinco.com)

3. Results

3.1 Interplay between midgut bacteria and the malaria parasite

In this study we aimed at investigating the bacteria diversity in the midgut of lab-reared *An. stephensi* during development and under different feeding regimes. We focused on determining;

1) The influence of malaria parasites on the mosquito microbiota.
2) Gut bacteria which could be exploited as vehicles for the generation of paratransgenic *Anopheles* mosquitoes.

3.1.1 Bacteria diversity of lab-reared *Anopheles stephensi* during development and under different feeding regimes

In order to determine the bacteria diversity in lab-reared *An. stephensi* mosquitoes during development and under different diet including *Plasmodium* infected blood, we first of all carried out a DGGE on total 16S rRNA obtained from the different midgut tissue samples (shown on Figure 22). The results of DGGE indicated that the *An. stephensi* eggs and midguts of larvae as well as the water in which larvae and pupae had developed had the highest bacteria diversity, while a significant reduction in midgut bacteria diversity during the mosquito development from larvae to adult was observed (Figure 25). There were neither any significant differences observed in the 16S rRNA band patterns between sugar-fed male and female mosquitoes nor between sugar-fed and blood-fed female mosquitoes. Also, there were neither any significant differences observed in the 16S rRNA band patterns between female mosquitoes skin-fed on human or mouse blood, nor between mosquitoes fed on non-infected blood or *Plasmodium*-infected blood (*P. berghei* or *P. falciparum*). Noteworthy, one prominent 16S rRNA band was observed in the 16S rRNA samples of all development stages and under all diet conditions. The respective band was excised from *An. stephensi* larvae and *P. falciparum* fed mosquito lanes (bands 1 and 2 on Figure 25). Sequencing following cloning showed nearest relation to *E. meningoseptica* (100%), a gram-negative rod-like bacterium of water and soil (Steinberg and Burd, 2010).

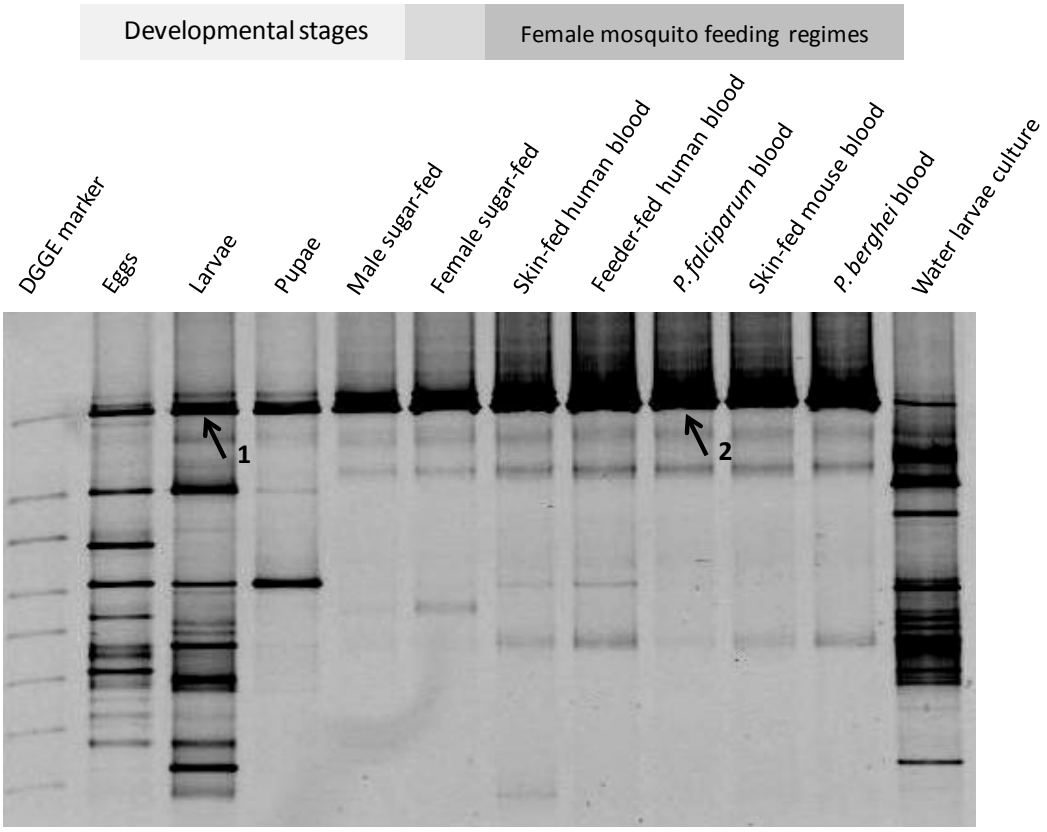


Figure 25: DGGE analysis of midgut microbiota from lab-reared *An. stephensi* in different developmental stages and following sugar, blood and *Plasmodium* feeds. The bands 1 and 2 were excised, their DNA extracted, cloned and sequenced and showed closest relation to *E. meningoseptica.*

Apart from investigating the midgut bacteria diversity, we also investigated the distribution of bacteria in the midguts of larvae and sugar-fed adult mosquitoes via semi-thin sectioning of the respective tissues following tissue embedding in Epon. The stained semi-thin sections revealed that the larvae midgut is homogenously filled with a gelatinous material interspersed with bacteria (Figure 26). The epithelial cells of the larvae gut form short microvilli on their apical sites. A peritrophic membrane, separating the meal from the midgut epithelial layer, is visible (Figure 26). The midgut epithelium of the adult, on the other hand, exhibits long microvilli protruding into the gut lumen. Bacteria can be predominantly found forming biofilms associated to the microvilli surface and were detected in all areas of the midgut (Figure 27). The fact that the peritrophic membrane is not visible indicates that the mosquito has not taken a meal prior to preparation (Figure 27). The data indicate that midgut microbes are evenly distributed over the midgut epithelium of larvae and adult and here preferentially adhere to the epithelial microvilli in the absence of the meal.

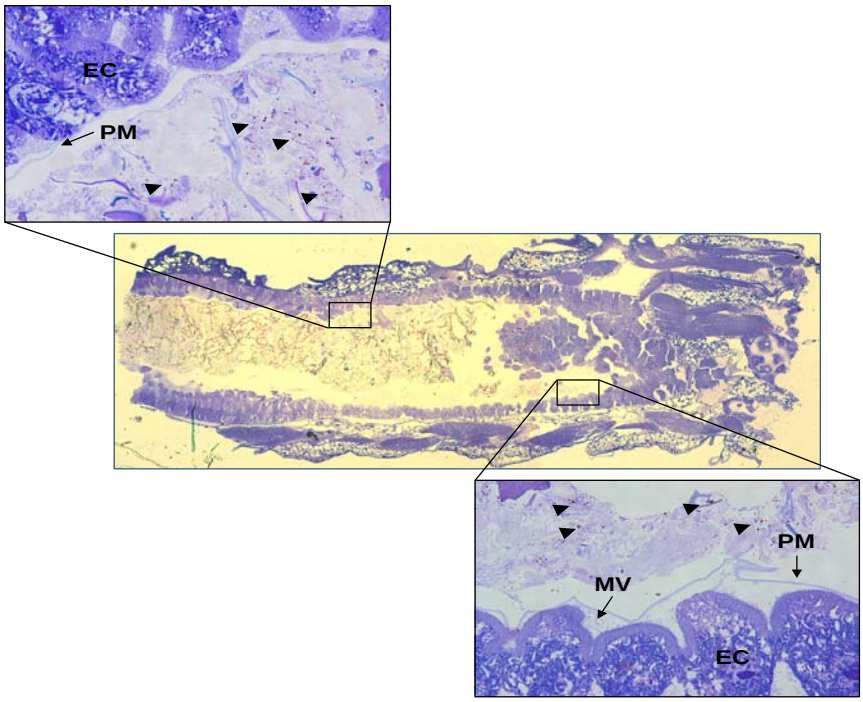


Figure 26: Longitudinal semi-thin section of an *An. stephensi* larvae midgut (picture taken by Gabriele Pradel)**.** The anterior pole is located on the left site of the section. The overview was taken at 100 x magnification, details of the midgut epithelium were taken at 1000x magnification. Arrows indicate bacteria. EC, epithelial cell; MV, microvilli; PM, peritrophic membrane.

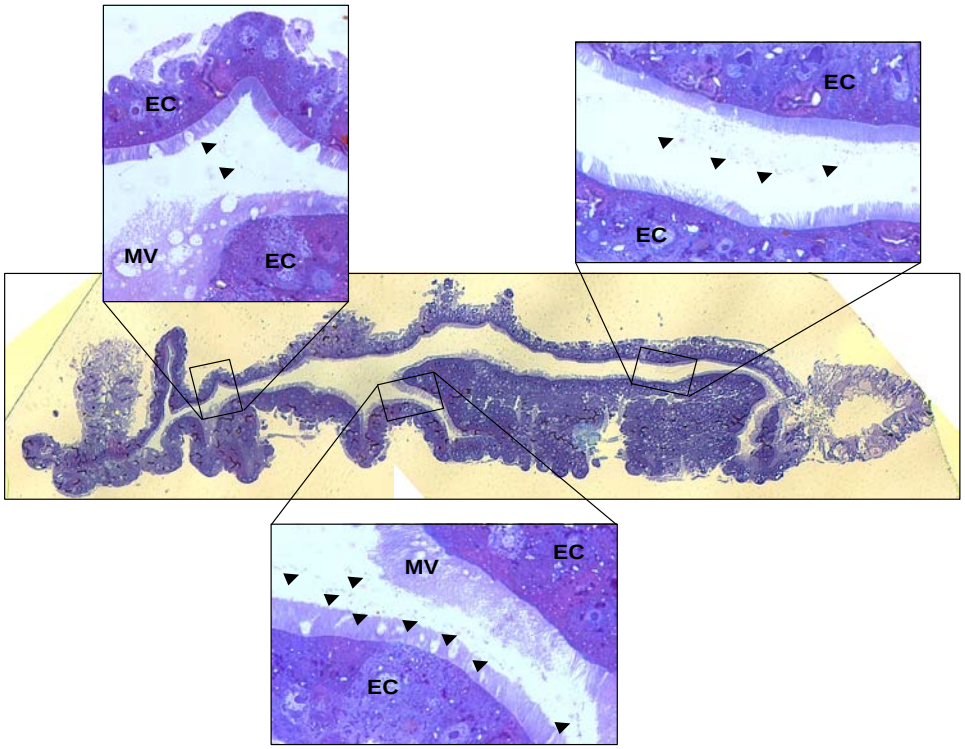


Figure 27: Longitudinal semi-thin section of a midgut of an adult female *A. stephensi* mosquito (picture taken by Gabriele Pradel)**.** The anterior pole is located on the left site of the section. The overview was taken at 100 x magnification, details of the midgut epithelium were taken at 1000 x magnification. Arrows indicate bacteria. EC, epithelial cell; MV, microvilli.

In order to further characterize the microbial diversity in the different developmental stages, we constructed 16S rRNA gene libraries for several experimental groups (Figure 22, marked with asterisks) and analyzed 50 clones of each library based on restriction fragment length polymorphism, sequencing and BLASTn analysis (Table 9). Sequences with similarity greater than 99.0 % were considered to belong to the same operational taxonomiy unit (OTU). As a result, 36 nearly full length 16S rRNA gene sequences were retrieved from the 8 libraries. Of those, 9 sequences were most closely affiliated with *E. meningoseptica* (phylum Bacteroidetes) (99% sequence similarity over > 1480 bp) (Figure 28, Table 9). Phylogenetic analyses placed DGGE- and clone library-derived sequences into one closely related phylogenetic clade along with the isolate obtained in this study and named as *E. meningoseptica* strain Che01. The closest relatives were *E. meningoseptica* phylotypes derived from other mosquitoes, i.e. *Culex quinquefasciatus*, the vector of lymphatic filariasis (NCBI accession number HQ154560), and the African malaria vector *An. gambiae* (Kämpfer et al., 2011), as well as from termites (*Reticulitermes speratus*, Cho et al., 2010), frogs (*Rana catesbeiana*, NCBI accession number GU180606) and the digestive fluid of carnivorous pitcher plants (*Nephentes* sp., NCBI accession number GQ360070). *E. meningoseptica* phylotypes were present in all developmental stages and in mosquito midguts from all feeding regimes. *E. meningoseptica* was further found in the water used for rearing of larvae, but not in tray water that had not come into contact with mosquitoes. Also, *E. meningoseptica* was present in the midguts of sugar-fed and blood-fed mosquitoes in the presence and absence of the malaria parasite, indicating that this bacterium is not affected by the mosquito diet.

The remaining 19 mosquito-derived sequences were affiliated with the α- (n = 7) and *β-Proteobacteria* (n = 3), the *Bacteroidetes* (n = 4), *Cytophaga* (n = 2), *Firmicutes* (n = 1), and the *Actinobacteria* (n = 2) (Table 9). The microbial midgut diversity decreased during mosquito development from egg to adult, and here the gut was largely dominated by *E. meningoseptica*. The microbial diversity of the water sample in which the larvae and pupae had developed was quite diverse with 7 different phylotypes, including *E. meningoseptica*. The fact that *E. meningoseptica* is present in all developmental stages as well as in the rearing water indicate that the bacteria are transmitted between developmental stages and generations. Furthermore, the bacteria diversity in lab-reared adult female *An. stephensi* mosquitoes fed on sugar or on human blood in the presence and absence of *P. falciparum* was very low and there were no significant dif-

ferences between the bacteria diversities in these mosquitoes. *E. meningoseptica* was the dominant bacterium in the midguts of sugar-fed and blood-fed mosquitoes in the presence and absence of the malaria parasite indicating that this bacterium is not affected by the mosquito diet.

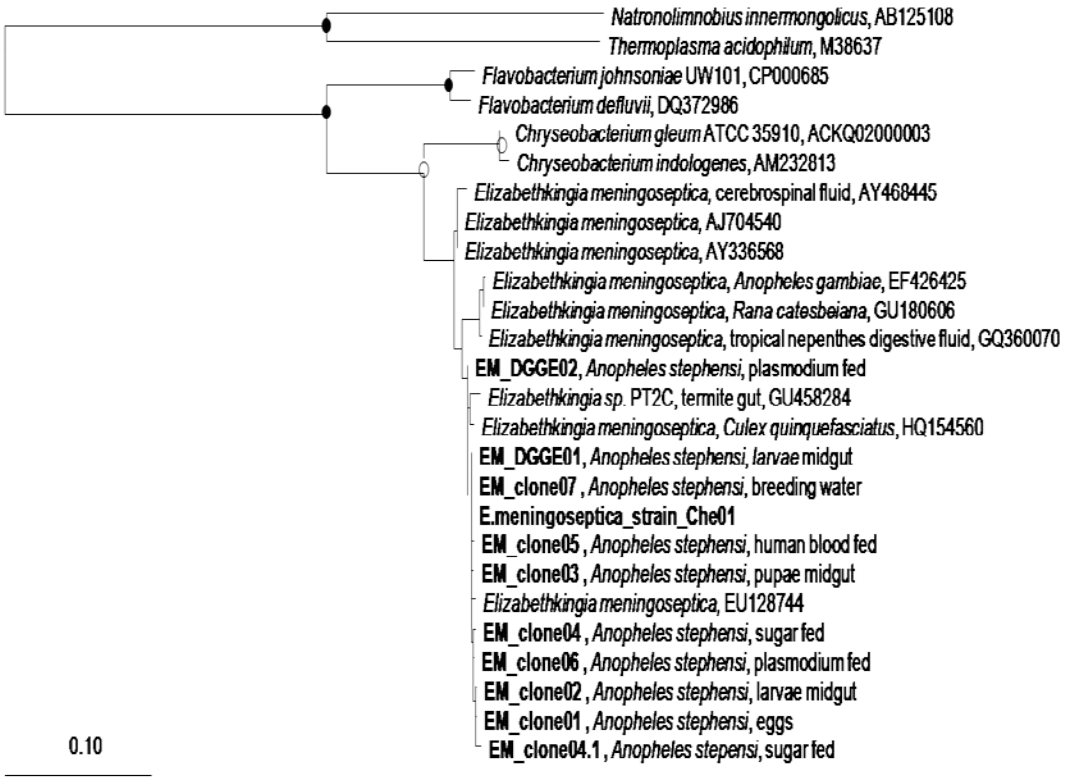


Figure 28: Neighbor-joining phylogenetic tree (constructed by Volker Glöckner) *E. meningoseptica* 16S rRNA gene sequences obtained in this study are shown in bold letters. Filled circles indicate bootstrap values over 90% open circles over 70%.

Table 9: 16s rRNA gene sequences from different developmental stages of *An. stephensi* and after different feeding regimes

Clone library source	Clone designation	Sequence length (bp)	Closest relative by BLAST (Accession number)	Sequence Similarity (%)	Prevalence in 16S rRNA gene library (%)	Order, Phylum/Subphylum
Eggs (n = 6 seqs)	ASE-2	1480	*Elizabethkingia meningoseptica* strain ATCC 13253 (NR_042267.1)	99	17.1	Flavobacteriales, Bacteroidetes
	ASE-33	1469	*Flectobacillus major* (NR_044736.1)	96	4.9	Cytophagales, Cytophaga
	ASE-1	1487	*Pelomonas saccharophila* strain DSM 654 (NR_024710.1)	99	43.9	Burkholderiales, Betaproteobacteria
	ASE-11	1491	*Sediminibacterium salmoneum* strain NJ-44 (NR_044197.1)	94	26.8	Sphingobacteriales,Bacteroidetes
	ASE-21	1494	*Undibacterium pigrum* strain CCUG 49009 (NR_042557.1)	97	4.9	Burkholderiales, Betaproteobacteria
	ASE-44	1499	*Brevibacillus formosus* strain DSM 9885 (NR_040979.1)	97	2.4	Bacillales, Firmicutes
larvae mg (n = 9 seqs)	ASLM-1	1480	*Elizabethkingia meningoseptica* strain ATCC 13253 (NR_042267.1)	99	12.1	Flavobacteriales, Bacteroidetes
	ASLM-12	1481	*Flectobacillus major* (NR_044736.1)	94	18.2	Cytophagales, Cytophaga
	ASLM-2	1486	*Microbacterium dextranolyticum* strain DSM 8607 (NR_044934.1)	99	21.2	Actinomycetales, Actinobacteria
	ASLM-3	1452	*Bradyrhizobium japonicum* strain 3I1b6 (NR_036865.1)	88	15.2	Rhizobiales, Alphaproteobacteria
	ASLM-5	1491	*Nubsella zeaxanthinifaciens* strain TDMA-5 (NR_041478.1)	95	12.1	Sphingobacteriales Bacteroidetes
	ASLM-9	1481	*Lishizhenia caseinilytica* strain UST040201-001 (NR_041043.1)	85	3.0	Flavobacteriales, Bacteroidetes
	ASLM-13	1449	*Rhizobium daejeonense* strain L61 KCTC 12121 (NR_042851.1)	98	3.0	Rhizobiales, Alphaproteobacteria
	ASLM-17	1448	*Rhizobium huautlense* strain SO2 (NR_024863.1)	97	9.1	Rhizobiales, Alphaproteobacteria
	ASLM-42	1448	*Mesorhizobium loti* strain NZP 2213 (NR_025837.1)	99	6.1	Rhizobiales, Alphaproteobacteria
pupae mg (n = 5 seqs)	ASPM-12	1480	*Elizabethkingia meningoseptica* strain ATCC 13253 (NR_042267.1)	99	88.1	Flavobacteriales, Bacteroidetes
	ASPM-4	1448	*Rhizobium daejeonense* strain L61; KCTC 12121 (NR_042851.1)	98	2.4	Rhizobiales, Alphaproteobacteria
	ASPM-6	1486	*Microbacterium dextranolyticum* strain DSM 8607 (NR_044934.1)	99	2.4	Actinomycetales, Actinobacteria

