Blocking *Plasmodium* development in the mosquitoes by human antibodies

Dissertation zur Erlangung des akademischen Grades des
Doctor rerum naturalium
(Dr. rer. nat.)
im Fach Biologie
eingereicht an der Lebenswissenschaftlichen Fakultät der Humboldt
Universität zu Berlin
von

Anna Maria Weyrich

Kommissarischer Präsident der Humboldt-Universität zu Berlin Prof. Dr. Peter Frensch

Dekan der Lebenswissenschaftlichen Fakultät Prof. Dr. Christian Ulrichs

Durchgeführt am Max Planck Institut für Infektionsbiologie in der Abteilung
für Vektorbiologie, Berlin

Gutachter*innen:

Prof. Dr. Elena A. Levashina
Prof. Dr. Arturo Zychlinski
Prof. Dr. Hedda Wardemann

Tag der mündlichen Prüfung: 25.04.2022

Zusammenfassung

Malaria ist eine Krankheit, die durch den Protozoen *Plasmodium* verursacht und von *Anopheles* Moskitos durch infektiöse Stiche übertragen wird. Diese Übertragung kann durch verschiedene Interventionsstrategien blockiert werden. Eine relativ neue Strategie, die bisher nur im Labor getestet wurde, ist der Einsatz genetisch veränderter Moskitos, die den Parasiten nicht auf einen neuen menschlichen Wirt übertragen können. Ein Ansatz ist die Entwicklung von Moskitos, die mit Antikörpern ausgestattet sind, und zwar mit Antikörpern aus der Maus, die gegen relevante Oberflächenproteine des Parasiten, dem Circumsporozoite Protein (CSP) gerichtet sind. Es ist jedoch nach wie vor unklar, auf welches Entwicklungsstadium man abzielen soll und welche Antikörper für diesen Ansatz effizient sind.
Hier zeige ich, dass in Stechmücken, die mit einem humanen Anti-CSP-Antikörper ausgestattet sind, die Sporogonie der Oozysten in Abhängigkeit von der Parasitendichte blockiert wird und somit die Sporozoitenlast in den Mücken signifikant verringern. Insbesondere Antikörper, die sich an die 'Repeat Region' des CSP binden, können die Sporozoitenlast in der Stechmücke verringern. Des Weitern, zeigen diese Stechmücken nur geringfügige Defekte in der Larvenentwicklung und im Überleben. Diese Ergebnisse, bestätigen die zuvor beschriebene Bedeutung von CSP während der Sporogonie und unterstreichen die Effizienz von humanen, 'Repeat Region' bindenden Anti-CSP-Antikörpern bei der Beeinträchtigung der Parasitenentwicklung auch in einem anderen Wirt. Darüber hinaus zeigen Stechmücken, die mit humanen Anti-CSP-Antikörpern ausgestattet sind, eine geringe (bei Infektionen mit hoher Parasitendichte) bis gar keine (bei Infektionen mit niedriger Parasitendichte) Entwicklung von Sporozoiten, was sie zu einem vielversprechenden Instrument für Maßnahmen zur Blockierung der Malariaübertragung macht.
Ich habe weitere Einblicke in den Mechanismus gewährt, durch den Anti-CSP-Antikörper die Parasitenentwicklung in der Mücke stören, und gezeigt, dass Oozysten ein effizientes Ziel für diesen Ansatz sind. Bei natürlichen Infektionen beträgt die festgestellte Parasitenlast in der Regel weniger als 30 Oozysten pro Mücke, sodass die hier verwendeten Mücken in einer natürlicheren Umgebung potenziell resistent sind. Darüber hinaus habe ich ein neues Instrument zur Verfügung gestellt, das ein schnelleres Screening von Antikörpern im Mückenkontext durch Injektion von "single chain Fabs" in die Hämolymphe der Mücke ermöglicht. Somit könnte dieser Ansatz eines Tages zu alternativen Strategien bei der Bekämpfung der Malaria-Übertragung führen.

Abstract

Malaria is a disease caused by the protozoan parasite *Plasmodium* and transmitted by *Anopheles* mosquitoes trough infectious bites, these transmission events can potentially be blocked by different intervention strategies. A relatively new strategy which has been so far only tested in the laboratory is the use of genetically modified mosquitoes unable to transmit the parasite to a new human host. One approach in particular is the design of mosquitoes equipped with antibodies, namely murine derived antibodies directed against relevant parasite surface proteins. However, it remains unclear which developmental stage is targeted and which antibodies are useful for this approach.

Here I show that in mosquitoes equipped with a human derived anti-CSP repeat binding antibody, oocyst sporogony is blocked in a parasite density dependent manner, and that only repeat binding antibodies can decrease sporozoite loads in the mosquito, additionally these mosquitoes do not show any significant defects in both development and survival.

These results confirm the previously described importance of CSP during sporogony and highlight the efficiency of human derived repeat binding anti-CSP antibodies in interfering with parasite development even in a different host. Additionally, mosquitoes equipped with human derived anti-CSP antibodies show little (in high parasite density infections) to no (in low parasite density infections) sporogonic development, making them a promising tool for malaria transmission blocking interventions.

I provided additional insights into the mechanism by which anti-CSP antibodies interfere with parasite development in the mosquito showing that oocyst are an efficient target for this approach in field infections the detected parasite load in usually less than 30 oocysts per mosquito, therefore the mosquitoes used here are potentially resistant in a more natural setting. Additionally, I provided a new tool allowing a faster screening of antibodies in a mosquito context by injection of single chain Fabs into mosquito hemolymph. Taken together, this approach could one day give rise to alternative strategies in tackling malaria transmissions.

Table of Contents

List of Figures

List of Tables

Abbreviations

A. coluzzii *Anopheles coluzzii*

A. gambiae *Anopheles gambiae*

Ae. agypti *Aedes agypti*

P. berghei *Plasmodium berghei*

P. falciparum *Plasmodium falciparum*

A Ampere

Aapp Anopheline antiplatelet protein

ABTS 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)

BF Blood feeding

BSA Bovine Serum Albumin

CelTOS cell-traversal protein for ookinetes and sporozoites

COPAS Complex object parametric analyzer and sorter

CRISPR Clustered regularly interspaced short palindromic repeats

CSP Circumsporozoite protein

DAPI 4',6-diamidino-2-phenylindole

DEPC Diethyl pyrocarbonate

DI deionized water

DNA Deoxyribonucleic acid

dNTP Deoxyribonucleotide triphosphate

dpi days post infection

ELISA Enzyme-linked immunosorbent assay

FACS fluorescence-activated cell sorting

Fc Fragment crystalizable

fw forward

GPI Glycophosphatidylinositol

HDR homology dependent repair

HPD Horseradish peroxidase

IgG Immunoglobulin G

kDa kilo Dalton

Lp Lipophorin

mAb monoclonal antibody

NHNJ non-homologous end joining

PBS Phosphate buffered saline

PCR Polymerase chain reaction

PFA Paraformaldehyde

PPO Prophenoloxidases

PVDF Polyvinylidene fluoride

rev reverse

RNA Ribonucleic acid

RNAi Ribonucleic acid interference

RT room temperature

scFab single chain fragment of antigen binding

scFv single chain fragment variable

SDS Sodium Dodecylsulfate

SPECT sporozoite microneme protein essential for cell traversal

ssRNA single stranded RNA

TALEN transcription activator-like effector nucleases

TEM Transmission electron microscopy

TEP1 Thioesthercontaining protein 1

TRAP Thrombospondin-related anonymous protein

Tris tris(hydroxymethyl)aminomethane

TSR thrombospondin repeat

V Voltage

1 Introduction

1.1 Malaria

Vector born diseases and, in particular, mosquito-borne diseases have been shaping human populations throughout history [14]. Malaria, a disease caused by a parasitic protozoan of the genus *Plasmodium*, is of particular importance worldwide. *Plasmodium* parasites have been infecting humans for approximately 10.000 years, killing nearly half of the human population ever-existing [30]. The genus *Plasmodium* includes several species, of which a few infect humans, such as *P. falciparum P. vivax P. ovale* and *P. malariae* [78]. Other well studied species such as *P. berghei*, *P. chabaudi*, and *P. yoelii* infect rodents and are, therefore, a frequently used tool in drug and vaccine research [119]
In a natural setting the parasites are transmitted by *Anopheles* mosquitoes, but not all *anopheline* mosquitoes are able to efficiently transmit *Plasmodium* [60]. Even though both parasite and vector have been studied intensively, in 2019, there were still 229 million malaria cases and 409 000 deaths worldwide. The majority of the deaths are children in sub-Saharan Afrika [195].
Upon parasite transmission, some but not all infections turn clinical. The main symptoms caused by *Plasmodium* vary among cases with uncomplicated malaria-causing flu-like symptoms, and include fever and sometimes anemia. In severe cases, malaria symptoms include cerebral malaria, respiratory failure, acute renal failure and severe anemia. Those symptoms can result in death [16]. Depending on the parasite, the incubation period of *Plasmodium* varies between 9-40 days [192]. Even though several *Plasmodium* species can infect and cause symptoms in humans, *P. falciparum* is responsible for the majority of cases and deaths [195]. The distribution of *P. falciparum* cases is spread all over the world. They are, however, concentrated in the Sub-Saharan Africa region. The prevalences of *P. falciparum* coincides with the predicted prevalence of the main vector *Anopheles gambiae* and *A. coluzzii* (Figure 1).

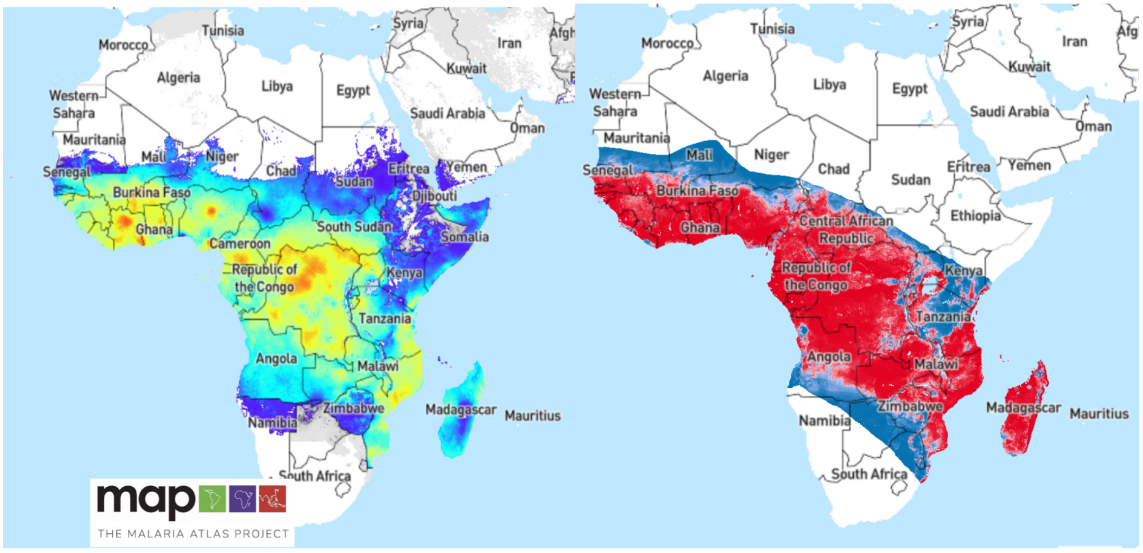

Figure 1. Distribution of malaria-causing parasites and their major vectors around the world. *P. falciparum* parasite rate from 0.01 and 1 (colors from blue to red). White are areas without *Plasmodium* infections between 2009 – 2019. The right side depicts the mean modelled relative probability of occurrence for *A. gambiae* species complex, on a scale 0 to 1.0 (blue to red).

Currently, there are several strategies in place to control malaria. Drugs targeting the parasites have been shown to be efficient tools in limiting deaths, especially in combination therapies using at least two drugs. Currently, there are several antimalarial drugs available, such as artesunate and piperaquine [178]. Achievements in treatment are, however, undermined by a rise in resistance mainly in Asia and recently also in Africa [180]. In addition, mosquito transmission is impeded by the use of insecticide-treated bed nets and indoor residual spraying [195]. The increase in resistance to insecticides in mosquitoes also limits the efficiency of insecticide-treated bed nets [147][146]. The most recent approach approved by WHO is a vaccine protecting mainly children from infections. Unfortunately, case numbers stagnate for five years even with the strategies in place. Therefore, there is still a need for new methods[195].

1.1.1 *Plasmodium* life cycle

Plasmodium life cycle takes part in two distinct environments, and two transmission events are essential for its maintenance. Female *Anopheles* mosquitoes are the vector for *P. falciparum* and infections with the parasite occur during a blood meal. Once a naive female takes a blood meal from an infected human with an established blood-stage infection, this female can take up *P. falciparum* gametocytes.

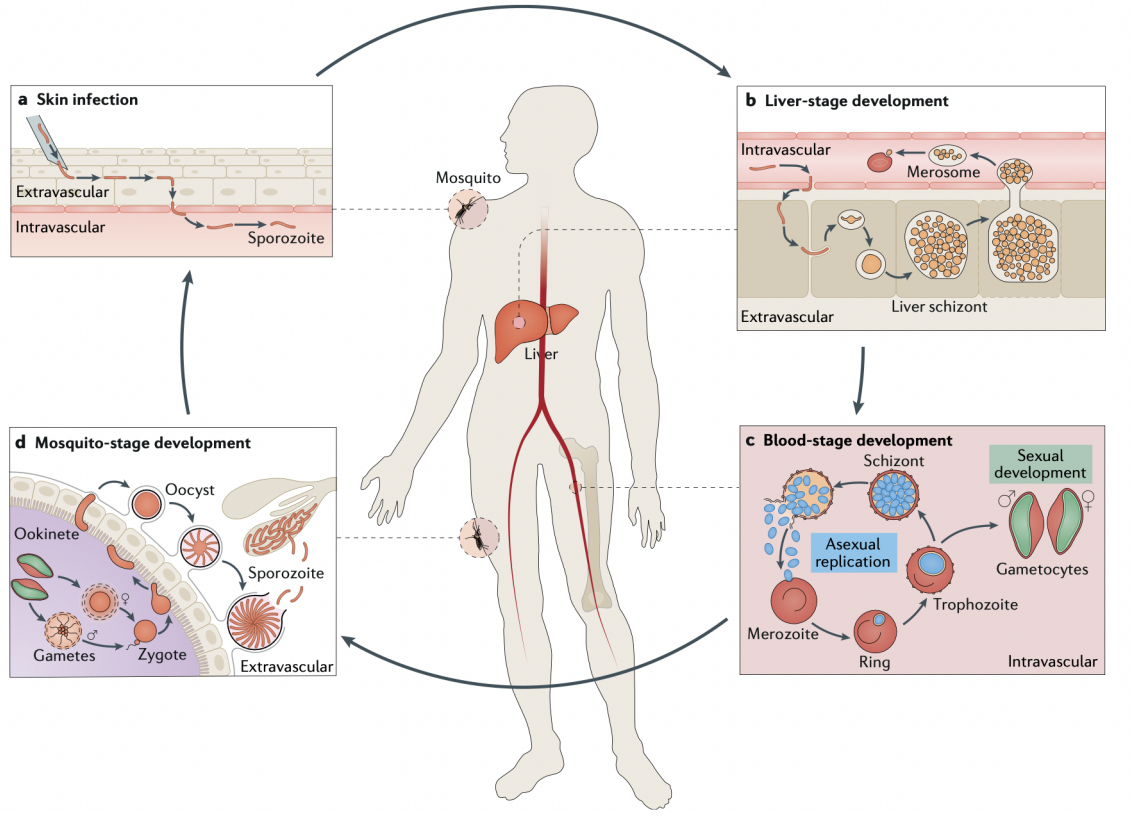

Figure 2. ***Plasmodium falciparum*** **life cycle in the vector and the human host.** Infected mosquitoes inject salivary gland sporozoites into the skin of a new host during probing for blood. During the probing, saliva is injected into the skin containing sporozoites. They enter the bloodstream (a.). In the liver, sporozoites infect hepatocytes and form liver schizonts. Merozoites are released into blood vessels from the merozoite and infect red blood cells (b.). The asexual replication cycle starts with merozoite invasion, followed by the ring stage, the trophozoite and a schizont. With the rupture of the schizont, a new cycle begins. A small proportion of parasites enter sexual development and from gametocytes (c.). Mosquitoes ingest gametocytes. In the mosquito gut, they form gametes, and fertilization occurs. The newly formed zygote develops into an ookinete which invades the gut and forms an oocyst underneath the basal lamina. In the oocyst, sporozoites are formed and, upon rupture of the cyst, released into the mosquito circulatory system (hemolymph). Sporozoites can attach to the salivary glands and infect them. They accumulate in the duct and can be transmitted to a new host during a bite (d.) Figure from [184].

Inside the mosquito, parasites are confronted with such environmental changes as a drop in temperature / pH and an increase in xanthurenic acid [21]. These changes activate release from red blood cells of male and female gametocytes that fuse and form a zygote. The zygote develops further into a motile ookinete. Within 24 hours, the parasite changes its form several times from a round immature to an elongated ookinete [155][67]. The mature ookinete attaches to the midgut wall and traverses midgut cells. Interaction with the mosquito basal lamina and, in particular, the laminin is crucial for the transformation of motile ookinetes into the sessile oocyst [2]. It takes between 9–12 days until the oocyst is fully mature and sporozoites are formed [158]. The detailed process of oocyst maturation and

sporozoite formation is explained more in the following subsection.
Upon rupture of the oocyst capsule, sporozoites are released into the hemolymph. The hemolymph is an open circulatory system, and sporozoites are transported passively through the system. During this migration, the number of sporozoites decreases, due to their loss in other tissues [77] [69]. The attachment to salivary glands, however, is active, and several proteins are essential for attachment as well as invasion and traversal of salivary gland cells [69] [166] [56] [6]. After attachment, the sporozoites traverse the salivary gland acinar cells and accumulate inside the duct [139]. Oocyst sporozoites are not infectious to the mammalian host [182] and lack many proteins needed for a successful infection. Once they have invaded salivary glands, a change in the transcriptional program occurs, and the parasite becomes infectious [106] [112]. Sporozoites reside in the salivary ducts until they are injected into the skin during the next blood meal. Traversal of acinar cells along with specific destruction by sporozoites leads to a deficiency in blood-feeding due to inhibitors of blood clotting factors [149] [51] [175]. This defect results in several probing attempts, thereby increasing the chances of sporozoites being injected into the skin of a new mammalian host [151].
In the skin, the sporozoites migrate through the epithelium until they reach blood vessels or lymph [183]. In the vessel, they are passively transported via flow to lymph nodes or liver parenchyma. Sporozoites do not immediately infect hepatocytes. Firstly they migrate through the sinusoidal barrier, traverse cells with low heparin sulfate content and only then actively infect hepatocytes with higher heparin sulfate density [42][61]. Several hosts and parasite factors are required for a successful traversal and infection. It is believed that the circumsporozoite protein (CSP) is required for binding to heparin sulfate [12][205]. Traversal of the cells is facilitated by the sporozoite microneme protein essential for cell traversal (SPECT), sporozoite microneme protein essential for cell traversal 2 (SPECT2) and cell traversal protein of ookinetes and sporozoites (CelTOS). SPECT1 and SPECT2 are microneme pore-forming proteins secreted by the parasite and required for disruption of the host cell membrane allowing traversal and invasion of these cells [87] [102] [94] whereas CelTOS is considered to allow moving through the cytoplasm and exiting the cell [96]. Another protein important for hepatocyte traversal and invasion is the thrombospondin–related protein anonymous protein (TRAP) [26]. Similar to CSP, TRAP is located on the sporozoite plasma membrane but anchored via a transmembrane domain. This connection allows interaction with the actinomyosin through an unknown protein. TRAP is essential for the gliding motility of the sporozoites [166]. The receptor for TRAP is considered to be integrins [55].
For the invasion of hepatocytes by *Plasmodium* expression of CD81 on the hepatocyte is required, and antibodies against CD81 inhibit *P. falciparum* invasion of hepatocytes [157]. CD81 presence allows for *P. yoelli* and *P. berghei* sporozoites to invade cells [150] and absence of the receptor led to increased cell traversal [115]. The scavenger receptor class B type 1 (SR-B1) is used by *P. vivax*, it can, however, also be used by *P. berghei* for invasion. CD81 and SR-B1 on the host cell interact with the parasite protein P36 [115].
After successful infection of the hepatocyte, the parasite resides inside a parasitophorous vacuole and develops into a spherical exoerythrocytic form. Through asexual replication, thousands of merozoites are formed. Inside a vesicular structure called merosome, the parasites are released into the liver sinusoids and transported

into the blood vessels. The release of merozoites into the bloodstream establishes the blood stage of *P. falciparum* infection. Merozoites invade red blood cells, form a parasitophorous vacuole, and another round of asexual schizogony occurs. Within the 48 h asexual cycle, the parasite undergoes a series of transformations. *Plasmodium* starts remodelling its host cells during the so-called "ring stage". During the 'trophozoite stage', the parasite growths and haemozoin, product of haemoglobin digestion, accumulates in the cell [107]. Additionally, knobs are formed on the outside of infected red blood cells. Parasite proteins located on the knobs enable cytoadherence to other host cells [81] and adherence to microvasculature is considered to be an important contributor to the pathogenesis of *P. falciparum* [162].
Another round of schizogony occurs during the schizont stage, forming merozoites which are released into the bloodstream and the cycle repeats [105][118]. The red blood cells rupture during this process, and the numerous repeated cycles lead to a significant loss in red blood cells and a release of cell debris, causing the symptoms of malaria such as anaemia and fever [76].
A small subset of parasites, however, takes a different path, undergoes sexual commitment and develop into gametocytes [27]. Immature gametocytes sequestrate in the spleen, and bone marrow [95] until they reach the mature infectious stage V [160]. The mature gametocytes can stay in circulation for a few days until, during a blood meal, they are taken up by female mosquitoes, and the cycle continues.