	ASPM-16	1442	*Rhizobium daejeonense* strain L61; KCTC 12121 (NR_042851.1)	97	4.7	Rhizobiales, Alphaproteobacteria
	ASPM-43	1492	*Aquabacterium parvum* strain B6 (NR_024874.1)	99	2.4	Burkholderiales, Betaproteobacteria
Adult female mg, sugar-fed (n = 3 seqs)	ASFM-1, ASFM -34	1480, 1484	*Elizabethkingia meningoseptica* strain ATCC 13253 (NR_042267.1)	99	84.6 13.2	Flavobacteriales, Bacteroidetes
	ASFM-30	1496	*Sphingobacterium siyangense* strain SY1 (NR_044391.1)	98	2.2	Sphingobacteriales, Bacteroidetes
Adult female mg, human blood-fed (n = 3 seqs)	ASHBF-27, ASHBF-5	1486, 1481	*Elizabethkingia meningoseptica* strain ATCC 13253 (NR_042267.1)	98	97.5	Flavobacteriales, Bacteroidetes
	ASHBF-42	1452	*Asaia siamensis* strain S60-1 (NR_024738.1)	99	2.5	Rhodospirillales, Alphaproteobacteria
Adult female mg, *Pf*-fed (n = 2 seqs)	ASPF-2, ASPF-16	1485, 1480	*Elizabethkingia meningoseptica* strain ATCC 13253 (NR_042267.1)	96	100	Flavobacteriales, Bacteroidetes
Water larvae culture (n = 7 seqs)	WLP-21	1477	*Elizabethkingia meningoseptica* strain : ATCC 13253 (NR_042267.1)	99	2.4	Flavobacteriales, Bacteroidetes
	WLP2	1466	*Flectobacillus major* (NR_044736.1)	96	73.7	Cytophagales, Cytophaga
	WLP-9	1494	*Roseateles depolymerans* strain 61A (NR_036813.1)	99	4.8	Burkholderiales, Betaproteobacteria
	WLP 10	1527	*Legionella fallonii* strain LLAP10 (NR_036805.1)	94	11.9	Legionellales, Gammaproteobacteria
	WLP 1	1445	*Mesorhizobium loti strain* NZP 2213 (NR_025837.1)	99	2.4	Rhizobiales, Alphaproteobacteria
	WLP-19	1473	*Rhizobium selenitireducens* strain B1 (NR_044216.1)	95	2.4	Rhizobiales, Alphaproteobacteria
	WLP-32	1428	*Asticcacaulis taihuensis* strain T3-B7 (NR_042933.1)	97		Caulobacterales, Alphaproteobacteria
Water before breeding (n = 1 seq)	WBF-7	1491	*Methylibium petroleiphilum* PM1 strain PM1(NR_041768.1)	97	100	Burkholderiales, Betaproteobacteria

3.1.2 Isolation, identification and characterisation of *E. meningoseptica*

Due to the fact that *E. meningoseptica* phylotypes were found to be dominant in our 16s rRNA gene libraries, we attempted to isolate the bacteria from the midgut of female *An. stephensi* mosquitoes. Midgut content of pooled female mosquitoes was cultured on LB agar plates and an isolate was identified by 16S rRNA sequencing to be *E. meningoseptica* and was named *E. meningoseptica* Che 01.

In order to characterize the isolate, a sample was prepared for transmission electron microscopy. The electron micrographs confirmed the isolate as a rod with a cell wall typical for gram-negative bacteria (Figure 29A). Higher magnifications revealed the double membrane structure of the cell wall (Figure 29B). Transmission electron microscopy thus confirmed that the isolate contained the gram-negative rod *E. meningoseptica* and that it was devoid of any contaminations by other midgut microbes.

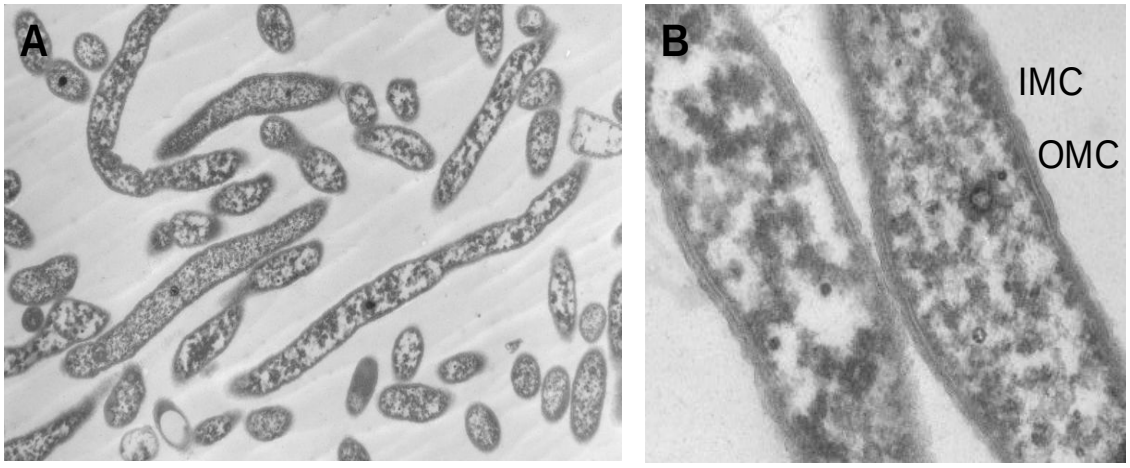


Figure 29: Transmission electron micrograph of cultured *E. meningoseptica* isolated from the *A. stephensi* midgut. (A), shows the rod-like structure of the bacterium. (B), shows the bacterium cell wall. IMC, inner membrane complex; OMC, outer membrane complex. Bar 1 µm (A), 0.5 µm (B).

Also, because a multi-resistance of *E. meningoseptica* to antibiotics was previously reported (Lin et al., 2004; Hung et al., 2008), we tested the resistance of Che 01 to selected antibiotics, using the standard disk diffusion assay. Che 01 showed resistance to ampicillin, kanamycin and streptomycin at concentrations of up to 250 µg/ml (Table 10). Furthermore, it was resistant to gentamicin at concentrations of up to

62.5 µg/ml while concentrations of 125 µg/ml and 250 µg/ml resulted in inhibition zones of 10.0 and 13.5 mm diameters, respectively. Water, which was used as a negative control, did not inhibit growth of Che 01.

Table 10: Resistance of *E. meningoseptica* to selected antibiotics

Antibiotics tested	Concentration [µg/ml]	Inhibiting areola [mm]
Ampicillin	≤ 250	0
Kanamycin	≤ 250	0
Streptomycin	≤ 250	0
Gentamicin	≤ 62.5	0
	125	10.0 ± 0.0
	250	13.5 ± 0.71
Water (negative control)	-	0
Gentamicin (positive control)	10 mg/ml	25 ± 1.41

3.1.3 Antibacterial and antifungal activities of organic extracts from *E. meningoseptica*

The dominance of *E. meningoseptica* in our 16S rRNA gene libraries led us to suspect that the bacterium may produce antimicrobial substances ensuring its dominance over other midgut inhabitants (bacteria and fungi). To prove this, Che 01 was cultured for 48 h in solid and liquid media to produce secondary metabolites which were extracted using different organic solvents. The extracts were subsequently tested against gram-positive *Staphylococcus aureus* strain 8325 and gram-negative *Escherichia coli* strain 536 as well as against the yeast *Candida albicans* strain S314, using the standard disk diffusion assay. The diameters of the inhibition zones were measured, and compared to solvents alone (negative control) as well as to gentamicin (antibacterial positive control) or nystatin (antifungal positive control). The screening showed that the ethyl acetate and acetone extracts exhibited antibacterial and antifungal activities against the three microbes tested in the assays (Table 11), indicating that *E. meningoseptica* produces antimicrobial substances to defeat other mosquito midgut inhabitants thereby explaining its dominance.

Table 11: Antibacterial and antifungal activities of organic extracts from *E. meningoseptica*

Pathogen	Extract	Inhibition zone [mm]
Staphyloccocus aureus 8325	Agar ethyl acetate	14.5 ± 1.41
	Agar acetone	8.0 ± 0.71
	Broth ethyl acetate	11.0 ± 0.0
	Broth methanol	0
	Gentamicin* (positive control)	25.0 ± 0.0
Eschericha coli 536	Agar ethyl acetate	12.0 ± 0.0
	Agar acetone	8.0 ± 0.0
	Broth ethyl acetate	9.0 ± 0.0
	Broth methanol	0
Candida albicans S314	Agar ethyl acetate	10.0 ± 0.0
	Agar acetone	0
	Broth ethyl acetate	12.0 ± 0.0
	Broth methanol	0
	Nystatin* (positive control)	17.0 ± 0.0
Negative control	Agar ethyl acetate (without bacteria)	0
	50 vol% methanol	0

*For positive controls, gentamicin was tested at 10 µg/µl and nystatin was tested at 25µg/µl.

3.1.4 Antiplasmodial and gametocytocidal activity of organic extracts of *E. meningoseptica*

E. meningoseptica extracts were tested for their effect on *P. falciparum* parasites of strain 3D7. First, the extracts were tested for their effect on growth of blood stage parasites, using the Malstat viability assay as previously described (Aminake et al., 2011). The ethyl acetate extract from broth and agar cultures exhibited moderate activities against the *P. falciparum* asexual blood stages in the assays with IC_{50} concentrations of 0.25 mg/ml and 0.89 mg/ml, respectively, while the acetone extract showed very low activity (Table 12). Chloroquine was used as a reference control in the assays and inhibited parasite growth with an IC_{50} value of 14.4 nM and the solvent control (0.5% methanol) had no inhibitory effect on the parasites.

Table 12: Antiplasmodial activity of organic extracts from *E.meningoseptica* against *P. falciparum* blood stages

Extracts tested	IC_{50}
Broth ethyl acetate	0.25 ± 0.014 mg/ml
Agar ethyl acetate	0.89 ± 0.049 mg/ml
Agar acetone	2.9 mg/ml*
Chloroquine (negative control)	14.4 ± 2.19 nM

Tested in triplicate; *, tested once.

We also investigated the effect of the extract from *E. meningoseptica* on the development of gametocytes, thus on the stages that are taken up during the mosquito blood meal and which then undergo gametogenesis in the mosquito midgut. The gametocytocidal effect of a compound would block transmission of parasites that escaped the blood stage killing and thus counteract the propagation of malaria parasites by the mosquito. The broth ethyl acetate extract of *E. meningoseptica* reduced the number of mature *P. falciparum* NF54 gametocytes by 58% at IC_{50} concentrations, when compared with the 1% methanol negative control (set to 100%). A similar concentration of the agar ethyl acetate extract had no effect on gametocyte development. The antimalarial primaquine was used as a positive control in the test and exhibited a gametocytocidal activity of 55% (Table 13).

Table 13: Gametocytocidal activity of organic extracts from *E. meningoseptica* against *P. falciparum* gametocytes

Extracts tested	Concentration	Gametocytemia [‰]	Rel. GC no. [%]
Broth ethyl acetate	0.25 mg/ml	27.3 ± 0.74	58
Agar ethyl acetate	0.25 mg/ml	43.3 ± 3.10	92
Primaquine (positive control)	3 µM	25.7 ± 5.51	55
Methanol (negative control)	1 vol%	47.0 ± 6.56	100

Tested in triplicate; GC, gametocyte.

3.2 Changes in the transcriptome during the initial phase of malaria parasite transmission from human to mosquito

Here, we investigated the changes in the in *P. falciparum* gametocyte transcriptome during the first half hour following gametocytes activation in the mosquito with principle aim of identifying plasmodial proteins which could be exploited as targets for transmission blocking strategies.

3.2.1 Determination of transcriptome changes by Suppressive Subtractive Hybridization (SSH)

Changes in the transcriptome of malaria parasites during the initial phase of transmission from the human to the mosquito was determined by SSH on mRNA isolated from mature non-activated gametocytes and from gametocytes at 30 min after in vitro activation, which had completed gametogenesis (Sologub et al., 2011). A subtracted cDNA library was constructed and the resulting cDNA was assigned to the respective plasmodial genes.

We identified a total number of 126 genes, for which expression levels changed in the gametocytes during activation. The majority of genes can be assigned to six major ontology groups (Figure 30, Table 14): 22 genes (17.5%) have putative functions in signaling, 18 genes (14.3%) are assigned to processes linked to cell cycle and gene regulation, 11 genes (8.7%) can be linked to the cytoskeleton or the inner membrane complex (IMC), 10 genes (7.9%) have putative functions in protein trafficking, stabilization and degradation (proteostasis), eight genes (6.4%) are linked to general metabolic functions, and 16 genes (12.7%) are proteins of the cell surface or PVM. Furthermore, 15 genes (11.9%) can be assigned to other functions, including protein synthesis and processing. A total of 26 genes (20.6%) encode for proteins with unknown function, nine of which have sequences for transmembrane domains and five of which encode for a signal peptide.

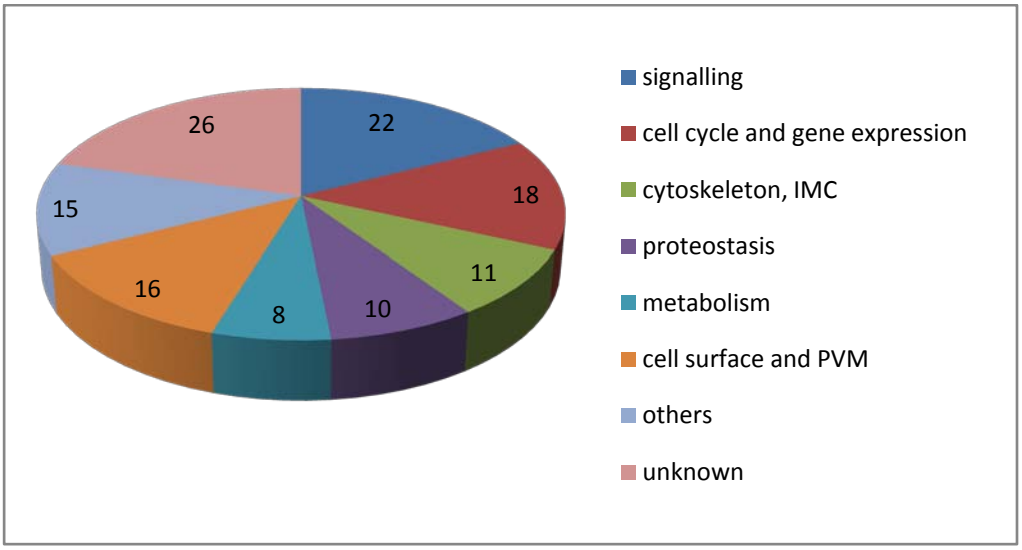


Figure 30: Depiction of ontology groups of *P. falciparum* genes with changes in transcript expression during gametocyte activation. The changes in transcript abundance were determined by SSH between mRNA of mature non-activated gametocytes and gametocytes at 30 min p.a.. A total number of 126 genes were identified (the numbers of genes per group are indicated).

3.2.2 Comparison of genes identified by SSH with *Plasmodium* proteome data

We compared the genes with changes in transcript with *P. falciparum* and *P. berghei* gametocytes transcriptome data which already exists (Florens et al., 2002, Silvestrini et al., 2005, Silvestrini 2010, Khan et al., 2005). This analysis was done by Gunnar R. Mair. The results showed that about 60% (76/126) of the SSH genes have been detected in the currently available *P. falciparum* gametocyte proteomes, while for 40% of the genes, there is as yet no protein evidence (Table 15). Also, almost identical numbers apply to the 113 orthologs of the rodent malaria parasite *P. berghei*; the 13 *P. falciparum* genes without *P. berghei* orthologs include amongst others the three *etramp* genes, *pfs16* and *resa*.

Table 14: Genes of the *Plasmodium falciparum* gametocyte transcriptome as identified by SSH (comparing mature non-activated gametocytes and gametocytes at 30 min p.a. Anotation done by Gabriele Pradel**)**

Plasmodb ID	Protein	Predicted features	pred. MW
Signaling			
PF3D7_0214600	serine/threonine protein kinase		204.3
PF3D7_1356800	serine/threonine protein kinase ARK3		475.7
PF3D7_0302100	serine/threonine protein kinase PfCLK-4/SRPK1	Phosphorylation of SR splicing factors	157.4
PF3D7_1230900	atypical protein kinase, RIO family		111.5
PF3D7_1238900	calcium/calmodulin-dependent protein kinase, PK2		58.9
PF3D7_0624000	hexokinase, HK		55.3
PF3D7_0515300	phosphatidylinositol 3-kinase, PI3K		255.9
PF3D7_1113100	protein tyrosine phosphatase, PRL		25.1
PF3D7_1403900	phosphatase		35.2
PF3D7_1464600	phosphatase	SP	170.5
PF3D7_0210600	conserved Plasmodium protein	SP, calcium-lipid-binding domain CaLB	53.7
PF3D7_1238100	calcyclin binding protein	Calcyclin-binding domain	266.4
PF3D7_1442200	GTP binding protein		118.7
PF3D7_1358600	zinc finger protein	Zinc-Finger domain	118.3
PF3D7_0927200	zinc finger protein	Zinc-Finger domain	189.7
PF3D7_1004300	zinc finger, C3HC4 type		131.7
PF3D7_1433400	conserved Plasmodium membrane protein	7 TMs, PHD C4HC3 zinc-finger-like motifs	682.5
PF3D7_1463900	conserved Plasmodium membrane protein	SP, 7 TMs, EF hand calcium-binding domain	127.1
PF3D7_1233600	asparagine and aspartate rich protein 1, AARP1	Zinc-finger domain, Ring/U-Box	146.3
PF3D7_0530200	triose phosphate transporter iTPT	SP, 7 TMs	59.8

PF3D7_0303900	conserved Plasmodium protein	SP, phosphatidyl-ethanolamine-binding protein PEBP do-main	23.2
PF3D7_0818200	14-3-3 protein	Regulative protein, binds signaling enzymes like kinases or TM receptors	30.2
Cell cycle and gene expression			
PF3D7_1445900	DEAD/DEAH box ATP-dependent RNA helicase		60.0
PF3D7_1019000	eukaryotic translation initiation factor subunit eIF2A		280.8
PF3D7_1417200	NOT family protein	Regulation of transcript	519.8
PF3D7_1103800	CCR4-NOT transcription complex subunit 1, NOT1	4 TMs	392.0
PF3D7_0622900	ApiAP2 protein	Transcription factor	237.4
PF3D7_0420300	ApiAP2 protein	Transcription factor	400.2
PF3D7_0615400	ribonuclease		323.2
PF3D7_0811300	CCR4-associated factor 1, CAF1		200.2
PF3D7_1034900	methionine-tRNA ligase		101.2
PF3D7_1469700	conserved Plasmodium protein	Homology to the transcriptional mediator subunit Med6	24.6
PF3D7_0513600	deoxyribodipyrimidinephotolyase		129.2
PF3D7_1105500	centrin-4 (CEN4)		20.1
PF3D7_1018500	PHF5-like protein	mRNA splicing factor RDS3 domain	12.5
PF3D7_0827800	SET domain protein SET3	Chromatin regulator, nuclear protein, in male gametocytes	283.6
PF3D7_1115200	SET domain protein, SET7	Chromatin regulator	94.3
PF3D7_1360900	polyadenylate-binding protein	RNA-binding domain	44.8
PF3D7_1011800	QF122 antigen	KH domain type 1, RNA binding	131.6