1.1.2 Sporogony and sporozoite invasion of salivary glands

Parasite development in the vector is a highly complex process involving several morphological stages. In particular, the formation of the infectious sporozoites and the subsequent invasion of mosquito salivary glands, are not well studied even though it is the longest developmental process in the vector. Most information available on oocyst development and invasion of salivary glands is based on electron microscopy of *P. gallinaceum* and *P. berghei*.
Oocysts are sessile forms of the parasite located between the midgut epithelium and the basal lamina. The cyst is protected by a capsule and the cytoplasm is surrounded by the parasite plasma membrane. Early oocyst starts growing in size and accumulates nutrients supplied by the mosquito [44]. The growth process is accompanied by multiple rounds of asexual divisions of the nucleus while the nuclear envelope preserves its structure and is not disrupted (schizogony) [66]. This is followed by the segregation of the oocyst cytoplasm into small islands called sporoblasts. This segregation is due to a retraction of the plasma membrane while the capsule surrounding the oocyst remains intact (Figure 3). Around ten days post-infection, the oocyst starts to form vacuoles by retracting the inner membrane into the cyst. In parallel, cleft formation occurs caused by vacuoles arising from the endoplasmatic reticulum [158]. Finally, sporozoites bud from the sporoblast. The oocyst sporozoites reside inside the cyst protected by the capsule and accumulate in the periphery [123] [120] [171]. The exit of sporozoites from the oocyst differs among species, with *P. falciparum* showing single sporozoites escaping through small wholes, whereas in *P. berghei* the oocyst shows removal of the wall and sporozoites exiting the cyst after it is fully opened and the wall destroyed [135].

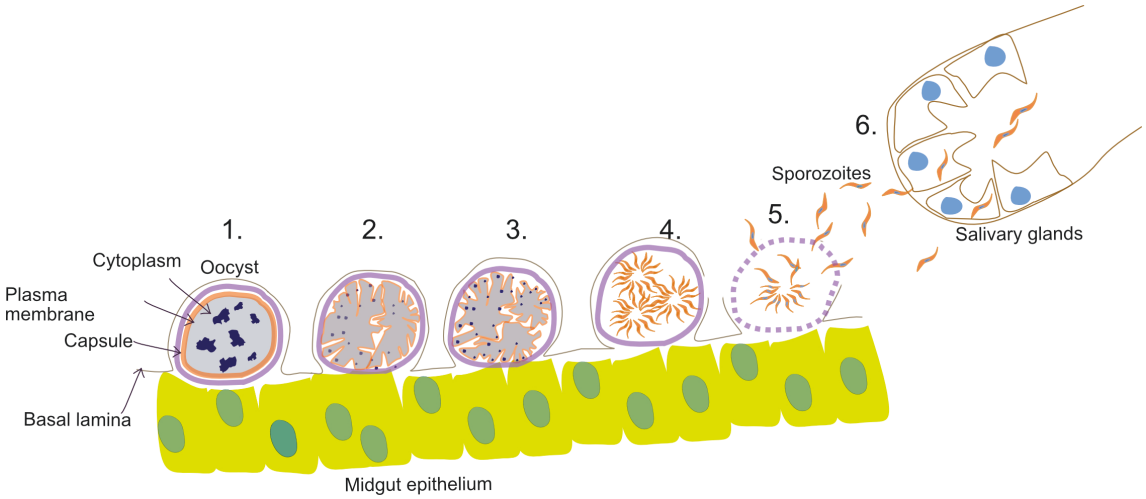

Figure 3. ***P. falciparum*** **oocyst development.1.** Schizogony starts with DNA replication in the oocyst. **2.**Retraction of the plasma membrane initiates sporoblast formation. **3.** The newly-formed nuclei align in the sporoblast periphery and sporozoite budding begins. **4.** Sporozoite budding and individualization leads to sporozoites accumulation in the oocyst. **5.** Sporozoites are released into the mosquito hemolymph and travel to the salivary glands. **6.** Sporozoites attach to the glands, invade the cells and accumulate in the duct.

Only a few proteins have been so far identified to be essential for oocyst development. It is known that the fatty acid synthesis is essential, and blocking of the protein FabI, as well as FabB/F, leads to deficiency in sporogony [181]. Depletion of an inner membrane complex protein called PbIMC1a in *P. berghei* also affects sporozoite formation by causing unusually shaped sporozoites with impaired motility [98]. Lipophorin (Lp), a mosquito lipid transporter, is another critical protein for oocyst development. Lipophorin depletion reduces oocyst size and decreases the overall sporozoite loads. Moreover, the formed sporozoites show low mitochondrial membrane potential and are not efficient in infecting the next mammalian host [44]. CSP is essential for successful sporogony, and it has been hypothesized that CSP drives retraction of the plasma membrane [121] [59]. Sporozoite release from *P. berghei* oocysts is an active process requiring CSP and a serine repeat antigens (SERAs) protease. It was proposed that the serine protease cleaves CSP on the cyst wall once it is matured and allows sporozoites to exit the cyst [10] [188]. For *P. falciparum* there are indications that adhesion proteins are necessary for the exit of sporozoites from oocysts [143].

Sporozoites freshly released from the oocyst are not infectious and need to mature before they can actively infect a new mammalian host. Sporozoites are transported through the hemolymph of the mosquito. There are, however, indications for chemotactic responses in sporozoites [6] directing the sporozoite to salivary glands. Mosquitoes have two glands consisting of three lobes each. Once the sporozoites have reached the glands, they attach to the basal lamina and traverse this barrier. There is evidence that sporozoites preferably invade the distal lateral lobes. Sporozoites puncture the salivary gland membrane, enter the cells and migrate through the cytoplasm [139]. Majority of sporozoites accumulate in the acinus cells and a few resides in the duct until they are transmitted during a blood meal [193].

1.1.3 Circumsporozoite protein (CSP) the major surface protein of sporozoites

One protein is of particular interest during the late mosquito stages and transmission to the human host. The circumsporozoite protein (CSP) is the major surface glycoprotein of *Plasmodium* [202] [133]. The expression of *CSP* starts as early as day six post-infection and the protein is located at the oocyst plasma membrane[141] [72]. The overall CSP organization is similar among all *Plasmodium* species. It consists of a signal peptide, an N-terminus, a repeat region followed by a C-terminus and a glycosylphosphatidylinositol (GPI) anchor, which connects the protein to the plasma membrane [189].
The so-called 'Region I' is located at the N-terminus and is believed to contain a conserved proteolytic cleavage site[41]. Region I has been proposed to function as a regulatory unit of the protein, mainly by hiding the C-terminus. Additionally, depletion of the N-terminus increased the midgut sporozoite numbers but decreased the salivary glands sporozoites [43][100]. Region I was reported to be involved in the attachment of sporozoites to different cells, in particular, the salivary glands [129].

The repeat region of CSP is present in all *Plasmodium* species. However, the amino acid composition and the number of repeats differ between species, but within each species, the amino acid sequence is the same Figure 4[97].For *P. falciparum* NF54 strain, there are 42 tandem repeats of N-(AV)-(ND)-P (see UniProtKB - P19597). The repeat region itself appears not to be essential for sporozoite formation, but depletion leads to defects in sporozoite maturation, causing sporozoite death before oocyst egress. Depletion of both repeat region and N-terminus cause the same defect as CSP depletion, full inhibition of sporozoite formation due to a lack of plasma membrane retraction [59]. The repeated sequence is also needed for a successful traversal of hepatocytes in the human host[124][177].

The C-terminus contains region III, an adhesive thrombospondin repeat (αTSR) region, and a GPI anchor. It is hypothesized that the αTSR region is masked by the N-terminus and is only exposed in the last part of the sporozoite journey through the mammalian host [43]. The αTSR region in combination with the repeat region is essential for sporozoite formation [59] and for attachment to hepatocytes [34][61]. Parasites with deletions of the CSP GPI-anchor did not produce sporozoites, and the phenotype could not be rescued by insertion of a transmembrane region [189]. CSP is constantly shed from the sporozoite surface, and gliding sporozoites leave a CSP trail behind. Therefore, CSP might also be important for sporozoite gliding[165] However, there is indecisive evidence surrounding the role of CSP in motility as well as in salivary gland invasion [48][191] [172][129][43].

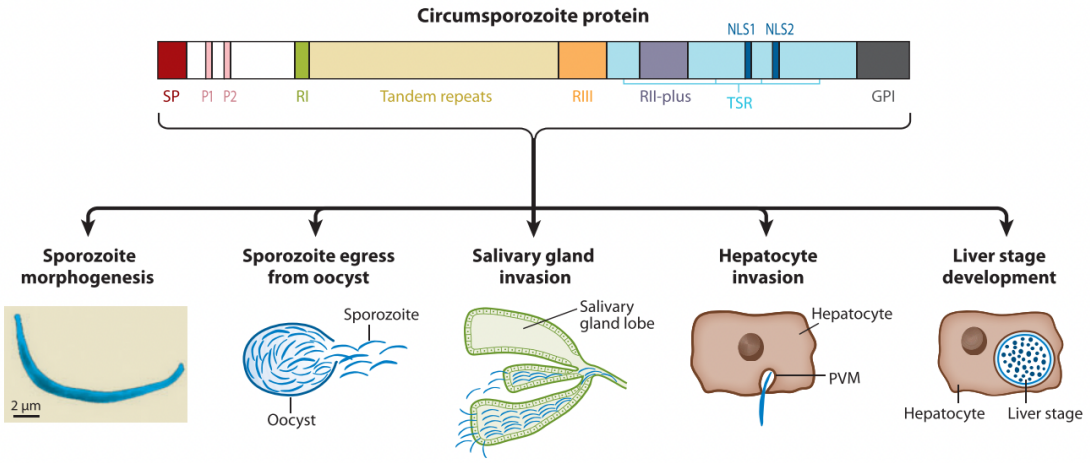

Figure 4. ***Circumsporozoite protein*** **is required for several steps during the parasite development.** Depletion of CSP abolishes sporozoite formation and egress from oocysts[121] [189]. Invasion of salivary glands can be decreased by blocking CSP [129] or depletion of the N-terminus[43]. Antibodies against CSP can also inhibit hepatocyte traversal infection[148][124][177]. Figure from [11]

Several functions of CSP are not yet associated with a particular region of the protein. Even though CSP is an essential component of the sporozoite development in the mosquito, not many interaction partners have been identified, nor could the conformation of CSP during its journey through both mosquito and mammalian host be fully understood. Most of the experiments on CSP were done with the murine parasite *P. berghei* since this parasite can easily be genetically modified [92]. Therefore, many of the previously identified functions of CSP for *P. berghei* could not yet be confirmed for *P. falciparum*.

1.2 Malaria vaccines

First approaches to develop a vaccine protecting against *P. falciparum* were undertaken in the early 1960s. They were based on the knowledge that repeated exposure to irradiated sporozoites can induce a protective immunity towards *Plasmodium* in mice [132], and humans [38]. Several methods have been utilized with the goal to generate a vaccine able to protect and prevent death. Until now, only one vaccine was efficient enough to get approved and is now in a pilot experiment, used in endemic areas[5]. Recently, this vaccine called RTS,S was approved by the WHO in particular for children living in high-risk areas [196].
RTS,S contains a partial sequence of CSP, in particular, the last 18 NANP repeats and the C-terminus except for the GPI anchor. However, there is room for improvement, since the protection of around 35% fades within four years to 2.5%, additionally, it does not efficiently inhibit infections of local strains [134].
Whether a naturally acquired immunity can convey sterile protection remains unclear for now [80]. CSP has been a major target for vaccine development. It is, as

mentioned previously, the dominant surface protein of sporozoites. Several clinical trials are ongoing aiming to make use of the efficient immune response against CSP [62][46][167].

Nevertheless, targeting CSP is not the only approach used in vaccine development. After all, the *Plasmodium* life cycle consists of multiple stages, and nearly all of them have been targeted by vaccine development approaches. There has been early proof that immunity against erythrocytic stages prevents symptoms and protects against severe malaria. [39]

As a consequence of this finding, other vaccine studies have tried to generate vaccines against blood and sexual stages. On the one hand, transmission-blocking vaccines, targeting the sexual stages with the purpose to inhibit transmission of *P. falciparum* to a new vector. The leading targets of such vaccines are Pfs48/45 and Pfs230 [199] [169] [114] [159]. Vaccines can also aim to protect individuals from severe forms of disease and prevent deaths. Potential targets are, for example, PfRH5. PfRH5 is a highly conserved merozoite protein essential for invasion of red blood cells [18]. Antibodies against PfHR5 inhibit merozoite invasion [54].

1.2.1 Anti-CSP antibodies

As described above, the circumsporozoite protein is essential for several developmental processes in the parasite life cycle and is expressed during mosquito as well as the early mammalian stages. Most importantly, CSP is exposed on the sporozoite surface during the vector to human transmission event. Such accessibility of this highly immunogenic protein facilitates a diverse immune response. In particular, the repeat region of CSP functions as a dominant B cell epitope and can induce antibody production [142][202][45].
This knowledge stimulated an increase in studies focusing on CSP based vaccines and antibodies. It ultimately led to the identification of the first inhibitory anti-CSP antibody 3D11[142] against *P. berghei* CSP (PbCSP). Shortly after the identification, the current "golden standard" murine-derived anti-PfCSP antibody 2A10 was generated [204].

Within the last 5 years, hundreds of anti-CSP antibodies were identified [177] [170] [127] [128] [137] [35] [138] [99]. Interestingly, even though many of these antibodies have similar affinities to the same epitope, the mechanisms of binding are different. The structure of the recombinant *PfCSP* repeats have been shown to form a spherical complex in combination with anti-repeat Fabs such as 311[136].
Antibodies interfere with epitopes through cell dependent or independent mechanisms. The majority of the identified anti-CSP antibodies are class IgG1 and IgG3 [79]. It was described that specific anti-repeat IgG1 or IgG3 antibodies activate the complement system [19] [103]. Opsonisation followed by phagocytosis has been reported to be induced by anti-CSP antibodies; however, this effect was observed us-

ing antibodies binding to the N-terminal region[58]. There are also cell-independent functions of anti-CSP antibodies. Upon contact with specific anti-CSP antibodies, sporozoites shed CSP and, upon movement, left behind a trail of CSP. This process is called the circumsporozoite precipitation reaction [165] [164]. In particular, repeat binding anti-CSP antibodies were shown to induce the death of sporozoites. In this particular case, the antibody triggers secretion of CSP and cell wounding proteins such as SPECT and SPECT2. Upon the circumsporozoite precipitation reaction, the CSP coat is removed from the sporozoite membrane, and the sporozoite is vulnerable to its own pore-forming proteins (SPECT and SPECT2), which subsequently lead to the death of the sporozoite [8]. A very common mechanism is the neutralization of sporozoites by antibodies. Neutralizing antibodies bind to epitopes of a protein and neutralize the functional activity of the protein by, for example, blocking interactions with receptors or binding partners but does not involve other cells [50]. In particular, repeat targeting antibodies were shown to be effective in binding and also neutralizing sporozoites [99][177] [124][127]
Neutralizing antibodies can also be used for passive immunization. Passive immunization is the transfer of protective antibodies, it differs from active immunization achieved by vaccination, in which the antibodies are produced by the individual. One of the recently identified antibodies, CIS43 [99], is now used in a clinical trial assessing its efficacy, safety and pharmacokinetics as an antimalarial antibody [65]. Anti-CSP antibodies are not only useful for vaccine development or passive immunization. They have been used in different approaches, for example, in the context of transgenic refractory mosquitoes [85].

1.3 *Anopheles* mosquito biology

1.3.1 *Anopheles* life cycle

The main vector for *P. falciparum* are mosquitoes of the genus *Anopheles*. Only a few species in this genus have been shown to be efficient transmitters of *P. falciparum*[194]. *Anopheles* mosquitoes belong to the family *Culicidae* and the order *Diptera*. They have a complex life cycle with four stages in two environments. The aquatic stages consist of eggs, four larvae instar and pupae (Figure 5). The eggs are deposited by female adults on water surfaces, and larvae emerge from the eggs approximately 1 to 10 days, depending on the water temperature[83]. During 1-2 weeks, larvae moult several times to go through four instar stages. During this period, larvae acquire nutrients and shape their future fitness as adults [176][113][130][200]. They undergo metamorphosis during the pupae stage. Pupae do not ingest any nutrients and are located underneath the water surface. The metamorphosis is completed after one day and adults emerge. The adult mosquito has reached its final size at the point of emergence, and no additional growth occurs afterwards. The size of adult mosquitoes is determined by the nutrition obtained as larvae[153]. There appears to be a correlation between the size of adult mosquitoes and the longevity [15]. Mosquito development is highly influenced by such environmental factors as nutritional state, temperature, and microbial environment[154] [198][40].

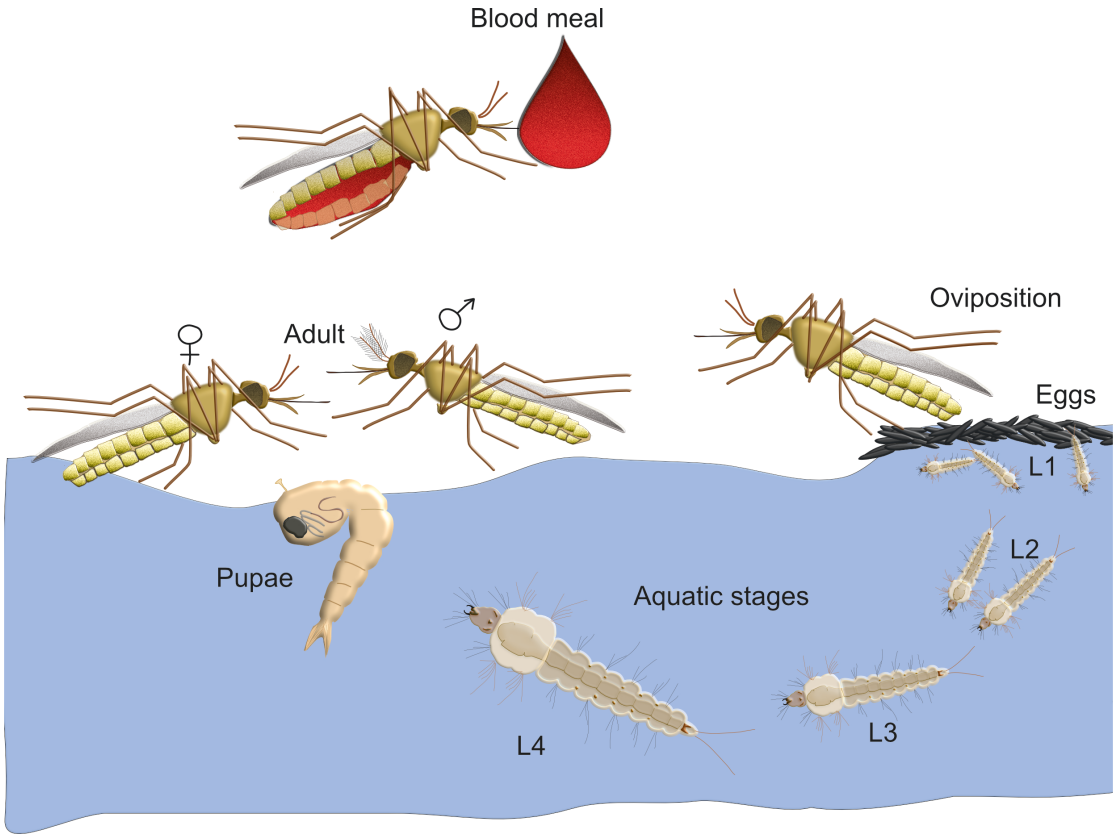

Figure 5. *Anopheles* life cycle *Anopheles* mosquitoes live in two different environments. The eggs are laid onto water surfaces and larvae hatch into the water. Here they undergo several moulting steps until they reach the pupal stage. After metamorphosis, adult mosquitoes emerge and mate. After mating, a blood meal is required for egg development.

1.4 Vector control strategies

The main vector control strategies focus on physical barriers to protect humans from mosquito bites. These methods, in combination with insecticides, are an effective measure to limit transmission [195]. Specifically, the use of insecticide-treated bed nets is one of the best strategies to protect humans from *Plasmodium* infections. However, insecticides are not as efficient as they were years ago. Within the last ten years, several countries reported resistance of mosquito populations to the main insecticide used.[195]. Therefore, there is a need for a better understanding of vector biology as well as vector-pathogen interactions.

1.4.1 Transgenic Mosquitoes

Transgenic insects have revolutionized research, in particular modification in the model organism *Drosophila melanogster* [163]. Modifications in insects are not restricted to *Drosophila*. Advancements in the field made it possible to modify vectors

such as *Aedes aegypti*[90], *Anopheles stephensi*[33], *Culex quinquefasciatus*[9], recently also for *Anopheles gambiae* [70] and *Anopheles funestus* [144]. With the first targeted mutagenesis of *A. gambiae* the main vector of human malaria was possible and also opened the door for new vector control strategies [161] [190].
Germline transformations are accomplished by injection of plasmids into mosquito eggs via microinjection [90]. The main mechanism of germline transformation in the past was P-transposon-based [68] for *D. melanogaster*. Transformation of *A. gambiae* was more difficult since most of the previously used transposable elements were absent from this species. The *piggyBac* transposable element was identified as an acceptable tool for *A. gambiae* germline transformation [70]. Other approaches such as TALEN mediated [161], or integrase-mediated transgenesis [22] have been since then applied for transformations. The most recent advancement in genome editing with the discovery of clustered regularly interspaced short palindromic repeat (CRISPR) associated endonuclease (Cas9) allowed precise insertions and deletions of genomic sequences also in mosquitoes [111][17].
Transgenic mosquitoes can be used in functional genomics studies, for example, to investigate specific gene functions or to tag proteins in order to study location and expression sites [140][185]. A relatively new idea is the use of genetically manipulated organisms to tackle vector-borne diseases [89] [190], for vector control and for managing insect pests [47]. In order to generate resistant mosquitoes it is essential to first understand mosquito physiology and identify promoters regulating protein expression in mosquitoes.