PF3D7_1347500	DNA/RNA-binding protein Alba 4, ALBA4		42.2
Cytoskeleton, IMC, Motor complex			
PF3D7_0422300	alpha-tubulin II	Cytoskeletal element	49.7
PF3D7_1246200	actin I	IMC component	41.2
PF3D7_0503400	actin-depolymerizing factor 1, ADF1		13.7
PF3D7_1020200	kinesin		99.1
PF3D7_0724900	kinesin-like protein		222.6
PF3D7_1246400	myosin A tail domain interacting protein, MTIP	IMC component	23.5
PF3D7_1251200	coronin	Actin-binding protein	69.0
PF3D7_0708000	cytoskeleton-associated protein	Cap-Gly domain	103.6
PF3D7_0515700	glideosome-associated protein 40, GAP40	10 TMs, IMC component	51.8
PF3D7_0918000	glideosome-associated protein 50, GAP50	SP, 2 TMs, IMC component	44.6
PF3D7_1351700	alveolin, ALV6	IMC component	151.2
Proteostasis (Protein trafficking, stabilization and degradation)			
PF3D7_0807500	proteasome subunit alpha type 6	Component oft he proteasome core particle	29.5
PF3D7_1466300	proteasome subunit RPN2	Proteasome non-ATPase regulatory subunit 1	132.8
PF3D7_0815800	Vacuolar sorting protein, VPS9	Ortholog of *Saccharomyces cerevisae* vacuolar sorting protein 9	214.7
PF3D7_1136400	conserved *Plasmodium* protein	TPR domain, mediates protein interactions and multi-protein complexes	107.1
PF3D7_0215400	conserved Plasmodium protein	WD40-domain protein, assembly and stabilization of MPCs	110.8

PF3D7_0818900	heat shock protein 70, HSP70-1		73.9
PF3D7_0917900	heat shock protein 70, HSP70-2		72.4
PF3D7_0306200	activator of Hsp90 ATPase, AHA1		41.6
PF3D7_0708400	heat shock protein 90, HSP90		86.2
PF3D7_0102200	ring-infected erythrocyte surface antigen, RESA	PHIST and DnaJ domains	126.5
Metabolism, general			
mal_mito_2	cytochrome c oxidase subunit I, cox1	SP, 12 TMs, component of the respiratory chain in mitochondria	53.0
PF3D7_1444800	fructose 1,6-biphosphate aldolase	Glycolysis pathway	40.1
PF3D7_0806800	vacuolar proton translocating ATPase subunit A	6 TMs	123.0
PF3D7_0922200	S-adenosylmethioninesynthetase, SAMS		44.8
PF3D7_0801900	conserved Plasmodium protein	FAD/NADP-binding domain	552.0
PF3D7_0403200	conserved Plasmodium protein	Possibly acetyl-CoA-synthetase	69.4
PF3D7_0511800	myo-inositol 1-phosphate synthase		69.1
PF3D7_0104400	4-hydroxy-3-methylbut-2-enyl diphosphatereductase, LytB	1 TM, protein export domain, non-mevalonate pathway	62.5
cell surface and PVM			
PF3D7_1035300	glutamate-rich protein; GLURP	SP, on parasite surface	141.1
PF3D7_1335300	reticulocyte binding protein 2, RH2b	1 TM, on parasite surface, involved in erythrocyte binding	382.9
PF3D7_1228600	merozoite Surface Protein 9, MSP-9	SP, on merozoite surface, involved in erythrocyte binding	86.6
PF3D7_1028700	merozoite TRAP-like protein, MTRAP	SP, 1 TM, belongs to TRAP family, in micronemes and on merozoite surface	58.1

PF3D7_0731500	erythrocyte binding antigen 175, EBA-175	SP, 1 TM, Duffy binding domain, on merozoite surface	174.6
PF3D7_0102500	erythrocyte binding antigen-181, EBA-181	SP, 2 TMs, Duffy binding domain, on merozoite surface	181.2
PF3D7_1035700	duffy binding-like merozoite surface protein, DBLMSP	SP, Duffy binding domain, SPAM	80.3
PF3D7_0202500	early transcribed membrane protein 2, ETRAMP2	SP, 1 TM, integral PVM protein	11.5
PF3D7_0423700	early transcribed membrane protein 4, ETRAMP4	SP, 2TMs, integral PVM protein	10.2
PF3D7_1033200	early transcribed membrane protein 10.2, ETRAMP 10.2	SP, 2TMs, integral PVM protein	38.9
PF3D7_1103500	CPW-WPC family protein	SP, adhesin, cysteine-rich CPW-WPC domain	67.3
PF3D7_0406200	Pfs16	SP, 2 TMs, integral PVM protein of gametocytes	16.7
PF3D7_0930300	merozoite surface protein 1, MSP1	SP, GPI anchor, EGF domain, on merozoite surface, part of MSP complex	195.7
PF3D7_1218800	conserved Plasmodium protein, PSOP17	SP, lectin domain, laminin G2 domain	39.6
PF3D7_0707300	rhoptry-associated membrane antigen, RAMA	GPI anchor, involved in erythrocyte binding	103.6
PF3D7_1021900	10b antigen	Ankyrin repeat region, PHAX RNA-binding domain, inter-acts with surface proteins like PfEMP1 (Y2H)	267.4
Others			
PF3D7_1103100	60S ribosomal protein P1		13.0
PF3D7_1027800	60S ribosomal protein L3		44.2
PF3D7_1408600	40S ribosomal protein S8e		25.1
PF3D7_1358800	40S ribosomal protein S13		17.3
PF3D7_0206200	metabolite/drug transporter	11 TMs, Major facilitator superfamily	62.7

PF3D7_1207700	blood stage antigen 41–3 precursor	SP	43.4
PF3D7_1457000	signal peptide peptidase, SPP	8 TMs	47.6
PF3D7_0320700	signal peptidase complex subunit 2, SPC2	2 TMs, SPC25 domain	21.0
PF3D7_0207700	serine repeat antigen 4, SERA-4	SP, papain family cysteine proteinase	108.7
PF3D7_1033800	plasmepsin VII	SP, 1 TM, aspartyl protease	52.3
PF3D7_0930000	procollagen lysine 5-dioxygenase	2 TMs	66.8
PF3D7_0817600	conserved Plasmodium protein		112.2
PF3D7_0403800	conserved Plasmodium protein	Alpha/beta-hydrolase	83.4
PF3D7_0709900	conserved Plasmodium membrane protein	4 TMs, alpha/beta-hydrolase	274.0
PF3D7_0311600	conserved Plasmodium protein, unknown function	SP, 1 TM, ribophorin-I domain,putativedolichyl-diphospho-oligosaccharide-protein	84.0
Unknown			
PF3D7_0904200	conserved Plasmodium protein	SP, 1 TM	34.0
PF3D7_1024800	conserved Plasmodium protein	SP, 2 TMs	171.1
PF3D7_1239400	conserved Plasmodium protein	SP	22.0
PF3D7_0730800.1	Plasmodium exported protein	SP	32.1
PF3D7_1316700	conserved Plasmodium protein	SP	73.4
PF3D7_0601900	conserved Plasmodium protein	1 TM	14.7
PF3D7_0704100	conserved Plasmodium membrane protein	6 TMs	425.3
PF3D7_1023000	conserved Plasmodium membrane protein	5 TMs	48.9
PF3D7_1439600	conserved Plasmodium protein	1 TM	22.6
PF3D7_1021700	conserved Plasmodium membrane protein	4 TMs	830.2

PF3D7_0417400	conserved Plasmodium protein	2 TMs, interacts with PF3D7_1021700 (Y2H)	822.4
PF3D7_1026600	conserved Plasmodium protein	Interacts with RNA-associated proteins (Y2H)	196.5
PF3D7_1362600	conserved Plasmodium protein	Interacts with splicing factor 3B subunit 2 (Y2H)	32.1
PF3D7_0202400	conserved Plasmodium protein	Pfg27 domain	141.9
PF3D7_1124200	conserved Plasmodium protein	UAS domain	49.3
PF3D7_1452400	conserved Plasmodium protein		107.5
PF3D7_1225600	conserved Plasmodium protein		93.1
PF3D7_1451200	conserved Plasmodium protein		176.2
PF3D7_1438800	conserved Plasmodium protein		84.0
PF3D7_1114600	conserved Plasmodium protein		39.6
PF3D7_1026100	conserved Plasmodium protein		23.9
PF3D7_1230100	conserved Plasmodium protein		61.5
PF3D7_1321000	conserved Plasmodium protein		80.6
PF3D7_0411000	conserved Plasmodium protein		179.7
PF3D7_1126700	conserved Plasmodium protein		110.1
PF3D7_0508900	conserved Plasmodium protein		370.2

IMC, inner membrane complex; PVM, parasitophorous vacuole membrane; SP, signal peptide; TM, transmembrane; Y2H, yeast-two-hybrid.

Table 15: Comparison of genes identified by SSH with *P. falciparum* and *P. berghei* gametocyte proteome data (analysis done by Gunnar Rudolf Mair).

			PF	*P. falciparum* GC				*P. berghei* male/female GC							total	
PF_ID	Gene Ontology	MW	troph	I & II	IV & V	V	I.II.IV.V	FG1	FG 3	FG (1+3)	MG1	MG3	MG (1+3)	FG +MG	FG.MG.PF	PB_ID
PF3D7_0210600	signalling	53.7	0	0	0	0	0	0	0	0	0	0	0	0	0	PBANKA_030750
PF3D7_0214600	signalling	204.3	0	0	0	0	0	0	0	0	0	0	0	0	0	PBANKA_031140
PF3D7_0302100	signalling	157.4	2	3	1	7	11	0	0	0	0	0	0	0	11	PBANKA_040110
PF3D7_0303900	signalling	23.2	0	4	4	11	19	0	0	0	0	0	0	0	19	PBANKA_040250
PF3D7_0515300	signalling	255.9	0	0	0	0	0	0	0	0	0	0	0	0	0	PBANKA_111490
PF3D7_0530200	signalling	59.8	0	0	0	3	3	0	0	0	0	0	0	0	3	PBANKA_124460
PF3D7_0624000	signalling	55.3	17	20	2	24	46	6	13	19	11	10	21	40	86	PBANKA_112290
PF3D7_0818200	signalling	30.2	18	21	14	25	60	11	20	31	9	11	20	51	111	PBANKA_071260
PF3D7_0927200	signalling	189.7	0	13	2	5	20	0	0	0	0	0	0	0	20	PBANKA_082800
PF3D7_1004300	signalling	131.7	3	5	1	0	6	0	0	0	0	0	0	0	6	PBANKA_120260
PF3D7_1113100	signalling	25.1	1	2	1	3	6	0	0	0	0	0	0	0	6	PBANKA_093450
PF3D7_1230900	signalling	111.5	0	0	0	0	0	0	0	0	0	0	0	0	0	PBANKA_144560
PF3D7_1233600	signalling	146.3	0	4	0	8	12	0	0	0	0	0	0	0	12	PBANKA_144820
PF3D7_1238100	signalling	266.4	7	9	3	9	21	2	6	8	8	7	15	23	44	PBANKA_145260
PF3D7_1238900	signalling	58.9	0	0	0	0	0	0	0	0	0	0	0	0	0	PBANKA_145340
PF3D7_1356800	signalling	475.7	0	0	0	0	0	0	0	0	0	0	0	0	0	PBANKA_113310
PF3D7_1358600	signalling	118.3	0	0	0	0	0	0	0	0	0	0	0	0	0	PBANKA_113490
PF3D7_1403900	signalling	35.2	6	9	5	12	26	×	×	×	×	×	×	×	26	×
PF3D7_1433400	signalling	682.5	0	0	2	0	2	0	0	0	0	0	0	0	2	PBANKA_101150
PF3D7_1442200	signalling	118.7	0	0	1	0	1	0	0	0	0	0	0	0	1	PBANKA_130610
PF3D7_1463900	signalling	127.1	0	0	0	0	0	0	0	0	0	0	0	0	0	PBANKA_132710
PF3D7_1464600	signalling	170.5	23	31	0	3	34	0	0	0	0	0	0	0	34	PBANKA_132800
PF3D7_0420300	cell cycle & gene expr.	400.2	0	0	0	0	0	0	0	0	0	0	0	0	0	PBANKA_052170
PF3D7_0513600	cell cycle & gene expr.	129.2	10	29	0	10	39	0	0	0	0	0	0	0	39	PBANKA_111330

PF3D7_0615400	cell cycle & gene expr.	323.2	0	0	0	0	0	0	0	0	0	0	0	0	0	PBANKA_123010
PF3D7_0622900	cell cycle & gene expr.	237.4	0	0	0	0	0	0	0	0	0	0	0	0	0	PBANKA_112180
PF3D7_0811300	cell cycle & gene expr.	200.2	0	2	1	2	5	0	0	0	0	0	0	0	5	PBANKA_142620
PF3D7_0827800	cell cycle & gene expr.	283.6	0	0	1	0	1	0	0	0	0	0	0	0	1	PBANKA_070290
PF3D7_1011800	cell cycle & gene expr.	131.6	41	22	6	17	45	4	11	15	14	0	14	29	74	PBANKA_121020
PF3D7_1018500	cell cycle & gene expr.	12.5	0	0	0	0	0	0	0	0	0	0	0	0	0	PBANKA_050270
PF3D7_1019000	cell cycle & gene expr.	280.8	7	0	0	0	0	0	0	0	0	0	0	0	0	PBANKA_050320
PF3D7_1034900	cell cycle & gene expr.	101.2	21	31	0	49	80	6	0	6	3	0	3	9	89	PBANKA_051870
PF3D7_1103800	cell cycle & gene expr.	392.0	7	12	0	23	35	0	0	0	0	0	0	0	35	PBANKA_094310
PF3D7_1105500	cell cycle & gene expr.	20.1	1	0	0	0	0	0	0	0	0	0	0	0	0	PBANKA_094140
PF3D7_1115200	cell cycle & gene expr.	94.3	0	0	0	0	0	0	0	0	0	0	0	0	0	PBANKA_093250
PF3D7_1347500	cell cycle & gene expr.	42.2	18	15	30	22	67	11	0	11	7	10	17	28	95	PBANKA_136030
PF3D7_1360900	cell cycle & gene expr.	44.8	8	11	11	12	34	×	×	×	×	×	×	×	34	×
PF3D7_1417200	cell cycle & gene expr.	519.8	5	6	0	6	12	0	0	0	0	0	0	0	12	PBANKA_102550
PF3D7_1445900	cell cycle & gene expr.	60.0	16	25	3	21	49	0	0	0	1	0	1	1	50	PBANKA_130970
PF3D7_1469700	cell cycle & gene expr.	24.6	0	2	0	3	5	0	0	0	0	0	0	0	5	PBANKA_133290
PF3D7_0422300	IMC, cy-toskeleton	49.7	11	25	13	30	68	0	0	0	0	0	0	0	68	PBANKA_052270
PF3D7_0503400	IMC, cy-toskeleton	13.7	6	5	1	8	14	0	0	0	0	1	1	1	15	PBANKA_110310

PF3D7_0515700	IMC, cytoskeleton	51.8	3	0	0	7	7	1	0	1	0	0	0	1	8	PBANKA_111530
PF3D7_0708000	IMC, cytoskeleton	103.6	0	0	2	12	14	0	0	0	1	0	1	1	15	PBANKA_080520
PF3D7_0724900	IMC, cytoskeleton	222.6	0	0	0	0	0	0	0	0	0	0	0	0	0	PBANKA_062240
PF3D7_0918000	IMC, cytoskeleton	44.6	14	12	1	23	36	0	0	0	0	0	0	0	36	PBANKA_081900
PF3D7_1020200	IMC, cytoskeleton	99.1	0	0	0	0	0	0	0	0	0	0	0	0	0	PBANKA_050430
PF3D7_1246200	IMC, cytoskeleton	41.2	23	28	13	29	70	12	19	31	6	6	12	43	113	PBANKA_145930
PF3D7_1246400	IMC, cytoskeleton	23.5	1	0	0	3	3	0	0	0	0	0	0	0	3	PBANKA_145950
PF3D7_1251200	IMC, cytoskeleton	69.0	1	0	0	0	0	0	0	0	0	0	0	0	0	PBANKA_146410
PF3D7_1351700	IMC, cytoskeleton	151.2	0	0	1	0	1	0	0	0	0	0	0	0	1	PBANKA_136440
PF3D7_0102200	proteostasis	126.5	33	3	1	0	4	×	×	×	×	×	×	×	4	×
PF3D7_0215400	proteostasis	110.8	0	0	1	0	1	0	0	0	0	0	0	0	1	PBANKA_031210
PF3D7_0306200	proteostasis	41.6	4	7	0	8	15	0	0	0	0	0	0	0	15	PBANKA_040460
PF3D7_0708400	proteostasis	86.2	54	60	17	82	159	7	0	7	8	10	18	25	184	PBANKA_080570
PF3D7_0807500	proteostasis	29.5	11	11	2	20	33	3	8	11	4	2	6	17	50	PBANKA_122310
PF3D7_0815800	proteostasis	214.7	0	3	0	0	3	0	0	0	0	0	0	0	3	PBANKA_071500
PF3D7_0818900	proteostasis	73.9	45	46	33	57	136	34	40	74	31	28	59	133	269	PBANKA_071190
PF3D7_0917900	proteostasis	72.4	45	40	42	51	133	24	46	70	16	16	32	102	235	PBANKA_081890
PF3D7_1136400	proteostasis	107.1	0	3	0	11	14	1	0	1	0	0	0	1	15	PBANKA_091220
PF3D7_1466300	proteostasis	132.8	23	26	3	34	63	4	9	13	4	0	4	17	80	PBANKA_132970
mal_mito_2	metabolism general	53.0	0	0	0	0	0	0	0	0	0	0	0	0	0	PBANKA_MIT0002
PF3D7_0104400	metabolism general	62.5	0	1	1	3	5	0	0	0	0	0	0	0	5	PBANKA_020870

PF3D7_0403200	metabolism general	69.4	0	0	0	0	0	0	0	0	0	0	0	0	0	PBANKA_100080
PF3D7_0511800	metabolism general	69.1	36	29	2	40	71	2	0	2	0	0	0	2	73	PBANKA_111140
PF3D7_0801900	metabolism general	552.0	0	0	1	0	1	0	0	0	0	0	0	0	1	PBANKA_122830
PF3D7_0806800	metabolism general	123.0	11	9	4	29	42	0	0	0	0	3	3	3	45	PBANKA_122380
PF3D7_0922200	metabolism general	44.8	25	16	0	4	20	1	2	3	0	0	0	3	23	PBANKA_082310
PF3D7_1444800	metabolism general	40.1	21	23	11	35	69	17	23	40	22	22	44	84	153	PBANKA_130860
PF3D7_0102500	cell surface & PVM	181.2	0	0	0	0	0	0	0	0	0	0	0	0	0	PBANKA_133270
PF3D7_0202500	cell surface & PVM	11.5	1	0	0	0	0	×	×	×	×	×	×	×	0	×
PF3D7_0406200	cell surface & PVM	16.7	3	9	34	17	60	×	×	×	×	×	×	×	60	×
PF3D7_0423700	cell surface & PVM	10.2	0	5	0	2	7	×	×	×	×	×	×	×	7	×
PF3D7_0707300	cell surface & PVM	103.6	3	0	0	0	0	0	0	0	0	0	0	0	0	PBANKA_080450
PF3D7_0731500	cell surface & PVM	174.6	0	0	0	0	0	0	0	0	0	0	0	0	0	PBANKA_133270
PF3D7_0930300	cell surface & PVM	195.7	77	0	3	0	3	0	0	0	0	0	0	0	3	PBANKA_083100
PF3D7_1021900	cell surface & PVM	267.4	11	26	4	31	61	0	0	0	0	0	0	0	61	PBANKA_112590/ PBANKA_141230
PF3D7_1028700	cell surface & PVM	58.1	0	0	1	11	12	0	0	0	0	0	0	0	12	PBANKA_051280
PF3D7_1033200	cell surface & PVM	38.9	11	16	0	4	20	×	×	×	×	×	×	×	20	×
PF3D7_1035300	cell surface & PVM	141.1	0	0	1	0	1	×	×	×	×	×	×	×	1	×
PF3D7_1035700	cell surface & PVM	80.3	10	0	0	0	0	×	×	×	×	×	×	×	0	×

PF3D7_1103500	cell surface & PVM	67.3	0	0	0	0	0	0	0	0	0	0	0	0	PBANKA_094340
PF3D7_1218800	cell surface & PVM	39.6	0	0	2	5	7	0	0	0	0	0	0	0	7	PBANKA_143440
PF3D7_1228600	cell surface & PVM	86.6	23	0	0	0	0	0	0	0	0	0	0	0	0	PBANKA_144330
PF3D7_1335300	cell surface & PVM	382.9	0	0	0	0	0	0	0	0	0	0	0	0	0	PBANKA_000220 + 10 more
PF3D7_0206200	others	62.7	0	0	0	2	2	0	0	0	0	0	0	0	2	PBANKA_030390
PF3D7_0207700	others	108.7	11	0	0	0	0	0	0	0	0	0	0	0	0	PBANKA_030480
PF3D7_0311600	others	84.0	0	0	0	5	5	0	0	0	0	0	0	0	5	PBANKA_040960
PF3D7_0320700	others	21.0	0	0	0	8	8	0	0	0	0	0	0	0	8	PBANKA_121780
PF3D7_0403800	others	83.4	0	0	0	0	0	0	0	0	0	0	0	0	0	PBANKA_100150
PF3D7_0709900	others	274.0	0	0	0	0	0	0	0	0	0	0	0	0	0	PBANKA_122050
PF3D7_0817600	others	112.2	0	0	0	0	0	0	0	0	0	0	0	0	0	PBANKA_071320
PF3D7_0930000	others	66.8	0	0	0	0	0	0	0	0	0	0	0	0	0	PBANKA_083070
PF3D7_1027800	others	44.2	17	9	14	18	41	0	0	0	0	0	0	0	41	PBANKA_051190
PF3D7_1033800	others	52.3	0	0	0	0	0	0	0	0	0	0	0	0	0	PBANKA_051760
PF3D7_1103100	others	13.0	2	2	5	4	11	1	0	1	0	0	0	1	12	PBANKA_094360
PF3D7_1207700	others	43.4	0	0	0	0	0	0	0	0	0	0	0	0	0	PBANKA_060620
PF3D7_1358800	others	17.3	8	6	4	9	19	8	5	13	1	0	1	14	33	PBANKA_113510
PF3D7_1408600	others	25.1	8	8	7	12	27	2	5	7	2	0	2	9	36	PBANKA_103390
PF3D7_1457000	others	47.6	0	0	0	0	0	2	0	2	0	1	1	3	3	PBANKA_132070
PF3D7_0202400	unknown	141.9	27	0	0	0	0	×	×	×	×	×	×	×	0	×
PF3D7_0411000	unknown	179.7	0	0	0	0	0	0	0	0	0	0	0	0	0	PBANKA_061240
PF3D7_0417400	unknown	822.4	0	0	1	0	1	0	0	0	0	0	0	0	1	PBANKA_071950
PF3D7_0508900	unknown	370.2	0	2	0	0	2	0	0	0	0	0	0	0	2	PBANKA_110850
PF3D7_0601900	unknown	14.7	0	0	0	1	1	×	×	×	×	×	×	×	1	×
PF3D7_0704100	unknown	425.3	0	0	0	0	0	0	0	0	0	0	0	0	0	PBANKA_080180

PF3D7_0730800.1	unknown	32.1	5	0	0	0	0	×	×	×	×	×	×	×	0	×
PF3D7_0904200	unknown	34.0	0	1	0	0	1	0	0	0	0	0	0	0	1	PBANKA_041720
PF3D7_1021700	unknown	830.2	0	0	0	1	1	1	0	1	0	0	0	1	2	PBANKA_050590
PF3D7_1023000	unknown	48.9	0	0	0	0	0	0	0	0	0	0	0	0	0	PBANKA_050720
PF3D7_1024800	unknown	171.1	4	33	15	33	81	0	0	0	0	0	0	0	81	PBANKA_050900
PF3D7_1026100	unknown	23.9	0	0	0	0	0	0	0	0	0	0	0	0	0	PBANKA_051030
PF3D7_1026600	unknown	196.5	0	0	0	0	0	×	×	×	×	×	×	×	0	×
PF3D7_1114600	unknown	39.6	0	0	0	0	0	0	0	0	0	0	0	0	0	PBANKA_093310
PF3D7_1124200	unknown	49.3	0	0	0	7	7	0	0	0	1	0	1	1	8	PBANKA_092410
PF3D7_1126700	unknown	110.1	0	1	0	0	1	0	0	0	0	0	0	0	1	PBANKA_092170
PF3D7_1225600	unknown	93.1	0	1	0	0	1	0	0	0	0	0	0	0	1	PBANKA_144050
PF3D7_1230100	unknown	61.5	0	0	0	0	0	0	0	0	0	0	0	0	0	PBANKA_144480
PF3D7_1239400	unknown	22.0	0	0	0	0	0	0	0	0	0	0	0	0	0	PBANKA_145390
PF3D7_1316700	unknown	73.4	0	0	0	0	0	0	0	0	0	0	0	0	0	PBANKA_141520
PF3D7_1321000	unknown	80.6	0	0	0	0	0	0	0	0	0	0	0	0	0	PBANKA_141930
PF3D7_1362600	unknown	32.1	0	0	0	0	0	0	0	0	0	0	0	0	0	PBANKA_113860
PF3D7_1438800	unknown	84.0	0	0	0	2	2	0	0	0	0	0	0	0	2	PBANKA_130270
PF3D7_1439600	unknown	22.6	0	6	0	10	16	1	0	1	0	0	0	1	17	PBANKA_130350
PF3D7_1451200	unknown	176.2	0	0	0	0	0	0	0	0	0	0	0	0	0	PBANKA_131490
PF3D7_1452400	unknown	107.5	0	0	0	0	0	0	0	0	0	0	0	0	0	PBANKA_131610

PF troph, peptide hits for gametocyte-less *P.falciparum* trophozoite (Silvestrini et al., 2005; 2010); I &II, stage I & II *P. falciparum* gametcoytes (Silvestrini et al., 2005; 2010); V, stage V *P. falciparum* gametocytes (Florens et al. 2002) ; I.II.IV.V, sum of all *P. falciparum* gametocyte data; FG1, peptide hits from highly purified female *P. berghei* gametocyte sample 1 (Khan et al., 2005); FG3, peptide hits from highly purified female *P. berghei* gametocyte sample 3 (Khan et al., 2005); FG(1+3), sum of both; MG1, peptide hits from highly purified male *P. berghei* gametocyte sample 1(Khan et al., 2005); MG3, peptide hits from highly purified male *P. berghei* gametocyte sample 3 (Khan et al., 2005);MG(1+3), sum of both; FG+MG, total number peptide hits from all four *P. berghei* gametocyte samples; total FG.MG.PF, total number of *P. falciparum* and *P. berghei* gametocyte peptide hits.

3.2.3 Genes with transcript changes regulated by DOZI and CITH

We further wanted to know which of the genes with changes in transcripts levels following gametocyte activation were regulated by DOZI and CITH. DOZI and CITH regulated genes are candidate genes for translational repression in non-activated gametocytes and may play important roles in zygote to ookinete development in the mosquito. To this end, we compared the *P. berghei* orthologs of the identified *P. falciparum* genes with a global gametocyte transcriptome analysis of *P. berghei* DOZI and CITH gene deletion mutants; destabilized mRNAs are candidates for translational repression in non-activated gametocytes (Mair et al., 2006; 2010). The comparison was done with the help of Gunnar R. Mair. A total of 11 of the SSH identified genes (9.7%) were identified regulated by DOZI and CITH (Table 16); 8 of those lack any gametocyte protein evidence. One of the destabilized genes was identified as a single peptide hit while the remaining two destabilized genes had 19 and 74 peptide hits in total.

Table 16: Genes identified by SSH with *P. berghei* orthologs showing destabilization in the absence of DOZI and CITH (analysis done by Gunnar R. Mair).

PF _ID	P	Protein	Predicted feature	MW	*P. berghei* ortholog gene ID	DOZI	CITH
PF3D7_1011800	74	QF122 antigen	KH domain type 1, RNA binding	131.6	PBANKA_121020	1.4	1.1
PF3D7_0724900	0	kinesin-like protein		222.6	PBANKA_062240	2.4	1.2
PF3D7_1033800	0	plasmepsin VII	SP, 1 TM, aspartyl protease	52.3	PBANKA_051760	2.1	1.3
PF3D7_1316700	0	conserved Plasmodium protein	SP	73.4	PBANKA_141520	2.1	1.4
PF3D7_1321000	0	conserved Plasmodium protein		80.6	PBANKA_141930	1.8	1.6
PF3D7_0303900	19	conserved Plasmodium protein	SP, phosphatidyl-ethanolamine-binding protein PEBP domain	23.2	PBANKA_040250	2.2	1.8
PF3D7_1103500	0	CPW-WPC family protein	SP, CPW-WPC domain	67.3	PBANKA_094340	1.3	2.3
PF3D7_1115200	0	SET domain protein, SET7	Chromatin regulator	94.3	PBANKA_093250	2.5	2.3
PF3D7_1207700	0	blood stage antigen 41-3 precursor	SP	43.4	PBANKA_060620	2.5	2.8
PF3D7_0904200	1	conserved Plasmodium protein	SP, 1 TM	34	PBANKA_041720	1.9	3.2
PF3D7_1362600	0	conserved Plasmodium protein	Interacts with splicing factor 3B subunit 2	32.1	PBANKA_113860	3.4	3.5

P, total number of peptides identified in *P. falciparum* and *P. berghei* gametocytes (Florens et al., 2002; Khan et al., 2005; Silvestrini et al., 2005; 2010); DOZI and CITH, $\log_2$ values of transcript abundance WT compared to DOZI-KO, or WT compared to CITH KO (Mair et al., 2010).

3.2.4 Quantification of transcript levels by qRT-PCR

In order to confirm transcript changes in gametocytes during activation and to determine if these genes become up or down regulated following gametocyte activation, qRT-PCR was conducted on a subset of the genes identified by SSH. Total RNA was isolated from immature (stages III and IV) and mature (stage V) gametocytes of *P. falciparum* strain NF54 and from activated gametocytes at 30 min post-activation (p.a.). Furthermore, we isolated RNA from mixed asexual blood stages of the gametocyte-less *P. falciparum* strain F12. Initially, the synthesized cDNA of each sample was tested for its stage-specificity by diagnostic RT-PCR, using primers for the asexual blood stage gene ama-1 (Peterson et al., 1989; Narum et al., 1994) and for the gametocyte-specific gene pfccp2 (Pradel et al., 2004). The tests confirmed that no pfccp2 transcript was present in the F12 sample, while no ama-1 expression was detected in the purified gametocyte samples of strain NF54 (Figure 31). The absence of ama-1 signals in the gametocyte samples and the absence of pfccp2 in the F12 asexual blood stage sample further showed that these were devoid of any contamination by genomic DNA (gDNA). An additional test for gDNA contamination was performed using primers specific for the gene hdac1, a gene expressed in all the stages studied. In all parasite samples, i.e. in samples of F12 asexual blood stages as well as in NF54 immature, mature and activated gametocytes, hdac1 transcript was present (Figure 31), as shown by diagnostic RT-PCR. In sample preparations lacking reverse transcriptase, on the other hand, no hdac1-specific PCR bands were detected.

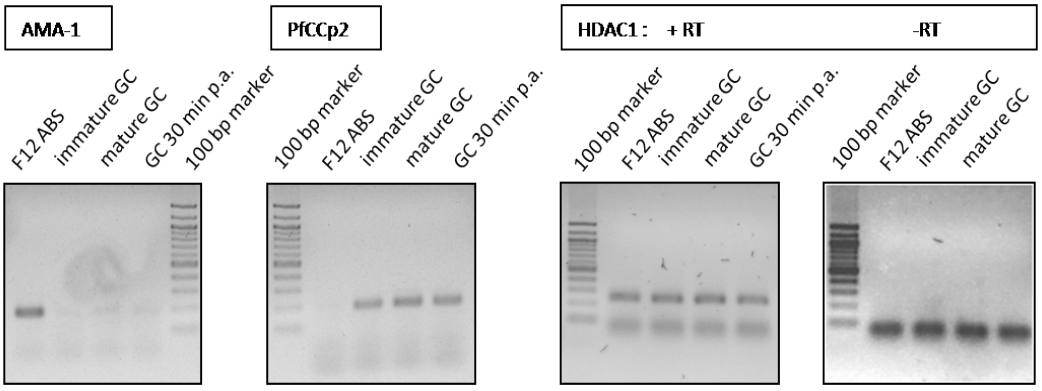


Figure 31: Diagnostic RT-PCR to check for gDNA contamination and stage specific purity of samples (PCR done by Matthias Scheuermayer).The diagnostic RT-PCR revealed the presence of stage-specific transcript in the asexual blood stages of the gametocyte-less strain F12 (F12 ABS) as well as in immature (stage III/IV) and mature (stage V) non-activated gametocytes (GC) and in gametocytes at 30 min p.a. in vitro. ama-1, marker for ABS; pfccp2, marker for GC. Transcript of gene hdac1 was detected in all stages investigated, while no hdac1-specific PCR product was detected in samples lacking reverse transcriptase (RT).

For qRT-PCR, we chose 34 genes from the SSH analysis for comparison of transcript abundance, with representatives from all ontology groups. The gene Pfs16 was selected as an internal control, because it is known to be highly expressed in gametocytes throughout development, while it is absent in the gametes (Sologub et al., 2011; Eksi et al., 2011; 2012). We also added two sexual stage-specific genes, actinII and pfs25 as external controls and the gene su α5, encoding for subunit (SU) α5 of the α-ring of the proteasome core particle (Aminake et al., 2012) was also included in the investigations to see if the proteosome plays an important role following gametocytes activation.

Transcript expression levels were measured via real-time RT-PCR and calculated by the $2^{-\Delta Ct}$ method (Livak et al., 2001; Sugden et al., 2009) in which the threshold cycle number (Ct) was normalized to the Ct of the endogenous control gene encoding for *P. falciparum* serylt RNA synthetase (PF3D7_0717700) as reference gene (Salanti et al., 2003, Wang et al., 2010). We considered transcript levels with $2^{-\Delta Ct}$ values below 0.5 as negligible. The results of the qRT-PCR are shown on Figure 33. Real-time RT-PCR revealed transcription in mature gametocytes for 29 out of the 34. Out of these, 20 genes showed increased transcript expression in gametocytes compared to asexual blood stage parasites.

We identified 8 genes i.e. PF3D7_0827800 (set3), Pf3D7_1246200 (actin1), PF3D7_1218800 (psop17), PF3D7_1239400, PF3D7_0704100, PF3D7_0417400, PF3D7_1321000 and PF3D7_1316700 for which transcript levels were increased in activated gametocytes as compared to asexual blood stages, immature gametocytes and non-activated gametocytes, indicating that these genes may play an important role downstream of gametocyte activation in the mosquito midgut. Twelve genes had an increased transcript expression in gametocytes compared to asexual blood stage parasites, and transcript levels either remained constant, decreased or increased following activation. These include PF3D7_0302100 (clk4), PF3D7_0818200 (14-3-3), PF3D7_1115200 (set7), PF3D7_0422300 (α-tubulin II), PF3D7_0708000, PF3D7_1103500 (encoding for a CPW-WPC protein), PF3D7_406200 (pfs16), PF3D7_1457000 (spp), PF3D7_0817600 (unknown protein termed PPLP6 because it was initially predicted to posses a MAC/PC domain which no more exist following the recent anotation), PF3D7_1225600, PF3D7_1438800 and PF3D7_1023000. Five genes were predominantly expressed in the asexual blood stages and showed a resurgence in transcript expression in gametocytes during maturation, i.e. PF3D7_1238900

(pk2), PF3D7_1136400 (encoding for a tetratrico peptide repeat region; TPR), PF3D7_1021700, PF3D7_1026600 and PF3D7_1251200 (coronin). For four genes, PF3D7_0818900 (hsp70-1), PF3D7_080700 (su α6), PF3D7_0807500 (gap50), and PF3D7_1444800 (encoding for fructose 1,6 bisphosphate aldolase, FBPA) expression was high in asexual blood stages and decreased during gametocyte development and following activation. Transcript expression for five genes, PF3D7_0815800 (vsp9), PF3D7_0215400 (encoding for a WD40 motif), PF3D7_1351700 (alv6), PF3D7_1033200 (etramp10.2), and PF3D7_0207700 (sera4), was negligible in gametocytes.

3.2.5 Changes in protein expression of SSH selected genes

In order to determine the changes in protein expression for selected SSH genes, we used polyclonal anti-sera which were already generated in our labouratory against some of the proteins. Also, we generated polyclonal antibodies against PF3D7_0818200 (14-3-3) in this study. This was done together with Olivier Levy (Diploma student). Full length of the 14-3-3 gene was cloned in the pGEX-4T1 plasmid and the GST-tagged protein was expressed in *E.coli* BL21 (DE3) RIL. The purified 56 KDa GST fusion protein (Figure 32A) was used to generate antibodies against *P. falciparum* 14.3.3 protein by immunization of mice. The recognition of 14.3.3 protein by the generated anti-sera was investigated by western blot analysis using parasite lysates of F12 asexual, immature gametocytes, mature gametocytes and activated gametocytes. Western blotting detected one prominent protein band in the F12 mixed asexual stages (F12), immature gametocytes (IMG), non anctivated mature gametocytes (naGC) and activated gametocyte (aGC) lysate with a molecular weight approximately 32 KDa (Figure 32B) which is 2 KDa bigger than the expected molecular weight of the 14-3-3 protein. Similar results have been obtained by Al-Khedery and colleagues (Al-Khedery et al., 1999) who reported that despite the fact that the *P. knowlesi* 14-3-3 and *P. falciparum* 14-3-3 have equal sizes, the *P. knowlesi* protein migrates at the right size of 30 KDa as compared to the *P. falciparum* counterpart which migrates on SDS-PAGE gel at a molecular weight of 32 KDa.