Several promoters have been identified in *Anopheles* mosquitoes allowing the expression of an exogenous gene. They can be divided into distinct groups. Promoters, such as *Polyubiquitin-c* allow multi-tissue expression at relatively high levels [3]. Others are tissue specific and allow consecutive expression in mosquito organs or cells such as the *hemolectin* promoter that drives gene expression in hemocytes [140]. Inducible tissue-specific expression in *Anopheles* is facilitated by several frequently used promoters, for example the *anopheline carboxypeptidase* (ACP) promoter, the *vitellogenein* promoter and the *lipophorin* promoter. The *anopheline carboxypeptidase* (ACP) promoter allows expression in the mosquito gut upon a blood meal[126]. The *vitellogenin* promoter is also activated upon blood-feeding and allows expression 24-48 h post blood meal in the mosquito fat body. Vitellogenin is secreted by fat body cells and transported to the ovaries [179]. A novel promoter used in transgenic mosquitoes is the *lipophorin* promoter. It was recently used for over expression of the immune factor TEP1 in the mosquito fat body [186]. Lipophorin is similar to vitellogenin in that it is a nutrient transport protein synthesized by fat body cells. Lipophorin is secreted into the mosquito hemolymph transporting lipids to different mosquito tissues[13]. In contrast to vitellogenin, lipophorin is constitutively expressed and its expression is up-regulated upon blood feeding [117]. Identification of these promoters facilitated specific modifications of the mosquito physiology.

The vector control approaches based on transgenic mosquitoes can be divided into two groups. The first approach is a population-suppression strategy aiming at decreasing and eliminating specific species in the field, thereby reducing the population of possible disease transmitters. The main approach used here is

sterile-insect-technique (SIT), based on generation of sterile males[20]. The sterile male technique relies on the usage of either radiation-sterilized males or genetically modified males carrying a self-limiting gene. The release of insects carrying a dominant lethal gene (RIDL) uses the tetracycline operon system. In the absence of tetracycline, expression of a toxin causes the death of the mosquito. RIDL can also be sex- and tissue-specific as in the fsRIDL system. The toxin has been recently expressed in the flight muscle of female mosquitoes and renders them incapable of flying (Figure 6 A and B) [63] [104]. For limiting *Aedes* populations, the sterile male tetracycline based system has been tested in the field. Reduction in population size has been achieved by releasing sterile males in the Cayman islands, Brazil and recently also in Florida (USA) [74][31][64].

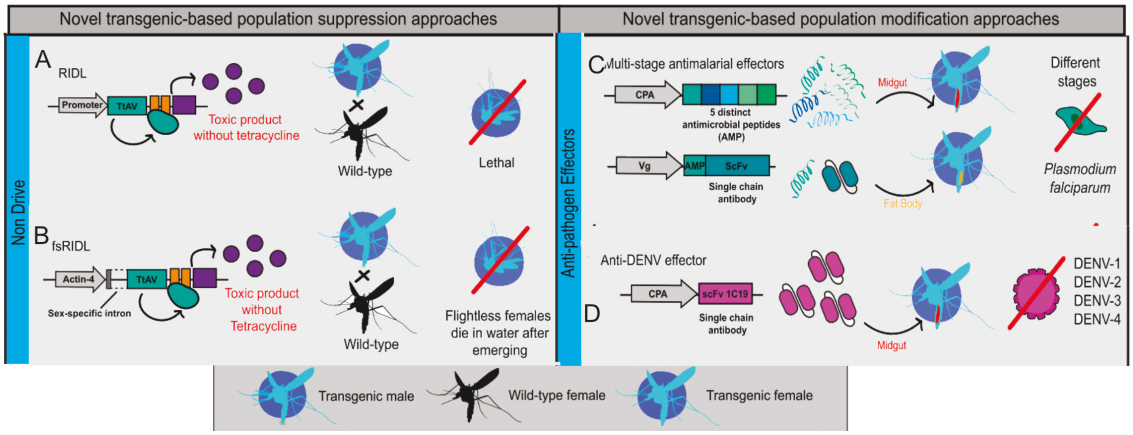

Figure 6. Population suppression and modification strategies tackling arboviruses and *Plasmodium* transmission. Population suppression strategies using (A) the release of insects carrying a dominant lethal gene (RIDL) in the absence of tetracycline toxic products are expressed in the mosquito causing death. (B) the fsRIDL incldues a sex-specificity and is only expressed in the flight muscles of females and prevents them from flying. (C) Antibody or antimircobial peptide expressing in fat body or midgut can limit plasmodium development in *Anopheles stephensi*. (D) Antibody expression can inhibit virus replication in *Aedes aegypti*. Modified from [187]

The second approach focuses on the replacement of native populations with parasite-resistant mosquitoes. Several reports have shown how partial resistance to parasites and viruses can be achieved in mosquitoes such as *Ae. agypti* [28] and *A. stephensi* [52] [85]. In the last decade, there have been many attempts to generate refractory mosquitoes to break the transmission chain of either malaria, dengue or Zika virus. This was approached by up-regulation or down-regulation of mosquito genes essential for parasite infection, as well as the up-regulation of immune factors targeting *Plasmodium* parasites (Figure 6C). Modifications of the mosquitoes immune system alone were not successful, and only partial reductions in parasite loads were achieved [101] [201] [53]. Additionally, this approach bears risk of high fitness costs. Upregulation and downregulation of mosquito immune genes can also affect other relevant physiological processes, for example pathways relevant for reproduction [201]. On its own, this method is not sufficient to generate refractory mosquitoes. Therefore, further tools are required to achieve full resistance to *Plasmodium* in mosquitoes.

1.5 Antibody expressing mosquitoes

As mentioned above, antibodies and, in particular, anti-CSP antibodies are efficient in neutralizing the parasite. Already in the early 2000, injection of full-length antibodies [191] and injection of viruses producing anti-CSP antibodies [48] reduced *P. gallinaceum* development in *Ae. aegypti*. With this knowledge, the first *A. stephensi* strains were generated targeting CSP [85]. Instead of full-length antibodies, single-chain fragment variable antibodies (scFvs) were fused to an antimicrobial peptide (Cecropin A). The idea behind this approach was to block sporozoite invasion of the salivary glands and to kill sporozoites by Cecropin A. Expression of the scFv-fusion protein was under the *vitellogenin* (Vg) promoter, facilitating relatively high expression levels in the fat body shortly after blood feeding [131][36]. In parallel, *A. stephensi* mosquitoes expressing antibodies against the ookinete protein Pfs25 to block ookinete invasion of the midgut and the *Plasmodium* chitinase 1 to limit oocyst formation were designed. The expression was in the mosquito midgut. This was achieved by using the *A gambiae carboxypeptidase A* (*AgCPA*) promoter. The first strain expressing anti-CSP antibodies decreased sporozoite loads in the salivary glands upon multiple blood feedings. However, even in combination with the two other strains decreasing ookinete invasion and oocyst formation [86] the development was never fully blocked.

The recent identification of the anopheline antiplatelet factor (Aapp) made an expression of exogenous proteins in the female salivary glands possible [203]. Soon after the first approach, murine-derived scFv anti-CSP antibody fused to RFP was expressed in *A. stephensi* salivary glands to limit transmission of sporozoites. Here transmission to mice was significantly reduced, and only 25% of mice got infected [168]. A similar result was observed in *A. coluzzi* expressing the human-derived scFv 125 in the salivary glands. Here, the effect was much stronger; only 10% of mice got infected, and the onset of parasitemia had a two days delay [177] indicating that human-derived anti-CSP antibodies are efficient in limiting parasites in the mosquito. Challenges remain to finding an optimal strategy to fully block parasite development. Therefore a better understanding of the anti-CSP antibodies in the mosquito context is essential.

2 Aim

The aim of this study was to characterize the role of CSP in the development of *P. falciparum* in the mosquito using transgenic mosquitoes expressing human anti-CSP antibodies in the fat body. Therefore I assessed the phenotype of the transgenic mosquito upon infection with *P. falciparum* to identify the developmental target site of the antibodies as well as the mechanism of antibody inhibition.
Secondly, I examined the early development and survival of the transgenic mosquitoes to identify potential trade-offs of the transgene expression.
Thirdly, I established a testing pipeline for new antibodies in the mosquito that allowed for screening for more efficient antibodies before designing new strains.

3 Material and Methods

3.1 Material

Table 1. Antibodies

Name	species	Target	Company
anti-mouse HPD	goat	mouse Fc	Thermo Fisher, Germany
anti-mouse-AF488	goat	mouse Fc	Thermo Fisher, Germany
anti-rabbit-AF555	goat	rabbit Fc	Thermo Fischer, Germany
anti-rabbit HPD	goat	rabbit Fc	Thermo Fisher, Germany
anti-FLAG	rabbit	FLAG tag	Thermo Fisher, Germany
anti-human IgG-HPO	goat	human IgG	Thermo Fischer, Germany
anti-PfCap380	rabbit	PfCap380	[88]
JH73 (anti-TEP1)	rabbit	AgTEP1	[110]
mAb 2A10	mouse	PfCSP	BEI resources
mAb 2A10 AF548	mouse	PfCSP	BEI resources
mAb 2A10 AF647	mouse	PfCSP	BEI resources
mAb 3D11	mouse	PbCSP	BEI resources
mAb 9E10	mouse	c-Myc tag	Thermo Fisher, Germany
mAb 9E10 AF647	mouse	c-Myc tag	Thermo Fischer, Germany
mAb 9E10 FITC	mouse	c-Myc tag	Thermo Fischer, Germany
scFab1210	-	repeat region (CSP)	kindly provided by S. Scally and JP. Julien
scFab1710	-	C-terminus (CSP)	kindly provided by S. Scally and JP. Julien
scFab5D5	-	N-terminus (CSP)	kindly provided by S. Scally and JP. Julien

Table 2. Biological Resources

Name	Origin
Anopheles coluzzii (Ngousso colony)	obtained from Institut de Biologie Moléculaire et Cellulaire (IBMC), Strasbourg, France [75]
Anopheles coluzzii S1 (*TEP1*S1*)	Originated from the Ngousso colony by selection of *TEP1*S1* homozygotes
Anopheles coluzzii Lp::125	Eric Marois and Maria Pissarev
Anopheles coluzzii Lp::2A10	Eric Marois and Maria Pissarev
Anopheles coluzzii Aapp::125	Eric Marois and Maria Pissarev
P. falciparum NF54 (Pf NF54)	from Prof. Dr. R.W. Sauerwein [49]
P. berghei ANKA PfCS(r)PbCS *eef1a::GFP-Luc*	Shahid Khan; Chris J. Janse (Leiden Malaria Research Group, LUMC)
P. berghei ANKA PfCS(r)PbCS *hsp70::mCh+eef1a::Luc*	Blandine Franke-Fayard; Chris J. Janse (Leiden Malaria Research Group, LUMC)