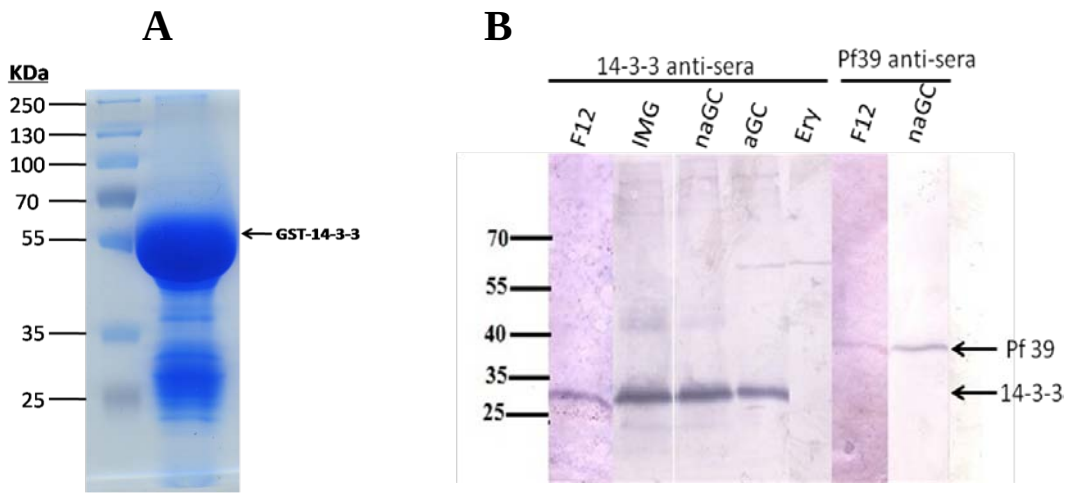


Figure 32: Purification 14-3-3 fusion protein and its protein expression in *Plasmodium* stages (Levy 2013)**.** (A), showing the 56 kDa GST-14-3-3 purified fusion protein on SDS-PAGE gel. (B), western blot analysis of protein expression in different parasite stages using polyclonal antisera against 14-3-3 protein. Pf39 was used as control. F12 represent mixed asexual blood stages, IMG; immature gametocytes, naGC; mature non- activated gametocytes, aGC; activated gametocytes, Ery; erythrocyte lysate, NMS; neutral mouse serum.

The 14-3-3 antibody together with already produced antibodies in our laboratory was then used to investigate the expression changes at the protein level. Antibodies against the proteins PK2, CLK-4, actinI and actinII, GAP50, proteasome SU α5, Pfs16, Pfs25, PPLP6 and 14-3-3 were used to immunolabel the respective proteins in samples of *P. falciparum* F12 asexual blood stages, non-activated NF54 gametocytes and gametocytes at 30 min p.a. via indirect immunofluorescence assay (Figure 34A). Asexual blood stage parasites were highlighted by MSP-1 labeling and gametocytes were highlighted by labeling with Pfs25 or Pfs230. The abundance of the respective proteins in gametocytes before and after activation was quantified by measuring the average fluorescence signal intensity of a total number of 15-20 plotted cells per setting. The results showed a significant up-regulation in the expression of PK2, actinII and Pfs25 following gametocyte activation (Figure 34B). While PK2 is also expressed in asexual blood stage parasites, actinII and Pfs25 cannot be detected in these stages. On the other hand, CLK4, proteasome SU α5, and PPLP6 were detected in asexual blood stage parasites and gametocytes during maturation, but the proteins were down-regulated in gametocytes at 30 min p.a. (Figure 34B). The 14-3-3 protein was highly expressed in asexual, gametocytes and 30 min. post activated gametocytes. While labeling of 14.3.3, CLK4 and SU α5 revealed a homogenous expression of these proteins in the parasite cytoplasm and nucleus, PPLP6-labeling exhibited a punctuated expression (Figure 34A).

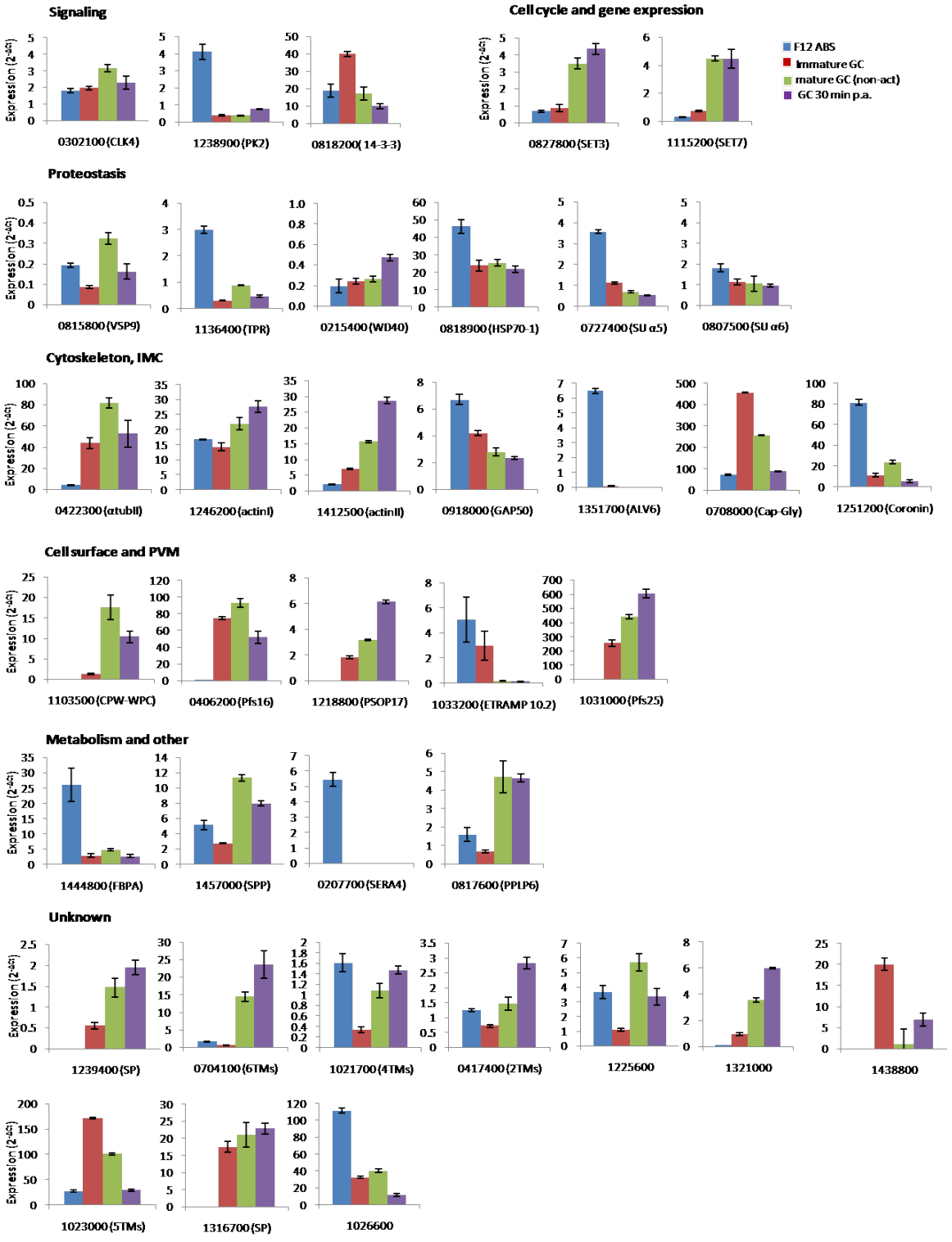
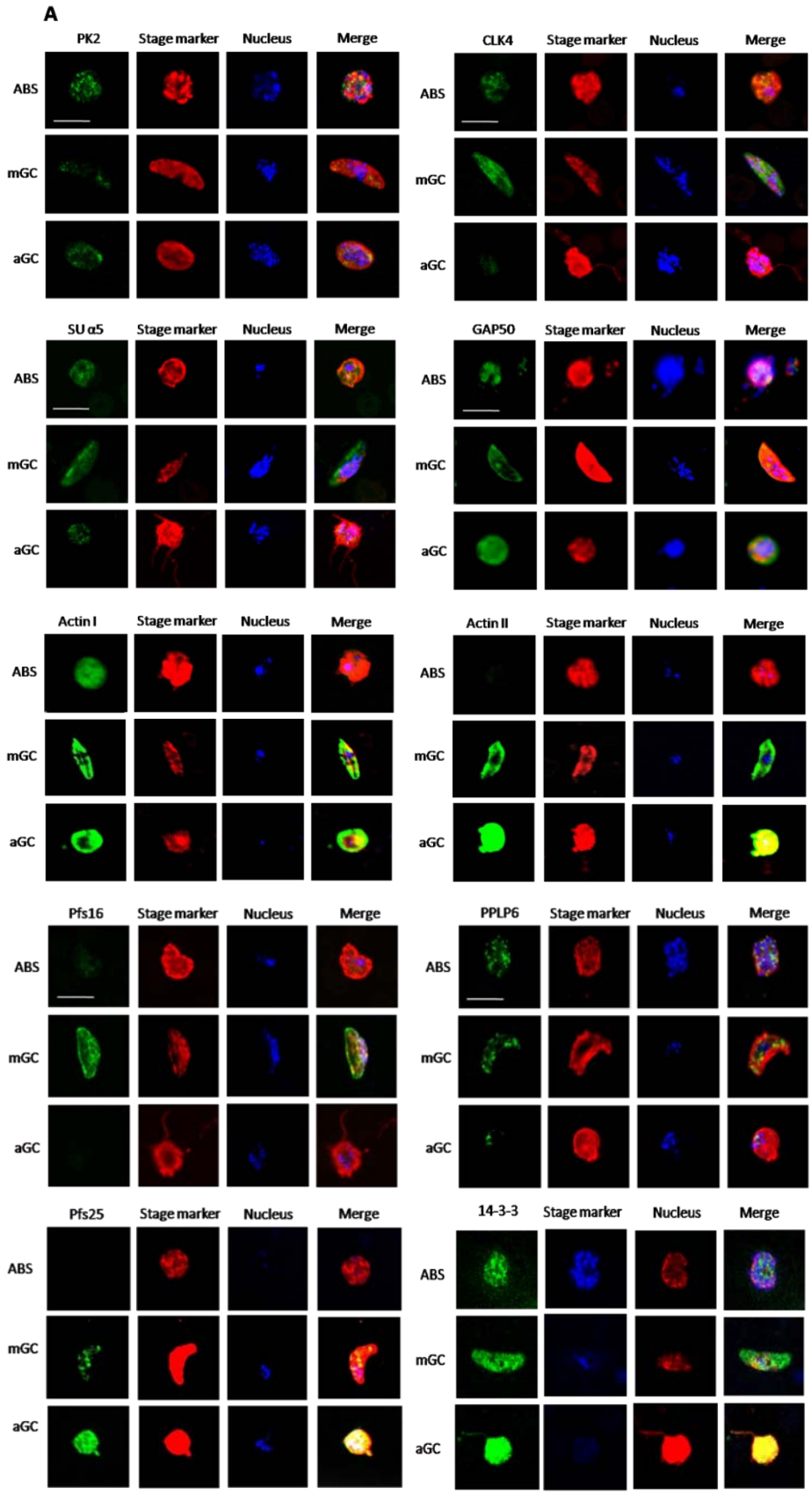
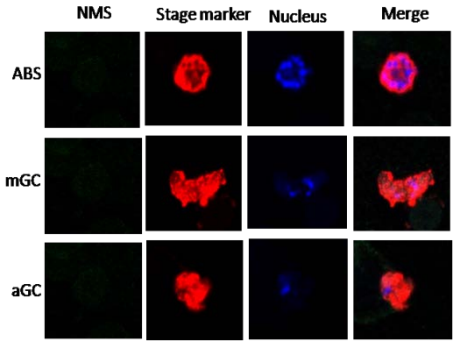


Figure 33: Changes in transcript expression levels during the formation and activation of *P. falciparum* gametocytes. Real-time RT-PCR analysis showed changes of transcript expression of 34 of the SSH-identified genes between ABS, immature and mature non-activated GCs and in GCs at 30 min p.a. in vitro. Transcript expression levels were calculated by the $2^{-\Delta Ct}$ method; the threshold cycle number (Ct) was normalized with the Ct of the gene encoding for seryl tRNA synthetase as reference.


A
PK2
Stage marker
Nucleus
Merge
CLK4
SU α5
GAP50
Actin I
Actin II
Pfs16
PPLP6
Pfs25
14-3-3
ABS
mGC
aGC



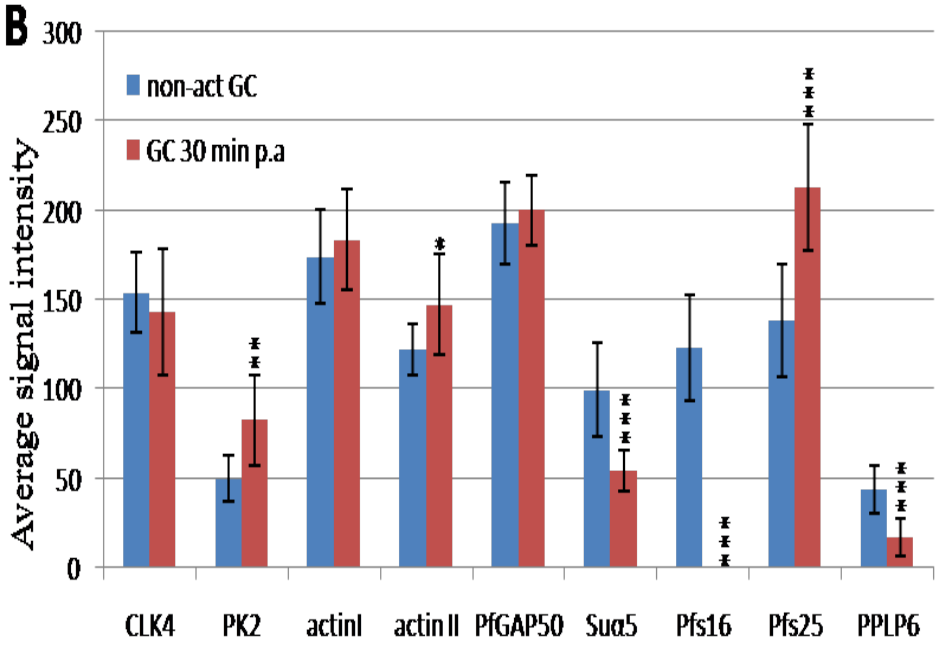


Figure 34: Changes in protein expression during formation and activation of *P. falciparum* gametocytes. (A) Immunofluorescence assays, using specific antibodies, detected the proteins of interest (in green) in asexual blood stage (ABS) parasites, in gametocytes (GC) and in gametocytes at 30 min p.a. (aGC). The parasite stages were highlighted with antibodies against stage-specific markers (in red; MSP1 for ABS; Pfs230 or Pfs25 for GCs and aGCs). Nuclei were highlighted by Hoechst nuclear stain (in blue). Bar, 5 µm. (B) Diagram showing the average signal intensity of the immunolabeled proteins in gametocytes before and at 30 min p.a.. Measurements were performed on 15-20 plotted cells per setting via quantitative confocal microscopy. Mean ± SD. *$p < 0.05$;**$p < 0.01$; ***$p < 0.001$ (student's t-test).

3.3 Role of antimicrobial molecules as transmission blocking agents

In this study, we evaluated the effect of antimicrobial molecules as transmission blocking agents to prevent the spread of malaria. The effect of harmonine, an antimicrobial defence molecule isolated from the hemolymph of the Asain ladybug *Harmonia axyridis* was tested again *P. falciparum* for its use as a transmission blocking drug. The transmission blocking effect of recombinant produced peptides (SM1, PcFK1, PcFK2) as transmission blocking agents were also investigated using the *P. berghei* model.

3.3.1 Transmission blocking effect of harmonine

Here, we tested the transmission blocking effect of harmonine which was isolated from the hemolyphm of the Asain lady bug, *Harmonia axyridis* and shown to display antimicrobial activities (Röhrich et al., 2012).

3.3.1.1 Antiplasmodial effect of harmonine

We initially tested the effect of the harmonine against the asexual stages of *P. falciparum* chloroquine-sensitive strain (3D7) and chloroquine-resistant strain (Dd2). The results showed that harmonine inhibits the growth of the asexual parasites with half-inhibitory concentration (IC_{50}) values of 4.8 and 7.6 µM for the chloroquine-sensitive strain 3D7 and the chloroquine resistant strain Dd2, respectively (Table 17). In comparison, chloroquine treatment resulted in parasite growth inhibition with IC_{50} values of 24 (3D7) and 158 nM (Dd2).

Table 17: Antimalarial and cytoxicity effect of harmonine

	IC_{50} (µM)	
	Harmonine	Chloroquine
***Plasmodium falciparum* strain**		
3D7 (asexual blood stages)	4.8 ± 0.2	0.024 ± 0.008
Dd2 (asexual blood stages)	7.6 ± 0.7	0.158 ± 0.001
NF54 (exflagellating microgametocytes)	5.8 ± 1.9	N/A
Human cells		
HeLa cells	37.0 ± 1.7	N/A

3.3.1.2 Effect of harmonine on gametocytes development and mosquito midgut stages

In order to determine the transmission blocking effect of harmonine *in vitro*, we initialy tested the toxic effect of the compound on gametocyte development. Harmonine at 4.8 µM (IC_{50} concentration) reduced the number of *P. falciparum* NF54 gametocytes to 18 % compared to 0.5 % DMSO control, 100 % reduction of gametocytes were achieved at a concentration 50 µM (Table 18). The positive control substance primaquine (IC_{50} activity on asexual blood stages 3 µM) reduced gametocyte numbers to 42%.

We further tested the effect of the harmonine on the ability of male gametocytes to exflagellate (microgemetogenesis). Exflagellation is the process by which male gametocytes become activated in the mosquito midgut after being taken up by the mosquito due to external stimuli. This process resuts in the formation of eight motile flagella-like structures which stick around red blood cells and female gametes. Exflagellation was inhibited by harmonine with an IC_{50} value of 5.8 µM (Table 17, Figure 35A).

The effect of harmonine on zygote formation was also investigated using the zygote development test. The compound reduced zygote formation to 17, 8.8 and 1.2 % at 5, 10 and 50 µM, respectively as compared to 1 % DMSO set to 100 % as control (Figure 35B).

Table 18: Gametocytocidal activity of harmonine

Compound	Concentration	Mean number of gametocytes per 1,000 RBCs
DMSO (negative control)	0.5 %	23.7 ± 2.5
Primaquine (positive control)	3.0 µM	10.0 ± 1.0
Harmonine	4.8 µM	4.3 ± 1.5
Harmonine	50.0 µM	0

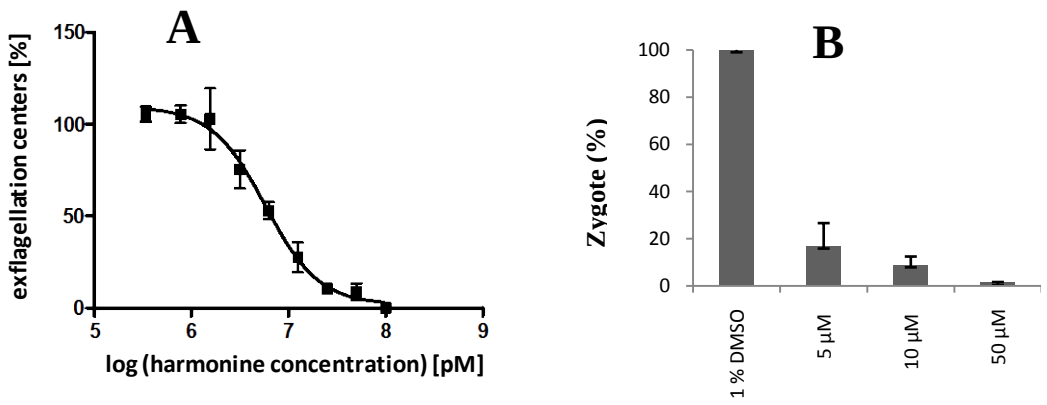


Figure 35: Effect of harmonine on microgametogenesis and zygote development. (A), Curve showing the inbitory effect of harmonine on the ability of male gametocytes to exflagellate.The relative number of exflagellation centers was determined in comparison to experiments without compound. From three independent experiments an IC_{50} of 5.8 ± 1.9 µM was determined. (B), the effect of different concentrations of harmonine on zygote formation. 1% DMSO was used as control.