Table 3. Chemicals

Chemical	Company
2-Propanol	Carl Roth, Germany
Agarose	Sigma-Aldrich, Germany
Anti-Fade Fluorescence Mounting Medium	Abcam, Germany
Bovine Serum Albumin, fraction V	Biomol GmbH, Germany
Chloroform	Sigma-Aldrich, Germany
dNTP-mix (2mM)	Thermo Fisher Scientific, Germany
Dodecylsulfate sodium (SDS), pelets	Severra
Ethanol, molecular grade	Carl Roth, Germany
Fetal calf serum (FCS)	Gibco Invitrogen, Germany
full-length recombinant CSP	kindly provided by the Wardemann lab
Giemsa concentrate (10x)	VWR, Germany
Giemsa staining buffer (1x)	VWR, Germany
Glycerol	Carl Roth, Germany
Glycine	Sigma-Aldrich, Germany
Liver Powder	NOW Foods, USA
Low melting point Agarose	Sigma-Aldrich, Germany
Mercury dibromoflourescein disodium salt	Sigma-Aldrich, Germany
Methanol	Merck, Germany
Milkpowder	Carl Roth, Germany
Paraformaldehyde	Alfa Aesa, Germany
Phosphate-buffered saline (PBS), premixed 10 X	Roche Diagnostics, Germany
Random Hexamer Primers ($0.2\mu g/\mu l$)	Fermentas, Germany
RNAse away	Thermo Fisher Scientific, Germany
RNAse-free PBS	Thermo Fisher Scientific, Germany
Sodium acetate	Carl Roth, Germany
Sugar, table sugar	LIDL, Germany
TRIS base	Sigma-Aldrich, Germany
Trition X 100	Sigma-Aldrich, Germany
Tuna Meal	
Tween	Sigma-Aldrich, Germany
Vanderzant vitamin mixture for insects	Sigma Aldrich, Germany

Table 4. Commercial Kits

Kit	**Company**
Ambion MEGAclear Kit	Thermo Fisher Scientific, Germany
Ambion MEGAscript T7 kit	Thermo Fisher Scientific, Germany
cOmplete™Mini Proteaseinhibitor-Cocktail	Merck, Germany
Pierce ECL® Western Blotting Substrate	Thermo Fisher Scientific, Germany
QIAquick PCR Purification	Qiagen, Germany
RapidOut DNA Removal Kit	Thermo Fisher Scientific, Germany
RNeasy Mini Kit	Qiagen, Germany

Table 5. Consumables

Consumable	Company
Beads, for TissueLyser LT, stainless steel, 5 mm	Qiagen, Germany
BRAND Malassez counting chamber	Thermo Scientific, Germany
Coverslips	Carl Roth, Germany
Eppendorf tubes, 1.5 ml and 2 ml	Eppendorf, Germany
Falcon tubes, 15 ml and 50 ml	Sarstedt, Germany
KIMBLE Dounce tissue grinder set 2 ml	Sigma Aldrich, Germany
MicroAmp Optical Adhesive Film	Applied Biosystems, Germany
Parafilm	Bemis, USA
Pasteur pipettes	Carl Roth, Germany
PCR tubes	Greiner Bio-One, Germany
Pipette filter tips, Biosphere, RNAse free, 10-1000 μl	Sarstedt, Germany
Pipette tips, 10-1000 μl	Sarstedt, Germany
Pipette tips, epTips, for epMotion, 50 μl	Eppendorf, Germany
Pizza Storer (pans for mosquito larvae rearing)	Sealfresh Square, UK
Rearing pans 3.5 l	Steward Catering, UK
Siliconized pipet tips	(Ref 613-0279, VWR)
Siliconized tubes	(Ref LW2420 Alpha Laboratories
Water, DEPC-treated	Thermo Fisher Scientific, Germany
96-Well ELISA Microplates	Greiner OneBio, Austria

Table 6. Enzymes

Enzyme	Company
Fast SYBR®Green PCR Master Mix	Applied Biosystems, Germany
FastDigest KpnI	Thermo Fisher Scientific, Germany
FastDigest NotI	Thermo Fisher Scientific, Germany
QuantiTect Sybr green	Qiagen, Germany
RevertAid® H Minus Reverse Transcriptase	Life Technologies, Germany

Table 7. Equipment

Equipment	Company
Aspirator, mechanical	Clarke, USA
Axio Observer	Zeiss, Germany
Binocular light source KL1500	LCD Leica, Germany
Binocular M80	Leica, Germany
C1, C2, C4 mosquito cages	Handmade
Centrifuge, 96-well plates, benchtop	Thermo Fisher Scientific, Germany
Centrifuge, benchtop	Eppendorf, Germany
Centrifuge, MiniSpin, benchtop	Eppendorf, Germany
ChemiDoc™MP	Bio Rad, Germany
COPAS™	Union BioMetrica, USA
Plate reader Infinite m200 Pro	TECAN, Switzerland
BD LSRFortessa™Cell Analyzer	BD Bioscience
epMotion, automated liquid handling system	Eppendorf, Germany
Freezer -20°C	Liebherr, Germany
Freezer -80°C	Liebherr, Germany
Fridge +4°C , by Mercateo	Siemens, Germany
Ice machine AF200	Scotsman, Germany
Incubator BK 160 (for mosquitoes)	Thermo Fisher Scientific, Germany
Maxi feeder, glas	Coelen glastechniek, Netherlands
Midi feeder, glas	Coelen glastechniek, Netherlands
Mosquito cages	Bugdorm, USA
Nanodrop 2000c	Thermo Scientific, Germany
Nanoject® II, microinjector	Drummond Scientific Company, USA
PCR Thermocycler MJ Mini	Bio-Rad, Germany
Pipette (2.5 μl , 20 μl , 200 μl, and 1000 μl)	Eppendorf, Germany
Pipetting aid Pipetus	Hirschmann Laborgeräte, Germany
Real Time PCR system StepOne Plus	Applied Biosystems, USA
Stereoscope	Leica, Germany
Thermo-mixer Mixmate	Eppendorf, Germany
Timer, Hand Clock	Carl Roth, Germany
TissueLyser® LT	Qiagen, Germany
UV Spectrometer	Eppendorf, Germany
Vortex-Genie2	Scientific Industries, USA
Water bath, GFL 1002	GFL, Germany

Table 8. Media and solutions

Media	**Composition/Company**
Complete ookinete media	RPMI, 0,5% Pen/Strept, 1% FCS, 1 % Xanthurenic acid, pH 8
Liver Food Mix	10% Vanderzant vitamin mixture for insects + 40% Liver Powder + 40% Tuna Meal
P.falciparum complete medium (asexual cultures)	RPMI 1641, 10% Human A+ serum, 2% hypoxantine liquid, 20 μg/ml gentamycin
P.falciparum complete medium (gametocyte cultures)	RPMI 1641, 10% Human A+ serum, 2% hypoxantine liquid, sterile filtered (0.22 μm)
RPMI 1641, with L-glutamine and 25mM HEPES	Gibco Invitrogen, Germany
SDS-Buffer	25 mM Tris, 192 mM glycine, 10% SDS
Squashing buffer	10 mM Tris-HCl pH 8,2, 1mM EDTA, 25mMNaCl and 200 μg /ml Proteinase K in H_2O
Sugar water	10% sugar in H_2O
TE-Buffer, low EDTA	Thermo Fisher Scientific, Germany
Transfer Buffer	25 mM Tris, 192 mM glycine, 20% Methanol

Table 9. Plasmids

Name	**purpose**	**officical name**	**Target**
pVB-06	dsRNAi production	pLL17	T*EP1*
pVB-07	dsRNAi production	pLL100	*LacZ*

Table 10. Primer

Name	**Sequence 5' to 3'**	**Target**
VB2166	*GCA TCA CGT TCA CCA ACT ACG CGT T*	*Aapp::125* fw
VB2167	*CCT CCT CCG AGA TCA GCT TCT G*	*Lp::125* rev
VB2168	*GAC TTG TCA TCG TCG TCC TTG TAA TC*	*Aapp::125* rev
VB2169	*GCC CGG CGA AAC GGT GAA G*	*Lp::2A10* fw
VB2170	*GGG TTC GGG ATG GGC TTA CC*	*Lp::2A10* rev

3.2 Methods

3.2.1 Mosquitoes

Anopheles coluzzii Ngousso *TEP1*S1* colony originated from the Ngousso colony [75] by selection of *TEP1*S1* homozygotes.

Transgenic mosquito lines *Lp::125*, *Lp::2A10* and *Aapp::125* were designed and generated in 2015 by Maria Pissarev (Vector Biology Unit, MPIIB) and Dr. Eric Marois (IBMC, Strasbourg, France). The transgene was inserted via the *Streptomyces* phage $\phi C31$ integrase system into the genome of the line called XK, carrying the docking sites on the X chromosome. Generated transgenic lines were backcrossed with wild-type *Ngousso* mosquitoes. *Lp::125* mosquitoes used for Figure 8,Figure 9 and Figure 10 were heterozygotes *Lp::125 +/-*). For all other experiments homozygote *Lp::125 +/+* were used.

3.2.1.1 Mosquito rearing

All colonies were maintained at 28 +/- 2°C and 80 +/- 5% humidity and 12 h day/night cycle with two 30 min twilight periods. Larvae were reared in plastic containers filled with 100-400 ml deionized H_2O. Mosquito larvae were fed 2.5 ml liver food mix daily per pan. Normal rearing conditions were approximately 300 larvae per pan. Adults were maintained on 10% sucrose. For maintaining the colony, adult females were fed on human blood using an artificial membrane feeding system [108] [109]. The blood was filled into a pre-heated 38°C glass maxi-feeder, which was kept warm by circulating water connected to a 39°C water bath. Mosquitoes were allowed to feed for 15-30 min on human blood. Two to three days post-feeding, a wet filter paper was provided as an oviposition jar. The deposited eggs were removed after 24 h and transferred into a pan filled with deionized H_2O.

3.2.1.2 Development and survival experiments

Freshly hatched larvae from all strains used for experiments were sorted by COPAS ^{TM}, and larvae (n=250) were put in a single pan containing 400 ml deionized H_2O. Every day, 2.5 ml of liver food were added per pan. Pupae were counted and collected every 24 h. At the peak of pupation, 20 female and 20 male pupae were put into one cage. Dead adults were counted every 24 h. The wings of dead mosquitoes were mounted on the glass slide, and their length was measured using a stereoscope. Adults were supplied with a cotton pad soaked in 10% sugar solution in H_2O. The cotton pad was exchanged every three days. After a blood meal 6 days after emergence, the number of fed females was counted. Three days after the blood meal, an oviposition site was inserted, and 24 h later, the number of deposited eggs was counted.
The survival data were analyzed using the R package *survminer* 0.4.9. Wing size was measured using ImageJ Fiji. Larval development data were analyzed using a recently developed model for larval dynamics (Estupinan et al. in preparation).

Additionally, a nutritional stress setup was used to compare the fitness of

transgenic lines. Here, different densities of larvae were reared in the same volume of water supplied with the same amount of food per pan. The following table Table 11 describes the number of larvae and the corresponding food per larva available. The same protocol for measuring development as explained above was used.

Table 11. Feeding regime per day for different nutritional conditions

Density	**per pan**	**per larvae**	Liverpowder	Tuna meal	Vitamine mix
100	2.5 ml	25 μl	200 μg	200 μg	100 μg
250	2.5 ml	10 μ l	80 μ g	80 μg	40μg
500	2.5 ml	5 μl	40 μg	40 μg	20 μg

3.2.1.3 Mosquito rearing for infections

Freshly hatched larvae (n= 3.000) from both wild-type and transgenic line were sorted by COPAS ^{TM}and mixed together in a pan. Larvae were reared together in the same environment. Pupae were sorted into wild-type and transgenic via fluorescence. Each group was either put into a separate small cage (for *P. berghei* infections) or in a compartment of a C2 or C4 cage (*P. falciparum* infections).

3.2.2 Parasites

3.2.2.1 *P. berghei*

Parasite cryostabilate aliquots were thawed on ice. Afterwards, 200 μl of the aliquot was injected intraperitoneal (i.p.) into the female CD1 mice. Mice parasitemia was assessed 3-7 days after inoculation in the blood smear. When parasitemia reached 2-8%, the parasitized blood was injected i.p., into a new mouse. On the day of infection, the parasitemia was either counted by blood smears or by FACS.

Exflagellation assay

The complete ookinete media (13 μl) was pipetted on a glass slide, and 2 μl of infected mouse blood were added. The drop was covered with a square coverslip and incubated at 20°C for 10 min. The slide was analyzed using a microscope and 40X magnification. When the first exflagellation event was detected, the number of exflagellation events was counted in 10-15 randomly chosen fields. The mean of exflagellations per field was calculated.

Mosquito infections

Mosquitoes were starved for 4-5 h. Mice with at least 0.5% gametcytemia were anesthetized for mosquito feeding. For each experiment, 1-2 mice were used, and mosquitoes were allowed to feed on every mouse three times for 15 min until mosquitoes were fully engorged. Unfed females were removed from the cage, and fresh sugar cotton pads were supplied. Mosquitoes were offered an additional blood meal from naive mice 7 days post-infection. Samples were collected at 14, 18, and 25 days post-infection (dpi) by aspiration into 70% Ethanol and subsequent washing with

PBS (3 x 10 min). Mosquitoes were kept at 20°C and 70% humidity with a 12/12 h day-night cycle after infections with *P. berghei*.

3.2.2.2 *P. falciparum*

NF54 asexual cultures (parasitaemia >2%) were harvested by centrifugation for 5 min at 1,500 rpm, washed with fresh red blood cells and diluted to 1% total parasitaemia in 6 ml complete gametocyte medium at 4% hematocrit in 25 cm^2 cell culture flasks. Gametocyte cultures were incubated at 37°C with 3% O^2 and 4% CO^2. The medium was changed daily for 15-16 days on heated plates to reduce temperature drops. On day 14 after establishment, gametocytaemia was checked by Giemsa stain of blood smears, and parasite exflagellation was measured.

Quantification of gametocytaemia by Giemsa staining

Two weeks after the establishment of NF54 gametocyte cultures, two μl of the culture was smeared onto a glass slide and left to dry for 30 s at 37°C , to avoid inducing exflagellation due to temperature decrease. Smears were fixed in 100% methanol for 60 s at room temperature and stained with 1x Giemsa solution for 7 min exactly. Quantification of stage V gametocytes was performed by counting the observed parasite stages in a visual field under a light microscope (40x magnification) and expressed as a proportion of the total number of all parasite stages detected (i.e. number of asexual and non-stage V gametocytes combined).

Quantification of gametocyte exflagellation

Two weeks after initiation of the NF54 gametocyte cultures, 4 μl of culture were added to 7 μl of FCS on a glass slide at incubated for 10 min at room temperature in a humidified chamber. A coverslip was placed on top, and exflagellation events were counted for 5 min under a light microscope in 10-20 microscope fields (40x).

Mosquito infections

Mosquitoes for infections were kept in C2 or C4 infection cages. Mosquito females were starved for 4 h before infectious blood-feeding, then transferred into the BSL3 laboratory for infections. Mosquitoes were fed on membrane feeders containing mixed *P. falciparum* $4.5x10^6$ gametocytes per 2 ml of blood. All experimental groups were provided with infectious blood from the same feeder for comparisons. After the infectious blood meal, unfed females were removed from the cage. For all experiments, an additional blood meal was offered seven days post-infection to boost the sporozoite production. Samples were taken at 10, 11, 12, 14 and 18 dpi. Mosquitoes were collected in 70% Ethanol and washed three times with PBS.

3.2.2.3 Oocyst counting

Mosquitoes were dissected on slides in 1% mercurochrome solution in MiliQ-purified water. Midguts were incubated for 10 min in the solution, mounted on the slide, and the midguts were analyzed under a light microscope with a 10x objective.

3.2.2.4 Sporozoite isolation

Mosquitoes (10-15 females) were aligned on a slide in PBS. The heads were carefully pulled off the mosquito, with the salivary glands remaining attached to the head. The heads were then transferred onto a new slide covered with PBS, and the heads were carefully removed. The salivary glands were then transferred to a glass vial containing 400 μl RPMI + 1% BSA. The tissue was smashed with a pistol, and the sporozoites counted using a Malassez counting chamber.

Sporozoite counting

The sporozoite suspension was loaded at 1:10 dilutions in the dissection medium into a Malassez counting chamber and left for sedimentation for at least 10 min. Sporozoites were counted in 25 fields two times. The mean number of sporozoites per mosquito was calculated.

3.2.2.5 Transmission electron microscopy

Midguts of *P. falciparum* infected mosquitoes were dissected and fixed with 2.5% Glutaraldehyde for 1 h at room temperature. After washing three times with PBS, the samples were stained with DAPI (1:4,000) for 1 h at room temperature. Following another round of washing, the samples were inspected for oocysts by a fluorescence microscope. Only infected midguts were then immobilized with a 2% Low melting point agarose mix and further prepared at the microscopy core facility by Christian Goosmann under the supervision of Dr Volker Brinkmann.

3.2.2.6 Mosquito injections

Mosquitoes were reared as explained above, and pupae were sorted into groups. Female mosquitoes were placed on ice 24 -48 h post-hatching and injected with 69 nl dsRNA for gene silencing using a Nanoject® II microinjector. After injection, mosquitoes were placed in cages and infected with *P. berghei* 4-5 days post-injection. For passive immunization of WT mosquitoes, females were injected with 386 ml of scFab and 3 days later infected with *P. falciparum*.

3.2.3 Molecular Methods

3.2.3.1 Immunoblotting

Mosquitoes were placed on ice. The proboscis was cut off, and the hemolymph was collected into 2 μl 1x Laemmli buffer. The hemolymph of $10-20$ mosquitoes of each group was pooled for further analysis. The samples were frozen at -20°C for 15 min, boiled at 95°C for 5 min and 15 μl of each sample loaded on the 15% SDS gel. Gels were run at 120 V for approximately 120 min. The gel was washed for 20 min in transfer buffer. The PVDF membrane was activated in 100% Methanol for 5 min and then washed with the transfer buffer. Four Whatman filters were incubated in 1x transfer buffer, and the transfer-sandwich was assembled between the sponge, 2x Whatman filters, gel, the activated PVDF membrane, 2x Whatman

filters, and sponge. The transfer apparatus was inserted into an ice bucket with ice and filled up with buffer. After a 90-min-long run at 300 mA, the membrane was blocked for 1 h with 5% milk powder in MiliQ-purified water and incubated with the primary antibody overnight 5% BSA in PBS at 4°C . The next day the membrane was washed 3 times for 15 min in PBS + 1% Tween. Incubated for 1 h at room temperature with the secondary antibody (anti-rabbit HRP 1:10,000/ anti-mouse HRP 1:10,000 in PBS 5% BSA) and washed three times x 15 min with PBS+1% Tween. The Pierce ECL® Western Blotting Substrate were mixed according to manufacturer recommendations, and the chemiluminescence was analyzed with a ChemiDoc-MP.

3.2.3.2 DNA extraction

Mosquitoes were collected in 200 μl squashing buffer and smashed using a pestle. The sample was shortly centrifuged and incubated at 50°C for 60 min, followed by proteinase K inactivation for 5 min at 95°C . The supernatant containing the extracted genomic DNA. was collected and diluted 1:10 for a PCR-based genotyping.

Table 12. Genotyping PCR Mix

Reagent	Volume
GoTaq MasterMix	12.5 μl
Primer fw	0.5 μl
Primer rev	0.5 μl
DNA template	2.5 μl
Nuclease-free H_2O	9 μl

Table 13. Genotyping PCR Cycler settings

Step	Temperature	Time	Repeat
Initial denaturation	95°C	2 min	-
Denaturation	95°C	40 sec	repeat
Annealing	61°C	30 sec	cycle
Extension	73°C	1 min	40x
Final Extension	73°C	5 min	
Refrigeration	4°C	-	-

3.2.3.3 Genotyping PCR Samples were loaded onto a 1.5% agarose gel supplemented with 1/1,000 ethidium bromide and run for 20-30 min at 100 V. The results were analyzed using a ChemiDoc^{TM}-MP.

3.2.3.4 ELISA

Mosquitoes were collected and collected in 200 μl of a proteinase inhibitor cocktail (cOmplete ^{TM}cocktails) on ice and smashed with a pestle. A 1:10–1:1,000 dilution series was prepared for the samples and the standard (2A10). Microplates with

96 wells were coated with 25 μl per well (0.2 μg /ml) full-length recombinant CSP overnight at 4°C . The following day, the plates were washed three times with MiliQ-purified water and blocked with 25 μl /well PBS+1% BSA at room temperature for 1 h. The plate was washed three times again with MiliQ-purified water, and 12.5 μl /well of diluted samples were transferred onto the antigen-coated plate. After incubation for 1,5 h at room temperature, plates were washed three times with MiliQ-purified water, the secondary antibody (1:1,000 of anti-mouse HPD) were added at 12.5 μl /well and incubated for 1 h at room temperature. The plate was washed three times with MiliQ-purified water and briefly let to dry. After adding 1 μl water/1 ml ABTS solution (25 μl /well), the absorbance was measured using a plate reader (BioRad) at 405 mm until the highest dilution reached an OD of approximately 4.0.

3.2.3.5 Immunofluorescence analysis

Mosquitoes were collected by aspiration into 70% ethanol, washed with PBS three times for 5 min. Mosquito midguts and heads with the attached salivary glands attached were dissected in PBS and immediately fixed with 4% PFA for 1 h, permeabilized by using a 1% Triton in 1% BSA for 1 h at 37°C . The next day they were washed 3 x with PBS (each time about 10 min) and then stained with the primary antibody mix overnight at 4°C . The samples were washed 3 times with 1% BSA in PBS for 10 min and incubated for 1 h at room temperature with the matching secondary antibody and the nuclear-staining dye DAPI (1:4,000). After 3 washes of 10 min in 1% BSA in PBS, the midguts and salivary glands were mounted in AquaPoly mounting medium. Antibodies used for Immunofluorescence analysis can be found in Table 1

3.2.3.6 Gene inactivation by RNA interference

dsRNA production

Plasmids pVB- 06 (TEP1) and pVB-07 (LacZ) (see Table 9 [23]) were linearized by restriction with the enzymes Fast digest *KpnI* or Fast digest *NotI* as manufacture recommendation and purified with a QIAGEN PCR purification kit. Afterwards, the efficiency was analyzed by electrophoresis. Single-stranded RNA (ssRNA) was synthesized using the MEGAscript T7 kit with the following protocol.