3.3.1.3 *In vivo* transmission blocking effect of harmonine

To determine the *in vivo* transmission blocking effect of harmonine, *An. stephensi* mosquitoes were fed on gametocyte cultures containing either 10 µM harmonine or 1% DMSO as control and the transmission blocking effect of the compound was determined by counting the amount of oocysts in the mosquito midgut following staining with mercurochrome. The results showed that the mean oocyte numbers were 1.0 ±1.6 and 5.1 ± 5.3% in the harmonine and control groups, respectively. Also, only 45 % of the mosquitoes were found to be infected in the harmonine group compared with 91 % in the control group (Table 19), indicating a significant reduction in parasite transmission ($p < 0.05$, Mann–Whitney test).

Table 19: *In vivo* transmission blocking activity of harmonine

Treatment of gametocytes	No. mosquitoes dissected	Infected mosquitoes [%]	Oocyst no. range	Mean oocyst no.	Inhibtion [%]
DMSO (1 %)	11	91	0 - 17	5.1 ± 5.3	100
Harmonine (10 µM)	20	45	0 - 6	1.0 ± 1.6	49.5

$p < 0.05$, Mann–Whitney test

3.3.1.4 Cytotoxic effect of harmonine

We also investigated the toxic effect of harmonine on human cells. The compound showed moderated toxicity to HeLa cells with IC_{50} values of 37.0 ± 1.7 µM (Table 17)

and a selectivity index (SI) of 7.7 and 4.8 for the CQ-senstive strain (3D7) and CQ-resistant strain (Dd2) respectively. Regarding the effect of the compound on the intergrity of RBCs, harmonine showed no significant effect on RBCs intergrity at concentrations of up to 100 µM as compared to 0.15% saponin which was used as positive control (Figure 36).

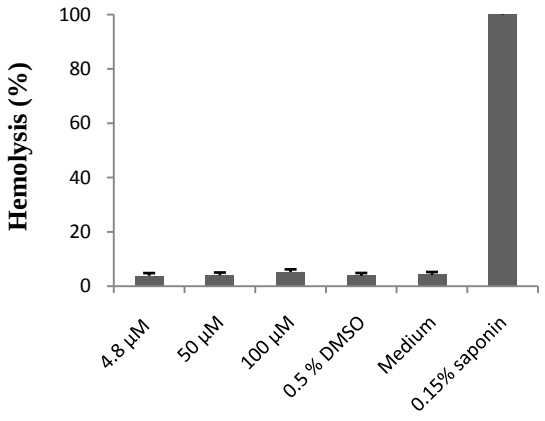


Figure 36: Hemolytic effect of harmonine at different concentrations. RBC at 5% hematocrit was plated in triplicate in 96 well plates and harmonine at different concentrations added. After 48 h of incubation at 37°C, the plate was centrifuged at 500 g for 2 min. A volume of 100 µl of the resulting supernatant was carefully transfered to another plate and the optical density was measured at 550 nm with a spectrophotometer (Multiscan Ascent). Medium supplemented with 0.15% saponin was used for lysis control and 0.5% vol. DMSO or medium only was used for negative control.

3.3.2 Transmission blocking effect of recombinantly produced peptides

The general idea for testing these recombinant peptides was to be able to determine a peptide expressed in plants with transmission blocking activity which can able used to generate a modified plant which is present in malaria endemic areas that expresses a transmission blocking agent on its nectar in a stable form.

3.3.2.1 Transmission blocking effect of SM1

We tested the transmission blocking effect of SM1, a peptide already shown to exhibit transmission blocking activity by binding either to the mosquito midgut or salivary gland (Ghosh et al., 2001; 2009).

The transmission blocking effect of different variants of the recombinant peptide was tested using the *P. berghei* system. We initially tested the effect of the recombinant GFP-fused SM1 proteins which was attached to the McoTi scafford (MC), with the expression of the recombinant protein targated either to the cytosol (15879) or the

Apoplast/ER (17620) of the tobacco plant (*Nicotiana benthamiana*). Neither the cytosolic nor Apoplast/ER targeted produced protein had a significant transmission blocking effect on *P. berghei* by either reducing oocyst or sporozoite counts *(*Figure 37)

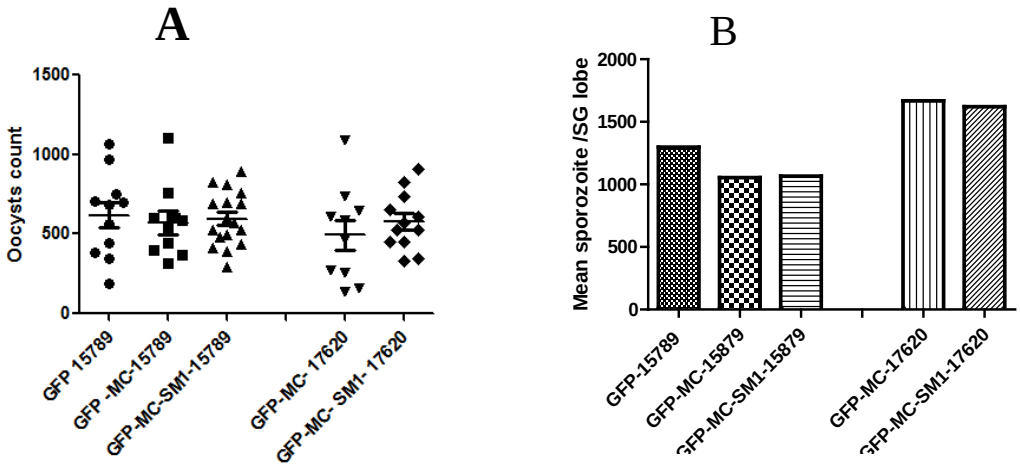


Figure 37: Transmission blocking effect of recombinant SM1 produced in tobacco. *An stephensi* mosquitoes were fed with 0.5mg/ml recombinant peptide together with a sugar solution containing food color for 2 days and then the mosquitoes were allowed to feed on *P.berghei* infected mice (1-10% parasitaemia). (A), shows the number of oocyst counted in mosquito midgut 7-12 d post infection.(B), the mean number of sporozoites per salivary gland lobe counted greater than 14 d post infection. The numbers 15789 indicate expression of the protein was targeted at the cytosol and 17620 the Apoplast/ER. GFP and GFP-MC served as controls. No significant differences were obtained between controls and peptide fed mosquitoes ($P > 0.05$ Mann-Whitney test).

Due to the fact that the tobacco-produced SM1 peptites had no transmission blocking effect and some preliminary results showed transmission blocking activity of the peptide produced in bacteria (personal communication, Heribert Wazecha). We decided to test the effect of the recombinant peptide produced in bacteria fused with either thioredoxin and McoTi or thioredoxin and agouti related protein (AGRP) or thioredoxin tandem repeats of 8 times SM1. Still, the results showed no significant reduction in Oocyst count between peptide fed mosquitoes and controls (Figure 38).

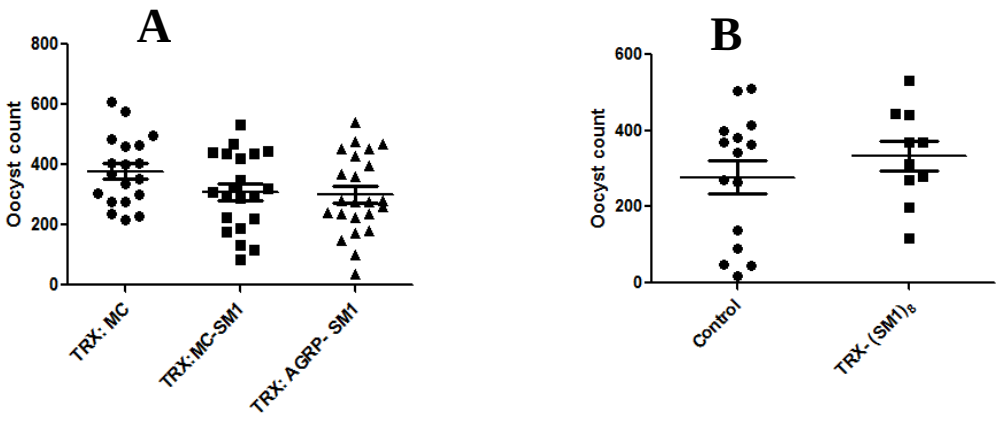
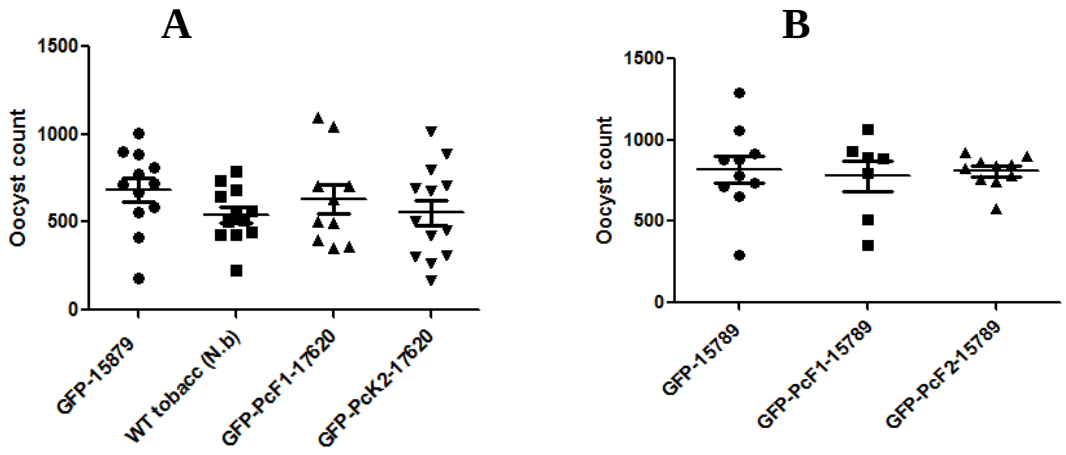


Figure 38: Transmission blocking effect of recombinant SM1 peptide produced in bacteria. (A), Oocyst count from dissected midguts of mosquitoes fed with 0.5mg/ml thioredoxin McoTi SM1 fusion (TRX:MC-SM1) peptide or thioredoxin agouti related protein SM1 fusion (TRX:AGRP-SM1) peptide.(B) Oocysts count from midgut of mosquitoes fed with 0.5mg/ml of thioredoxin containing 8 times tandem repeats of SM1 fusion peptide. No significant differences were obtained between controls and peptide fed mosquitoes (P > 0.05 Mann-Whitney test).

3.3.2.2 Transmission blocking effect of PcFK derivatives

Here, the transmission blocking activity of tobacco-produced recombinant *Psalmopoeus cambrigei falciparium* killers (PcFK1 and PcFK2) were tested. The peptides were first isolated from the venom of the Trinindad chevron tarantula *Psalmopoeus cambrigei* by Choi et al. and shown to exhibit antiplasmodial activity against the intra-erythrocytic stage of *P. falciparun in vitro* (Choi et al., 2004).

The transmission blocking effect of total extracts of PcFK1 and PcFK2 were tested. Both PcFK1 and PcFK2 had no significant transmission blocking effect on *P.berghei* in the mosquito. No significant reduction in either oocyst count or sporozoite count in the mosquito midgut and salivary gland respectively was obtained (Figure 39).



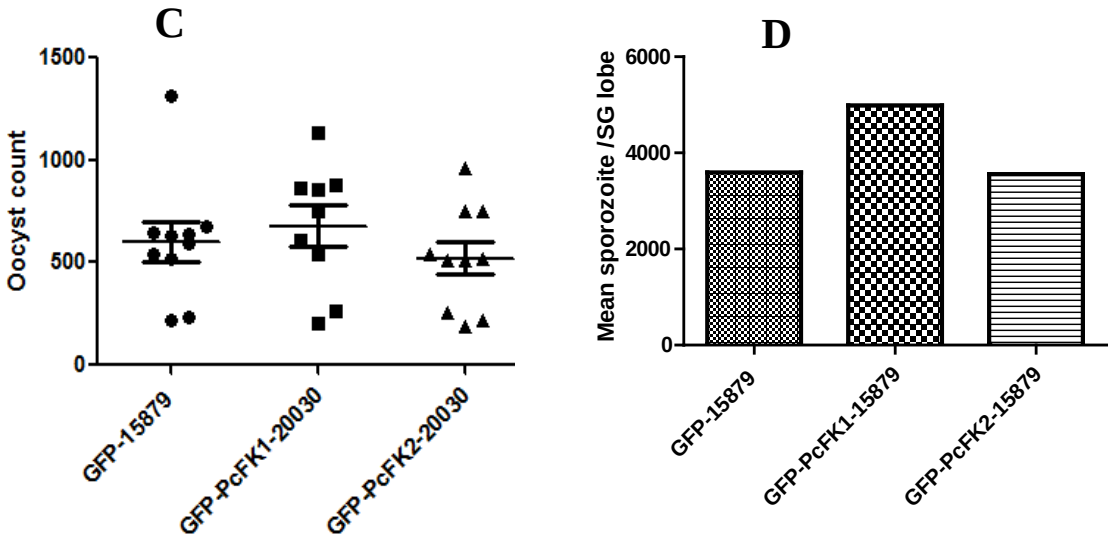


Figure 39: Transmission blocking effect of PcFK derivatives. (A), oocyst count following feeding of mosquitoes with PcFK recombinant extracts with peptide expression targeted to the Apoplast/ER (17620).(B), oocyst count following feeding of mosquitoes with PcFK recombinant extracts with peptide expression targeted to the cytosol.(C), oocyst count following feeding of mosquitoes with PcFK recombinant extracts with peptide expression targeted to the chloroplast (20030).(D), mean sporozoite per salivary gland lobe following feeding of mosquitoes with extracts whose peptide expression was targeted to cytosol (15879). No significant differences were obtained in oocyst or sporozoite counts ($p > 0.05$, Mann-Whitney test).

4. Discussion

4.1 Interplay between midgut bacteria and the malaria parasite

4.1.1 Bacteria diversity in *An. stephensi* during development and different feeding regimes

In order to investigate the interplay between the mosquito midgut microbiota and the malaria parasite, we initially used the culture independent 16S rRNA gene sequencing method to investigate the bacterial diversity in the midgut of the Asian malaria vector *An. stephensi* during development and under different feeding regimes with special focus on the presence of the malaria parasite in the gut. We showed that the diversity of the midgut microbiota drastically decreased during mosquito development from egg to adult. Such "sterilization" of the mosquito midgut has been reported before and was assigned to the sequestration of larval gut bacteria within the confines of the meconium and the meconial peritrophic membranes and also due to the bactericidal effect of molting fluid which is ingested during the process of metamorphosis from larvae to adult (Moll et al., 2001). However, this sterilization appears to be incomplete as some bacteria (*Acinetobacter*, *Bacillus*, *Enterobacter*, *Staphylococcus*, *Pseudomonas, Cryseobacterium (Elizabethkingia)* and *Serratia* species) are able to be transferred from larvae to adult (Rani et al., 2009). We found the highest bacteria diversity in *An. stephensi* larvae. The high bacteria diversity in larvae midgut is probably due to the fact that microorganisms represent an important food source to mosquito larvae (Merrit et al., 1992; Walker et al., 1988; Straif et al. 1998). It has been shown that *Anopheles* larvae stopped growing following the addition of antibiotics (Wotton et al., 1997). We also observed that the bacteria diversity in lab-reared adult female *An. stephensi* mosquitoes fed on sugar and blood in the presence and absence of the malaria parasite was very low. Low bacterial diversity in lab-reared and field collected *Anopheles* mosquitoes has been reported (Lindh et al., 2005; Rani et al., 2009). Furthermore, no significant differences were observed between the bacteria diversity in sugar, blood and *Plasmodium*-fed female mosquitoes as revealed by DGGE and 16s RNA gene analysis indicating that the malaria parasite probably does not affect the mosquito midgut bacteria diversity.

4.1.2 Dominance of *E. meningoseptica* in *An. stephensi* midgut

In corroboration with previous studies (Lindh et al., 2005; Rani et al., 2009; Dong et al., 2009; Boissiere et al., 2012) we identified the rod-shaped gram negative bacterium *E. meningoseptica* (previously known as *Chryseobacterium meningoseptica* or *Flavobacterium meningoseptica* (King 1959)) as dominant in all developmental stages of *An. stephensi*, i.e. in eggs and in the midguts of larvae, pupae and male and female adults. The bacterium still remained dominant in the midgut of blood-fed and malaria parasite-fed mosquitoes indicating that it is not affected by the diet of the mosquito. Interestingly, we were able to obtain and identify an isolate of *E. meningoseptica* from the midgut of female *An. stephensi* mosquitoes which we named *E. meningoseptica* Che 01. Phylogenetic analysis of *E. meningoseptica* Che 01 and the other *E. meningoseptica* phylotypes which were obtained in this study placed them in a single clade of *E. meningoseptica* phylotypes derived from other host-associated environments such as mosquitoes, termites, frogs and carnivorous plants (Kämpfer et al., 2010). In accordance with an isolate from the midgut of *An. gambiae* whose name was proposed as *Elizabethkingia anophelis*, the isolate showed natural resistance to a range of antibiotics (Kämpfer et al., 2010).

Several other studies have reported the dominance of *E. meningoseptica* in either field or lab-reared *Anopheles* mosquitoes. Dong and others (Dong et al., 2009) reported the dominance of *Chryseobacterium meningosepticum* (*E. meningoseptica)* within all five generations in the midgut of lab reared *An. gambiae* mosquitoes studied. They also observed that when *E. meningoseptica* became the dominant bacterium of the midgut flora, the growth of other bacterial species, that could be cultured on LB agar, was usually limited suggesting that the species may possess some competitive advantages in the gut environment. In a recent study, which investigated the midgut bacteria from lab-reared *An. gambiae* mosquitoes collected in natural breeding sites in Cameroon, the authors identified *Elizabethkingia* species to be present in >95% of the mosquitoes (Boissière et al., 2012). Also, Boissière et al. identified *Elizabethkingia* species in field collected mosquitoes, although at low densities. They then suggested that the predominance of *Elizabethkingia spp.* in the midguts of the insectary-reared mosquitoes reflects that the bacterium has found a thriving niche in this environment where competition with other bacterial species is limited (Boissière et al., 2012). Rani et al. also reported a dominance of *Serratia marcescens* and *E. meningoseptica* in lab-reared *An.*

stephensi mosquitoes (Rani et al., 2009). In an earlier study, Lindh and coworkers (Lindh et al., 2008) identified *E. meningoseptica* and *Pantoea stewartii* to be dominant in lab-reared *An. gambiae* mosquitoes.

We now show that organic extracts of metabolites from cultured *E. meningoseptica* exhibit activities against gram positive and negative bacteria and also against yeast *Candida albicans* which probably account for the dominance of the bacterium in the mosquito midgut. It should be noted that yeasts such as *Wickerhamomyces anomalus (Pichia anomala)* have been isolated from mosquito midgut (reviewed in Ricci et al., 2011) and could therefore be a competitor for food and nutrients with *E. meningoseptica* in the mosquito midgut. The differences in the antimicrobial activities of acetone and ethyl acetate extracts observed in this study is probably due to the difference in the polarity of the two solvents; since acetone is more polar than ethyl acetate, it would preferentially dissolve compounds with higher polarity.

We also demonstrated that the predominance of *E. meningoseptica* in the midgut of the female *An. stephensi* is not altered by the blood meal, because differences between the midgut microbiota of sugar-fed and blood-fed female mosquitoes were not observed. Furthermore, the gut microbiota was not altered by the presence of human or rodent malaria parasites, because feeds with *P. berghei*- and *P. falciparum*-infected blood did not result in any significant changes in the bacterial populations, indicating the malaria parasites do not represent a threat for *E. meningoseptica.* Also, the frequent isolation of the bacterium in other studies (indicated above) further confirmed that the bacterium is not affected by the mosquito diet.

E. meningoseptica extracts also had an effect on *P. falciparum* parasites *in vitro* and exhibited *in vitro* toxicity on gametocytes. The antiplasmodial and gametocytocidal activities of *E. meningoseptica* metabolites might enable the bacteria to harm the malaria parasites, once these have entered the mosquito midgut with the blood meal, thereby outrivaling a potential competitor for nutrition and space. Midgut bacteria have been shown to produce compounds with potential antimalarial activites. *Enterobacter* bacterium isolated from wild mosquitoes in Zambia has been shown to produce reactive oxygen intermediates that kill developing parasites in the midgut lumen, inhibiting the development of the parasite in the mosquito (Cirimotich et al., 2011). Noteworthy, it has also been shown that midgut bacteria can also stimulate the mosquito´s innate immune response to produce antimicrobial peptides which act on both the bacteria and the malaria parasite in the gut. Because mosquito gut bacteria drasti-

cally proliferate after the blood meal, the uptake of *Plasmodium*- infected blood triggers immune defense mechanisms in the mosquito that are directed against both bacteria and parasites (Meister et al. 2009). In a recent study by Eappen and colleagues, (Eappen et al., 2013), they were able to show that *Enterobacter cloacae* strongly induces the expression of a putative serine protease inhibitor in *An. stephensi* (*As*SRPN6) which is a vital component of the *E. cloacae*- mediated immune response that restricts *Plasmodium* development in the mosquito. Also, a global transcription profiling of septic and aseptic mosquitoes identified a significant subset of immune genes that were mostly up-regulated by the mosquito´s microbiota, but that also include several anti-*Plasmodium* factors (Dong et al., 2009). Antimicrobial factors which are also up-regulated by bacterial infections, which have implications on malaria parasites, include members of the fibrinogen-related protein FREP family (Dong and Dimopoulos 2009) and the gram-negative bacteria-binding protein (GNBP) family (Warr et al., 2008). It is still to be determined whether *E. meningoseptica* is also able to activate the mosquito´s innates response against the malaria parasite.

4.1.3 Transstadial and vertical transmission *E. meningoseptica*

The presence of *E. meningoseptica* phylotypes in all development stages and from the analysis of the bacterial content in the water used for rearing of the larvae before and after the water had come in contact with the mosquitoes led us to the conclusion that *E. meningoseptica* is transstadially transmitted and also vertically transmitted between mosquito generations. Transstadial and vertical transmission of bacteria has been shown to take place in *Anopheles* mosquitoes. Damiani and coworkers investigated the colonization of the African malaria vector *An. gambiae* by the α-proteobacterium *Asaia* and were able to show by fluorescent visualization of the bacterium that it was localized in the guts, salivary glands and reproductive organs of male and female mosquitoes (Damiani et al., 2010). The localization of the bacterium in the mosquito´s reproductive organ relates to vertical transmission routes whereby *Asaia* is transmitted from mother to offspring by a mechanism of egg-smearing. A study in *An. gambiae* using GFP-expressing *Pantoea stewartii* reported that the bacteria are not transferred from pupae to adults, but that the adult mosquitoes take up bacteria from the water they emerged from and transfer the same bacteria to the water during egg laying (Lindh et al., 2008). Other studies have suggested the transstadial transfer of bacteria from larvae to adults (Jadin et al. 1966, Pumpuni et al. 1996). *An. quadrimaculatus*

larvae were fed with a *Pseudomonas* sp., and the bacterial species was later isolated from adult guts, suggesting that transstadial transmission had occurred.(Jadin et al. 1966) Pumpuni et al. fed *An. gambiae* larvae with *Escherichia coli* HS5 and later recovered this species from one adult in an open system (Pumpuni et al. 1996). However, the mechanism by which *E. meningoseptica* is transmitted across generations is not known.

4.1.4 *E. meningoseptica* as a potential paratransgenic weapon

The fact that *E.meningoseptica* is well adapted to the mosquito midgut, is able to compete with other mosquito midgut inhabitants, is vertically or transstadially transmitted, is cultivable in the laboratory, is not affected by the diet of the mosquito makes *E.meningoseptica* a potential candidate vehicle for the generation of paratransgenic mosquitoes. Its also has an additional paratransgenic advantage by its ability to produce metabolites which act against the *Plasmodium* gametocytes which come in contact with the mosquito midgut during a blood meal. Paratransgenesis which is a novel tool to fight insect pests and vector-infectious diseases has been shown to be feasible in malaria-transmitting *Anopheles* mosquitoes (reviewed in Coutinho-Abreu et al., 2010). *E. coli* expressing cecropin A fusion protein has been shown to inhibited the development of *P. berghei* midgut stages when fed to lab-reared *An. stephensi* mosquitoes (Yoshida et al. 2001). Furthermore, Riehle at al. genetically engineered *E. coli* to display two anti-*Plasmodium* effector molecules, SM1 and phospholipase A2, on their outer membrane surfaces and showed that the bacteria significantly inhibited *P. berghei* development in *An. stephensi* mosquitoes (Riehle at al., 2007). However, the transgenic *E. coli* survived poorly in the mosquito midgut, excluding this bacterial species as a paratransgenic tool. Contrary to *E. coli*, however, the cultivable *E. meningoseptica* strain Che01 is well adapted to the mosquito gut and demonstrates dominance over other midgut inhabitants and thus might represent an ideal paratransgenic vehicle to deliver peptides with transmission blocking properties to the malaria vector.

4.2 Changes in the transcriptome during malaria parasite transmission from human to mosquito

4.2.1 Changes in gene expression by SSH following gametocyte activation

In this study, we initialy used the SSH method to determine changes in the transcriptome of the malaria parasites during the initial phase of transmission from the human to the mosquito. The SSH method amplifies differentially expressed cDNAs while simultaneously suppressing amplification of common cDNAs has been shown to be a suitable tool for identification of differentially expressed genes (Altincicek et al., 2007; Zhu et al., 2003). We identified a total number of 126 genes, for which expression levels changed in the gametocytes during activation with a majority of genes assigned to have functions in signaling, cell cycle and gene regulation, and proteostasis, or they are cytoskeletal and IMC or cell surface proteins. The regulated expression of proteins involved in signaling is not doubtful since the induction of gametogenesis by the external stimuli i.e. temperature drop and the mosquito-derived molecule XA results in the initiation of a number of signaling events in the parasite (McRobert et al., 2008; (reviewed in Kuehn and Pradel, 2010; Baker, 2011)). At least three effector pathways exist; a cGMP-dependent protein kinase (PKG) calcium-independent pathway that mediates rounding up of activated gametocytes, a calcium-dependent pathway that initiates microgamete formation and a calcium-dependent pathway that regulates emergence of activated gametocytes of both sexes (McRobert et al., 2008).

The differential expression of cell cycle and gene regulation protein is probably due to the fact that following gametocyte activation their gene products are highly needed. Following activation of gametocytes, they are release from the cell cycle arrest state and the male gametocytes enter the cell cycle and undergo a rapid transformation following three rounds of DNA replication during gametogensis (Janse et al., 1986). Janse et al., observed that during gametogenesis microgametocytes within 8-10 min synthesize DNA steadily and at a very high rate to more than the octoploid value (Janse et al., 1986).

It should be noted also that following activation of gametocytes in the mosquito, both male and female gametocytes round up and then escape from the enveloping erythrocyte. The egress from the enveloping RBC is crucial for gamete formation and requires the role of proteases to execute this task (reviewed in Wirth and Pradel, 2012) which probably accounts for the high number for regulated proteostasis genes. The role of proteases in gametocyte egress from RBCs has been shown indirectly through

the use of protease inhibitors on microgametogenesis. It was shown that exflagellation of activated *P. berghei* and *P. falciparum* gametocytes can be blocked by the cysteine/ serine protease inhibitors TLCK and TPCK (Torres et al., 2005; Rupp et al., 2008; Sologub et al., 2011).

The adoption of the crescent or banana shape of mature gametocytes and the rounding up of gametocytes following activation require the active participation of cytoskeletal and IMC proteins (Dixon et al., 2012; Kono et al., 2012). Also, following activation of gametocytes, they are able to form filamentous cell to cell connections which allow for communication between cells (Rupp et al., 2011). The high need of these proteins following activation is the probable reason for the regulated cytoskeletal and IMC protein expression.

The high expression changes of surface proteins following activation can be explained by the fact that these proteins cover the surface of gametes to protect them from the aggressive environment of the mosquito midgut. In a recent study, Simon and colleagues (Simon et al., 2013) showed how the IMC protein PfGAP50 is able to relocate to the gamete surface where it binds to factor H and uses surface bound FH to inactivate the complement system thereby preventing the gametes from complement lysis in the blood meal. Also, it is suspected that surface adhesion proteins form complexes which cover the macrogamete in the form of sticky coat and that they are involved in important processes during malaria transmission to the mosquito (reviewed in Kuehn and Pradel, 2010).

Noteworthy, about 60% of the SSH genes have been detected in the currently available *P. falciparum* gametocyte proteomes, while for 40% there is as yet no protein evidence indicating the identification of more gametocyte specific proteins in this study. We also found that only 8.7% of the SSH genes have *P. berghei* orthologs regulated by DOZI and CITH indicating that the majority of genes identified via SSH, which change in transcript expression levels during gametocyte activation, are not translationally controlled in non-activated gametocytes, while many such genes have been identified to function in zygote to ookinete transformation (Sebastian et al., 2012). Therefore it is probable that gene regulation is important for the early events of gametocyte activation, i.e. for gametogenesis and fertilization, while translational repression particularly plays a role for the expression of proteins important for ookinete development.

4.2.2 Changes in transcript levels by qRT-PCR

We wanted to confirm transcript changes in gametocytes during activation and determine if these genes become up or down regulated following gametocyte activation. We performed qRT-PCR on a subset of genes from the different ontology groups whose transcript levels changed following gametocyte activation as determined by SSH. We quantified the transcript levels from mixed asexual F12 gametocyte- less *P. falciparum* strain, immature (stages III and IV) and mature (stage V) gametocytes of *P. falciparum* strain NF54 and from activated gametocytes at 30 min post-activation.

The results showed transcription in mature gametocytes for 29 out of the 34 genes analyzed and 20 genes showed increased transcript expression in gametocytes compared to asexual blood stage parasites. The presence of gametocyte transcripts in most of the genes studied confirm the purity of the gametocyte samples used in the SSH method and also to some extent validates the usefulness of the method.

4.2.2.1 Genes with high transcript levels in activated gametocytes as compared to other stages

We identified 8 genes i.e. PF3D7_0827800 (set3), Pf3D7_1246200 (actin1), PF3D7_1218800 (psop17), PF3D7_1239400, PF3D7_0704100, PF3D7_0417400, PF3D7_1321000 and PF3D7_1316700 for which transcript levels were increased in activated gametocytes as compared to asexual blood stages, immature gametocytes and non-activated gametocytes, indicating that these genes may play an important role downstream of gametocyte activation in the mosquito midgut. Among these genes, only set3, actin 1 and psop17 have been studied previously to some extend. SET proteins have been identified as multifunctional proteins associated to different processes such as chromatin remodelling (Matsumoto et al., 1999), regulation of epigenetic marking of histones (Cervoni et al., 2002), transcription (Shikama et al., 2000) and viral DNA replication (Nagata et al., 1995).

SET3 has been described to accumulate in male gametocytes of *Plasmodium*, where it is suspected to contribute to a prompt entry and execution of S/M phases in the developmentally arrested male gametocytes once taken up into the mosquito (Pace et al., 2006). SET domains are assigned to chromatin dynamics and are often found in histone methyltransferases, thus they play a role in the epigenetic control of gene regulation. The *P. falciparum* genome encodes for at least nine SET-domain-containing genes which exhibit five different types of substrate specificities (Cui et al.,

2008). The up regulated expression of set3 following gametocytes activation is an indication of a role of the gene product in the epigenetic control of gene regulation during gamete formation.

ActinI forms part of the plasmodial motor complex (reviewed in Baum et al., 2008; Nono et al., 2012) and recently have been also reported to be present in gametocytes (Deligianni et al., 2011; Simon et al., 2013). PSOP17 (putative secreted ookinete protein 17) was previously reported to be expressed in mature gametocytes of *P. falciparum* (Florens et al., 2002; Silvestrini et al., 2010) and in ookinetes of *P. berghei* (Ecker et al., 2008). However, the function of the protein is not known.

In a recent study, *P. falciparum* RNA seq sequences from seven stages including asexual and sexual stages (ring, early and late troph, schizont, gametocyte II and V, and ookinete) also revealed high expression of PF3D7_1239400, PF3D7_0704100, PF3D7_0417400, PF3D7_1321000 and PF3D7_1316700 in matured stage V gametocytes as compared to the asexual stages (López-Barragán et al., 2011).

The fact that the transcript expression of the two control genes, PF3D7_1031000 (pfs25) and PF3D7_1412500 (actinII) was strong in gametocytes compared to asexual blood stage parasites and increased during gametocyte activation validates the qRT-PCR results. Pfs25 is expressed in the vesicular structures during gametocyte maturation then it relocate to the surface of macrogametes where it is highly expressed following activation and then subsequently present on the parasite surface until the ookinete stage (Rener et al., 1983; Quakyi et al., 1987; Scholz et al., 2008). ActinII is a sexual stage-specific actin isomer and has been shown to be highly expressed in the sexual stages (Wesseling et al., 1989) and in *P. berghei* actinII protein has been reported to play a role during microgametogenesis (Deligianni et al., 2011).

4.2.2.2 Genes with high transcript levels in gametocytes compared to asexual stages

Twelve genes were identified with increased transcript expression in gametocytes compared to asexual blood stage parasites, and transcript levels either remained constant, decreased or increased following activation. These include PF3D7_0302100 (clk4), PF3D7_0818200 (14-3-3), PF3D7_1115200 (set7), PF3D7_0422300 (α-tubulin II), PF3D7_0708000, PF3D7_1103500 (encoding for a CPW-WPC protein), PF3D7_406200 (pfs16), PF3D7_1457000 (spp), PF3D7_0817600 (encoding an for unknown protein termed PPLP6), PF3D7_1225600, PF3D7_1438800 and

PF3D7_1023000. The *P. falciparum* mRNA splicing kinase CLK-4 (also termed SRPK1) is expressed in the asexual blood stages and gametocytes (Dixit et al., 2010; Agarwal et al., 2011) with expression levels shown to be higher in matured gametocytes (López-Barragán et al., 2011). Also, a gene knock-out of the orthologous protein in *P. berghei* failed to exflagellate upon gametocyte activation suggesting a role in microgamete formation (Tewari et al., 2010). The proteins 14-3-3 belong to a family of highly conserved molecules present virtually in all eukaryotes and are known to regulate a large variety of cellular processes such as core metabolism, cell cycle, protein localization and trafficking and signal transduction (reviewed in van Heusden, 2005). They mediate these events through interaction with a large number of target proteins. In *Plasmodium*, it has been shown that dematin; a component of the erythrocyte membrane skeleton is recuited by the malaria parasite following infection (Lalle et al., 2011). The internalised dermatin interacts with *Plasmodium* 14-3-3 and the dematin-14-3-3 interaction is strictly dependent on phosphorylation of dematin at specific serine residues (Lalle et al., 2011). Also, in a recent study, Dastidar and colleagues (Dastidar et al., 2013) identified *Plasmodium* 14-3-3 as a protein that binds selectively to *P. falciparum* histone H3 phosphorylated on Ser28. Noteworthy *P. falciparum*14-3-3 has been shown to be expressed in gametocytes (López-Barragán et al., 2011).

Transcript of set7 is destabilized in the absence of DOZI and CITH pointing at a translational repression of set7 in gametocytes and suggesting a role in epigenetic control mechanisms of the ookinete. Pfs16 is a transmembrane protein of the gametocyte PVM (Eksi and Williamson, 2011; Silvestrini et al., 2005; Baker et al., 1994; Bruce et al., 1994). After the PVM has ruptured during the egress of the activated gametocyte from the host cell, Pfs16 is not detectable any longer in the sexual stage parasite (Sologub et al., 2011) which explains why there is a decrease of transcript following activation. Alpha-tubulin II is an isoform of tubulin which has been shown to be expressed in asexual blood stage parasites, in gametocytes and in microgametes (Schwank et al., 2010; Fennell et al., 2008; Rawlings et al., 1992). Targeted gene modification studies in *P. berghei* indicated that α-tubulin II plays an important role for microgametogenesis (Kooij et al., 2005). The fact that α-tubulin II transcript levels decrease during gametogenesis lets us conclude that the α-tubulin II filaments needed for the formation of the microgametes are already present in the mature gametocytes prior to activation. The presenilin-like signal peptide peptidase (SPP) was previously reported to be involved in merozoite invasion and cleavage of the erythrocyte cy-

toskeletal protein band 3 (Li et al., 2008). A recent study, however, disagrees with these findings, and reported that SPP is an ER-resident protease required for growth of the erythrocytic stages (Marapana et al., 2012). SPP is considered as a potential drug target of liver and blood stage parasites (Marapana et al., 2012; Li et al., 2009; Parvanova et al., 2009). SPP has also been shown to be highly expressed in matured gametocyte (López-Barragán et al., 2011) which is in confirmity with our results. The combined data therefore indicate that SPP is present in the liver, blood and gametocyte stages, pointing to a multi-stage function of SPP.

The gene product of PF3D7_0817600 is an unknown protein which was initially predicted to posses a MAC/PF domain, similar to the MAC/PF domains of the previously described plasmodial perforin-like proteins PPLP1-5 ((reviewed in Wirth and Pradel, 2012), Sologub et al., 2011; Kaiser et al, 2004). However, recent annotation shows a lacked of the domain. We termed the protein PPLP6 in this study. The gene product of PF3D7_1103500 is a member of a family of nine secreted proteins with cysteine-rich CPW-WPC domains ((reviewed in Sutherland et al., 2009); Saeed et al., 2008). This domain is a conserved domain of about 61 residues in length that exhibits six well-conserved cysteine residues and six well-conserved aromatic sites. The PF3D7_1103500 gene ortholog in *P. berghei* is under translational control. Also, the expression of PF3D7_1103500 has been shown to be upregulated in ookinetes pointing to a role of the protein in zygote to ookinete development. In a recent study, Kangwanrangsan and coworkers (Kangwanrangsan et al., 2013) showed that P*lasmodium yoeli* cpw-wpc1 mRNA is transcribed in gametocyte while the protein is expressed mainly on zygote/ookinete surfaces which further suggests that members of the CPW-WPC family are under translational control in *Plasmodium.* The expression of the protein on zygote or ookinete surfaces makes them potential targets for transmission blocking vaccines. The genes PF3D7_1225600, PF3D7_1438800 and PF3D7_1023000 have been shown to be highly expressed in gametocytes (López-Barragán et al., 2011) which corroborates with our results. Their functions are not known.

4.2.2.3 Genes with high transcript levels in asexual blood stages

The qRT-PCR also revealed five genes whose expression was predominant in the asexual blood stages and show a resurgence in transcript expression in gametocytes during maturation, i.e. PF3D7_1238900 (pk2), PF3D7_1136400 (encoding for a tetra-

trico peptide repeat region; TPR), PF3D7_1021700, PF3D7_1026600 and PF3D7_1251200 (coronin). The calcium/calmodulin-dependent protein kinase PK2 was mainly investigated in the asexual blood stages of *P. falciparum* (Kato et al., 2008, Ward et al., 2004). A study of *P. falciparum* RNA seq sequences from seven stages (ring, early and late troph, schizont, gametocyte II and V, and ookenete) revealed that the gene is expressed in gametocytes and highly upregulated in ookinetes (López-Barragán et al., 2011). This result is in confirmity with our findings and therefore suggesting that the protein may be important in the midgut stages of the malaria parasite. The gene product of PF3D7_1136400 comprises a TPR domain, which is known to mediate protein-protein interactions and the assembly of multiprotein complexes (reviewed in Allan et al., 2011), but the function of the plasmodial TPR domain protein is not yet known. Coronin is a WD repeat containing actin binding protein that was first characterized in *Dictyostelium discoideum*, where it is essential for phagocytosis and motility (de Hostos, 1999). WD-repeat proteins are a large family found in all eukaryotes, implicated in a variety of functions ranging from signal transduction and transcription regulation to cell cycle control and apoptosis. The WD40 motifs act as a site for protein-protein interaction, and proteins containing WD40 repeats are known to serve as platforms for the assembly of protein complexes or mediators of transient interplay among other proteins (reviewed in Stirnimann et al., 2010). WD-repeat proteins of *P. falciparum* have hitherto been described as receptors for protein kinase C, and as components of the myosin-driven motor complex (Madeira et al., 2003; Foth et al., 2006, Tardieux et al., 1998). *P. falciparum* coronin has been shown to be associated with F-actin and therefore involved in actin dynamics (Tardieux et al., 1998). Therefore the gene product of PF3D7_1251200 (coronin) could be important in this process in asexual parasites and gametocytes as depicted by its transcript level in these stages.

For four genes, PF3D7_0818900 (hsp70-1), PF3D7_080700 (su α6), PF3D7_0807500 (gap50), and PF3D7_1444800 (encoding for fructose 1,6 bisphosphatealdolase, FBPA) expression was high in asexual blood stages and decreased during gametocyte development and following activation. SU α6 is a component of the plasmodial proteasome, a proteolytic complex composed of more than 33 SUs that is responsible for the degradation and recycling of ubiquitinated proteins. As an external control we thus investigated the transcript levels of another α-ring component, su α5, and revealed similar changes in the transcript levels of su α5 in asexual blood stage parasites and

gametocytes before and after activation. The fact that the transcript level following gametocytes activation was low for su α5 and su α6 suggest that protein re-cycling is important for asexual and sexual development, but that it does not play an important role for the rapid morphological changes during gametogenesis. The genome of *P. falciparum* encodes for a variety of chaperones, including heat shock proteins (HSPs) of the HSP70, HSP90 and DnaJ/HSP40 families (reviewed in Pavithra et al., 2007). HSP70-1 was previously described to be located in the cytoplasm and nucleus of the parasite blood stages (Kappes et al., 1993) as well as in the PV, pointing to a role in protein transport to the erythrocyte (Nyalwidhe and Lingelbach, 2006). Another proposed function of HSP70-1 includes the protein trafficking to the apicoplast (Ramya et al., 2007; Foth et al., 2003) , indicating that the chaperone has several essential functions and is important for multiple life-cycle stages.

The gene PF3D7_0807500 encodes for a transmembrane protein GAP50 which has been identified as part of the IMC of the parasite invasive stages, including the ookinete (Baum et al., 2008, 2006). Recently, GAP50 was also described as a component of the gametocyte IMC (Dearnley et al., 2012; Kono et al., 2012; Simon et al., 2013). The transmembrane protein links myosin with the outer membrane of the IMC and thus contributes to gliding motility of the parasite. Gap 50 is also suspected to be under translational control, thus the release of the translational repression and onset of protein synthesis during gametocyte activation might cause the detected decrease in the gap50 transcript level (Gunner Mair, unpublished observation). FBPA is an enzyme of glycolysis and in *Plasmodium* is also reported to be part of the motor complex, here linking TRAP to actinI (Baum et al., 2006; Buscaglia et al., 2006). Noteworthy in this context, during gametocytogenesis, malaria parasites shift from glycolysis towards mitochondrial respiration (van Dooren et al., 2006), which might explain the decrease in FBPA expression in gametocytes compared to asexual blood stage parasites.

Transcript expression for five genes, PF3D7_0815800 (VSP9), PF3D7_0215400 (encoding for a WD40 motif), PF3D7_1351700 (alv6), PF3D7_1033200 (etramp10.2), and PF3D7_0207700 (sera4), were negligible in gametocytes. The gene PF3D7_0815800 encodes for a homolog to the yeast vacuolar sorting protein VSP9 with a predicted function in protein-protein interactions, while PF3D7_0215400 encodes for a protein with a WD40 motif. The fact that their transcript levels were negligible in gametocytes and gametes suggest that they donot play important roles in

these stages. Furthermore, ALV6 is a member of the alveolin family, which comprises 7 proteins in *P. falciparum*, associated with the membranous sacs of the IMC (Gould et al., 2008). The fact that alv6 expression decreases during gametocytogenesis is fairly surprising, considering that gametocytes posses an IMC. It has to be elucidated, if the expression of any other ALV protein member is up-regulated in gametocytes. The early transcribed membrane proteins (ETRAMPs) are proteins of the plasmodial PVM (Spielmann et al., 2003; MacKella et al., 2011). In *P. falciparum* the proteins were shown to form complexes with the PVM protein exported protein 1 (EXP-1) (Spielmann et al., 2006). To date, etramp10.2 transcripts were found in *P. falciparum* trophozoites and the mixed asexual stages and the liver stages of *P. yoelii* (Spielmann et al., 2003; MacKella et al., 2011). Because EXP-1 is also present in the gametocyte PVM (Sologub et al., 2011), the expression of some of the ETRAMPs in these stages can be expected. It thus remains to be elucidated, if the other two SSH-identified ETRAMPs, ETRAMP2 and ETRAMP4, might play a specific role for gametocytes. Expression of serine repeat antigen SERA4 in the asexual blood stages has previously been described (Miller et al., 2002). The SERA family comprises 9 proteins with functions in asexual blood stage growth and host cell egress (reviewed in (Wirth and Pradel 2012; Rosenthal et al., 2011)).

4.2.3 Changes in protein expression of SSH gene

In order to determine if the changes in transcript level following qRT-PCR, are in conformity with that at the protein level, we selected some of the analyzed qRT-PCR genes and analyzed their protein expression levels using antibody against them via indirect immunofluorescence assay. We confirmed an up-regulation in the protein expression of PK2, actinII and Pfs25 following gametocyte activation indicating an important role of the protein following gametocytes activation in the mosquito midgut. Pfs 25 and ActinII have already been shown to be important in the development of the malaria parasite in the mosquito midgut (Vlachou et al., 2001; Deligianni et al., 2011) but the function of PK2 is not yet known. Also, CLK-4, proteasome SU α5, and PPLP6 were detected in asexual blood stage parasites and gametocytes during maturation, but the proteins were down-regulated in gametocytes at 30 min p.a. which corroborates with our qRT-PCR results. The down regulation of CLK-4, SU α5 and PPLP6 suggest that the proteins are not important following gametocyte activation in the mosquito. Pfs16 was almost undetectable in gametes following gametocyte activa-

tion. This is due to the fact that the protein is localized in the PVM and following gametocytes activation for 30 min, the parasites has egressed from the enveloping erythrocyte and the PVM is destroyed. Noteworthy, the transcript level was also significantly reduced following gametocyte activation as depicted by qRT-PCR. The slightly upregulation of GAP50 protein following gametocyte activation while the mRNA transcript level as depicted by qRT-PCR was low can be explained by the fact that translationally repressed gap50 transcript is present in the non-activated gametocytes and that the release of repression during activation leads to rapid translation and in consequence to a loss in transcript abundance and an increase in protein abundance. Also, GAP50 has been recently shown to relocate from the gametocytes IMC to the surface of gametes following activation where it is able to bind factor H and prevent the parasite from complement lysis in the mosquito midgut (Simon et al., 2013). In agreement with the qRT-PCR results on ActinI transcript, the protein is present in both, asexual blood stages and gametocytes, and a minor up-regulation in the activated gametocytes was detected. The 14-3-3 protein is also expressed in asexual blood stages, gametocyte and gametes as depicted by qRT-PCR and indirect immunofluorescent assay. The high expression of the protein in gametocytes and gametes suggest a role of the protein in these stages.

The overall data on the changes in the *P. falciparum* transcriptome before and after activation of gametocytes provide first insights into the regulation of gene expression following initiation of gametogenesis in the mosquito vector and form a base for further studies on genes important for the transmission of malaria parasites to the mosquito. Also, we identified novel genes involved in the sexual reproduction and future development of the mosquito midgut stages, which might serve as important targets for transmission blocking strategies.

4.3 Role of antimicrobial molecules as transmission blocking agents

In this part of the study we determined the effect of antimicrobial molecules which could be exploited in transmission blocking strategies to prevent the spread of malaria by the mosquito.

4.3.1 Harmonine

Since insects among all phyla in the animal kingdom seem to have succeeded best in life, accounting for more than one million different species (Vilmos et al., 1998), this is proof that they have developed a potent and efficient immune system to fight against pathogens. Therefore some of these substances produced by the innate immune reponse of insects could be exploited to kill pathoges causing diseases in humans. The Asian harlequin ladybird beetles *Harmonia axyridis* has been known for its high invasive nature in Europe which allows it to outpower and therefore dominate the most abundant native European ladybirds, *Coccinella septempunctata* and *Adalia bipunctata* (Roy et al., 2008). Its invasive success has been attributed to their enduring resistance against diverse pathogens due to its unprecedented expansion among genes encoding antimicrobial molecules (Vilcinskas et al., 2013). The Asian harlequin ladybird beetle release droplets of hemolymph containing deterrent alkaloids through their leg joints when threatened or attacked as a means of defence. The active compound called harmonine (17R,9Z)-1,17-diaminooctadec-9-ene) was isolated from the hemolymph and identified by mass spectrometry and shown to exhibit a broad-spectrum of antibacterial activities (Röhrich et al., 2012).We tested the compound against *P. falciparum* asexual stages as well as mosquito midgut stages. We showed that the compound exhibited significant effect on both asexual and sexual stages (gametocytes and zygotes) and in vivo transmission blocking assay showed a significant transmission blocking effect. The fact that the compound had an effect on both asexual and sexual stages implies it could serve as an important drug which is able to act against the erythrocytic stages, thereby preventing the clinical manisfestation of malaria and also against the sexual stages preventing the spread of the disease by the mosquito. Antimicrobial molecules isolated from the hemolymph of insects have also been shown to exhibit *Plasmodium* transmission blocking activities. Gomesin, an antimicrobial peptide isolated from the hemocytes of the spider *Acanthoscurria gomesiana* was also shown to inhibit the growth of intraerythrocytic forms of *P. falciparum*, *P. berghei* mature gametocytes, exflagellation of male gametes and the formation of ookinetes and exhibited transmission blocking effect in *in vivo* experiments (Moreira et al., 2007). Shahabuddin and coworkers reported that injection of defensins from *Phormia terranovae* (flesh fly) and *Aeschna cyanea* (dragon fly) into the heamolymph of *Aedes aegypti* negatively affected the development of *P. gallinaceum* oocysts and

sporozoites (Shahabuddin et al., 1998). Gambicin an antimicrobial peptide isolated from *An. gambiae* has also been shown to exhihibit a moderate effect on *P. berghei* ookinetes (Vizioli et al., 2001). Harmonine at concentration of upto 100 µM did not affect the intergrity of uninfected RBCs indicating that the compound only acts on infected parasitized RBCs. Harmonine also demonstrated moderate cytotoxicity to mammalian Hela cells. Since the compound is moderately toxic to human cells, we therefore suggest that the development and testing of derivaties may lead to the discovery of less toxic anti-malarial drugs with both parasitocidal and transmission blocking activities.

4.3.2 Recombinant produced peptides

The transmission blocking effect of a range of recombinantly produced peptides in tobacco was tested against *P. berghei.* Several recombinant proteins produced in plants can serve for medical purposes like vaccines (reviewed in Glenz and Warzecha, 2006). All recombinant peptides tested in this study showed no transmission blocking activity in *P. berhei*. Several reasons could account for this lack of activity; one reason could be the fact that the recombinant peptides produced with expression targeted into the different compartments in tobacco lead to missfolding thereby making them unable to execute their functions. Also, another reason could be that the concentration of peptides used in the study was not high enough to gain transmission blocking activities or the peptides do no actually act on the sexual stages of the malaria parasite.

Among the peptides tested, only SM1 has been shown to inhibit the development of *Plasmodium* in the mosquito. It has been shown that SM1 bind to both the luminal side of the midgut epithelium and the distal lobes of the salivary gland in order to inhibit *Plamodium* development in the mosquito (Ghosh et al., 2001). However, the mechanism of inhibition has only been shown in the mosquito salivary gland whereby the SM1 peptide competes with TRAP (Thrombospondin-related anonymous protein), a protein expressed in *Plasmodium* sporozoites for binding to saglin, a protein expressed on the suface of the mosquito salivary gland (Ghosh et al., 2009). The saglin/TRAP interaction is crucial for salivary gland invasion by *Plasmodium* sporozoites. When SM1 binds to saglin, TRAP is prevented from binding and therefore the sporozoites are unable to invade the mosquito salivary gland leading to inhibition of *Plasmodium* development. The receptor of SM1 in the mosquito midgut lumen is not yet known. The lack of activity of SM1 in our study is probably due to the improper folding of the

peptide. The experimental set up could also be a problem since in most studies where the transmission blocking activity of SM1 was tested, the peptide was either injected through the tail vein of an infected mouse and the inhibitory activity tested following feeding of mosquitoes on the treated mouse or the peptides were mixed together with *Plasmodium* infected blood which was not the case in our study.

5. Conclusions and future perspectives

This doctoral thesis provides novel insights on strategies to reduce malaria transmission in the mosquito. In summary;

1) We have identified the rod-shaped *E. meningoseptica* as a dominant midgut bacterium of lab-reared *An. stephensi*. *E. meningoseptica* is well adapted to the mosquito midgut, transmitted between developmental stages and generations and not affected by the diet of the mosquito. Also, extracts from *E. meningoseptica* exhibited antibacterial, antifungal and antiplasmodial activities. Most importantly, isolates of *E. meningoseptica* were cultivable, making the bacterium a potential candidate vehicle for the generation of paratransgenic *Anopheles* mosquitoes.

2) Following analysis of transcriptome changes during the initial phase of malaria transmission from the human to the mosquito, we have identified novel genes with high expression levels in activated gametocytes as compared to asexual blood stages, immature and mature gametocytes pointing to important roles downstream of gametocyte transmission to the mosquito.

3) We have shown the antiplasmodial and transmission blocking effect of the antimicrobial molecule harmonine, a defense compound isolated from the hemolymph of the Asian ladybug *Harmonia axyridis.* Harmonine thus represents a potential lead structure for the development of novel antimalarials.

Future experiments will include a further characterization of *E. meningoseptica* as a paratransgenic weapon. First, *E. meningoseptica* will be tested for its survival in the mosquito midgut by treating mosquitoes with antibiotics to remove the midgut microbiota and then introducing *E. meningoseptica* via the sugar pads or the blood meal, allowing us to monitor the survival over a period of time. Then the bacterium will be tested for its transformation competence. As proof of concept, we intend to transform the bacterium with a plasmid which will enable it to express GFP. The survival of the bacterium after transformation, the stability of the plasmid in the transformed bacterium and the expression of the GFP protein by *E. meningoseptica* will be determined. Also, the stability of the transformed GFP-*E. meningoseptica* in the mosquito midgut will also be evaluated following feeding to *An. stephensi* mosquitoes. If successful, the bacterium will then be genetically modified to express effector molecules such as inhibitory peptides or highly specific single chain antibodies and its transmission

blocking potential will be investigated using transmission blocking assays. Once the genetically modified *E. meningoseptica* is able to demonstrate transmission blocking effect in the mosquito, further experiments will be to determine the fitness of the para-transgenic mosquito in the laboratory. Then we will assess the safety of the genetically modified bacterium to the environment and the most suitable way of delivery of the bacterium to mosquitoes in the field.

Other future works will include the functional characterization of the genes with high transcript levels following gametocyte activation since their gene products may represent key targets for transmission blocking interventions. Firstly, specific antibodies will be generated to determine the expression pattern and localization of the gene products in mosquito midgut stages by indirect immunofluorescent assays. Secondly, mutant malaria parasites in which the genes of interest are disrupted will be generated. The phenotype of the different mutants will be investigated for its survival through the transmission stages of the malaria parasite in the mosquito vector by membrane feeding of mosquitoes with the mutant parasite lines. Disrupted gene products of mutant parasites with reduced malaria transmission in the mosquito may represent transmission blocking targets and will be investigated further for their potential as either transmission blocking drugs or transmission blocking vaccine targets. For determination if they represent transmission blocking drug targets, specific inhibitors targeting the gene products will be generated and testing on their ability to inhibit microgametogenesis through exflagellation inhibition assays. To determine if they represent transmission blocking vaccines targets, specific anti-sera against surface expressed gene products will be generated and tested for their transmission blocking effect through *ex vivo* membrane feeding assays.

Lastly, due to the fact that harmonine exhibited moderate toxicity to human cells, the compound thus represents a potential lead structure for the development of novel antimalarials. Futher experiments will be to synthesize harmonine derivatives and test them for their antimalarial and transmission blocking effect as well as their toxic effect on human cells. If a highly selective derivative with antimalarial and transmission blocking activity is obtained, its pharmacokinetic properties can then be determined before proceeding to clinical trials.

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7. Appendix

7.1 Abbreviations

%	percentage
°C	degree celcius
µ	micro
16s rRNA	16s ribosomal RNA
aa	amino acid
act	activated
ACTs	artemisinin combination therapies
AMA	apical membrane antigen
APAD	3-acetylpyridine adenine dinucleotide
APS	ammonium peroxyde sulfate
ATP	adenosine 5’-triphosphate
BLAST	basic local alignment search tool
bp	base pairs
BSA	bovine serum albumin
cDNA	complementary DNA
CO_2	carbon dioxyde
d	days
dATP	2’-deoxyadenosine-5’-triphosphate
ddH2O	doubled distilled water
DEPC	diethyl pyrocarbonate
DGGE	denaturing gradient gel electrophoresis
dH2O	distilled water
DMEM	Dulbecco's Modified Eagle Medium
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dNTP	deoxynucleotide triphosphate
DTT	dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
EGF	epidermal growth factor
g	gram
GC	gametocyte
GFP	green fluorescent protein
GST	Glutathione-S-transferase
h	hour
HEPES	*N*-(2-hydroxyethyl)-piperazine-*N*’-2-ethanesulfonic acid
IC_{50}	inhibitory concentration 50
IFA	immunofluorescence assay
IMC	inner membrane complex
IPTG	Isopropyl-β-D-1-thiogalactopyranoside
kDa	kilodaltons
l	liter
M	molar
mg	milligram

min	minutes
mRNA	messenger RNA
MTT	(3-(4, 5-Dimethylthiazol-2-yl)-2,5 Diphenyl tetrazolium bromide
MW	molecular weight
NBT	nitroblue tetrazolium
OD	optical density
OTU	operational taxonomic unit
p.a	post activation
PABA	paraaminobenzoic acid
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PVM	parasitophorous vacuolar membrane
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
RNase	ribonuclease
RNase H	Ribonuclease H
RT	room temperature
SDS	sodium dodecyl sulfate
sp.	species
SSH	suppressive subtractive hybridization
TBA	transmission blocking antigen
TBDs	transmission blocking drugs
TBVs	transmission blocking vaccines
TEMED	*N, N, N', N'*-tetramethylethylendiamine
TR	translational repression
Tris	tris-(hydroxymethyl)-aminomethane
U	unit
UV	ultraviolet
v/v	volume concentration
w/v	weight per volume
WBA	western blot analysis
XA	xathurenic acid

7.2 16s rRNA gene and SSH sequences

16s rRNA gene and SSH sequences obtained in this study can be found on the CD attached to this thesis.

Publications

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