Table 14. MEGAscript T7 kit

Volume	Reagent
x μl	DEPC treated H_2O
16 μl	mixed NTPs
4 μl	buffer (keep at 37°C before using)
x μl	linearized plasmid (2 μg)
4 μl	enzyme mix
total 40 μl	

Samples were gently mixed, shortly centrifuged and incubated at 37°C overnight. The remaining template DNA was destroyed by adding 2 μl DNAse I for 15 min at 37°C . The RNA samples were cleaned up by the Ambion MEGAclear Kit (Ambion AM1908) per manufacturer recommendations, including the precipitation step. The RNA pellet was re-suspended after ethanol washing in 21 μl of DEPC-treated H_2O. The concentration of ssRNA was measured using a UV spectrometer. Sense and anti-sense RNA were diluted to the final concentration of 3 μg /μl in DEPC-treated $H_2O$and mixed in equal volumes. For annealing, a 5 l beaker was filled with deionized $H_2O$and heated to boiling temperature. The ssRNAs were denatured for 5 min and left to anneal overnight. The next day the samples were analyzed by electrophoresis, aliquoted and stored at -20°C .

Mosquito injection
One day old mosquitoes were immobilized on ice for 5 min and injected with 69 nl of the corresponding dsRNA. The knockdown efficiency was analyzed by immunoblotting.

3.2.3.7 Flow Cytometry

The sporozoite samples were kept on ice in PBS. Sporozoites (n=10,0000) were diluted to 60 μl in 1% FCS in PBS in siliconised tubes. The primary antibodies anti-CSP (2A10) AF-568 and anti-c-Myc (9E10) AF-647 were diluted (1:500) in 1% FCS in PBS. Sporozoites were incubated with the primary antibody for 30 min at 4°C . Afterwards, the samples were topped up to 2 ml with 1% FCS in PBS, centrifuged at 9,300x g for 4 min at 4°C . The supernatant was removed, and the pellet was re-suspend in 100 μl 1% FCS in PBS containing 1:4,000 dilution of DNA stain (SYBERgreen). Samples were incubated for 10 min at 4°C in the dark, topped up to 2 ml with 1% FCS in PBS and centrifuged at 9,300 x g for 4 min at 4°C . Afterwards, the supernatant was removed and the pellet re-suspended in 200μl 1% FCS in PBS. The solution was transferred into a standard FACS tube and stored on ice in the dark till FACS acquisition. Samples were analyzed using a BD LSRFortessa^{TM} Cell Analyzer at the DRFZ core facility. For SYBRGreen excitation at 488 nm and filter 525/50 was used. For 2A10 AF-568 561 nm excitation and 610/20 filters. For 9E10 AF-647 excitation, 633nm and 670/30 filters were used.

3.2.4 Statistical Analysis

Statistical analysis and plots were performed using R and Rstudio (Version 1.2.1335) [145]. For the analysis and visualization of the average sporozoites per mosquito plots, a constant value of 0.1 was added to all values to enable the analysis and plotting on Log scale of zero values. All statistical tests used, exact p-values and posthoc tests can be found in the Supplements (Table 17). The ggplot.2 package was used to visualize all plots.

The dose dependent responses for Figure 15 were calculated using the drc

and nlme package. Several models were compared and the best fit chosen.
a: $f(x) = 0.814203 + (1 - 0.814203)exp(-exp(1.486996(log(x) - log(33.227485))))$
b: $f(x) = 0 + (0.360227 - 0)exp(-exp(-2.445245(log(x) - log(37.935526))))$
c: $f(x) = -0.013169 + (0.910249 + 0.013169)(exp(-x/189.602758))$
d: $f(x) = 0 + (0.097140 - 0)exp(-exp(-2.893782(log(x) - log(43.270846))))$

The model used in Chapter 2 was designed by Juan Estupiñán and Dr Paola Carrillo-Bustamante. The estimations were done by Juan Estupiñán.

Table 15. R packages used

Name	Version
base	4.0.4
dplyr	2.1.1
drc	3.0-1
ggplot2	3.3.5
ggpubr	0.4.0
graphics	4.0.4
grDevices	4.0.4
methods	4.0.4
modelr	0.1.8
nlme	3.1- 153
plyr	1.8.6
readxl	1.3.1
stats	4.0.4
survminer	0.4.9
tidyverse	1.3.1
utils	4.0.4
viridis	0.6.1

The code used can be found here:
GitlabAnnaWeyrich

3.2.5 Image Analysis

Image J Fiji Version: 2.1.0/1.53c

4 Chapter 1: Parasite density-dependent inhibition of sporogony in oocysts by anti-CSP antibodies

4.1 Aim

Antibody-expressing mosquitoes have been used in the past to generate resistant mosquitoes [168] [85] [52]. However, it remains unclear how the antibodies expressed in different mosquito tissues affect parasite development. It has been hypothesized that invasion of salivary glands is the primary mechanism of inhibition [91]. Since all previously published antibody expressing mosquito lines never entirely blocked the parasite development, I will investigate the means by which anti-CSP antibodies can interfere with the parasite development and compare the two parasites *P. falciparum* and *P. bergehi*.

4.2 Results

4.2.1 Transgene expression and secretion into the hemolymph

The two transgenic lines used in this project (Table 16) were previously generated in the laboratory. Two monoclonal antibodies (mAbs) were selected for these experiments. The mAb125 was isolated from naturally exposed human volunteers in Gabon. The expression of mAb 2A10 was previously described [177]. In this study, we used it as a control. It was initially prepared against the recombinant circumsporozoite protein (CSP) of *P. falciparum* and purified from supernatants obtained from mouse 2A10 hybridoma. It recognizes the minimal epitope $(NANP)_3$ of the *P. falciparum* CSP repeats and reacts with the variant repeat sequence (NANPNVDP-NANP) contained in the junction region of CSP of all *P. falciparum* isolates (BEI resources). Both mAbs were expressed as single-chain antibodies (scFvs). Their expression was regulated by the promoter of the gene encoding the mosquito lipid transporter *Lipophorin* (*Lp*). *Lp* is expressed throughout the larval stages and in adults where its expression is further upregulated after a blood meal ([117]). To facilitate analyses of the transgene expression, each scFv expressed a C-terminal tag: c-Myc for scFv-125; and V5 for scFv-2A10 (Table 16). The plasmids harbouring the *Lp::125* and *Lp::2A10* expression cassettes were injected into eggs of the docking line XK (Figure 29). Following the injection, the derived fluorescent larvae were isolated, and the obtained adults were backcrossed with the *A. coluzzii* Ngousso parental line. The transgenic mosquitoes express fluorescence proteins under the promoter of the *Drosophila Pax3* gene repeated three times (3 x Pax3), in the mosquito central nervous system, including the eyes (Figure 29). Unexpectedly, the resulting transgenic lines were homozygous lethal. As the insertion site was located on the X chromosome, the transgenic progeny contained only heterozygous females. Therefore, the lines were maintained by backcrosses with wild-type males.

Table 16. Transgenic mosquito lines

Name	Transgene	Promoter	expression site	tag	Marker
Lp::125	mAb125	*Lipophorin*	fat body	c-Myc	GFP
Lp::2A10	mAb2A10	*Lipophorin*	fat body	V5	YFP

To confirm the expression of scFabs and their secretion, I performed an immunoblotting analysis using hemolymph extracts of the transgenic and wild-type mosquitoes. For both transgenic lines, I observed a specific signal at 24 and 48 h post blood-feeding (Figure 7). As expected, no signal was detected in wild-type mosquitoes. These results showed detectable levels of scFvs expression after a blood meal and successful antibody secretion into the hemolymph.

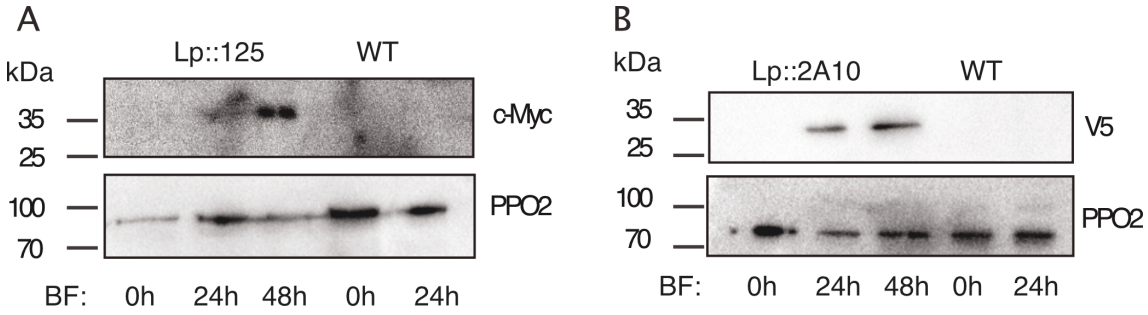

Figure 7. Transgene expression profile Immunoblotting analyses showing the expression of c-Myc-tagged scFv-125 in the wild-type (WT) and *Lp::125* mosquito lines (**A**) and of V5-tagged scFv-2A10 (**B**)in the wild-type (WT) and *Lp::2A10* mosquito lines at 0, 24 and 48 h post blood meal. Antibodies against the mosquito prophenoloxidase 2 (PPO2) were used as loading control

4.2.2 Expression of scFv-125 and scFv-2A10 interferes with salivary gland infection of *P. falciparum* sporozoites

I next examined how the expression of scFvs affected development of *P. falciparum*. In these experiments, wild-type and transgenic mosquitoes were fed with gametocyte cultures of *P. falciparum* NF54 and parasite loads were investigated in the dissected midguts (10 days post-infection (dpi)) and the salivary glands (14 and 18 dpi). In all lines, the majority of midguts (80%) were infected with oocysts (Figure 8A and C). These results suggested that expression of scFvs did not affect midgut colonization by oocysts. This observation is in line with the timeline of CSP expression, which starts after the ookinete to oocyst transformation. In contrast, scFv expression significantly decreased sporozoite invasion of the salivary glands at 14 dpi. While 50-60% of the salivary glands were infected with sporozoites in wild-type mosquitoes, only 20 and 10% of the salivary glands harboured sporozoites in the scFv-125 and scFv-2A10-expressing mosquitoes, respectively (Figure 8B and C). The proportion of infected salivary glands increased with time in all lines, bringing sporozoite prevalence to 70-80% in wild-type. However, the differences between the

wild-type and transgenic mosquitoes persisted (Figure 2B and C). Of note, the observed differences in salivary gland invasion were statistically significant for *Lp::125* but not for *Lp::2A10* line. These results may suggest a more potent inhibitory activity of scFv-125 than scFv-2A10 or result from the lower number of independent experiments performed for *Lp::2A10*.

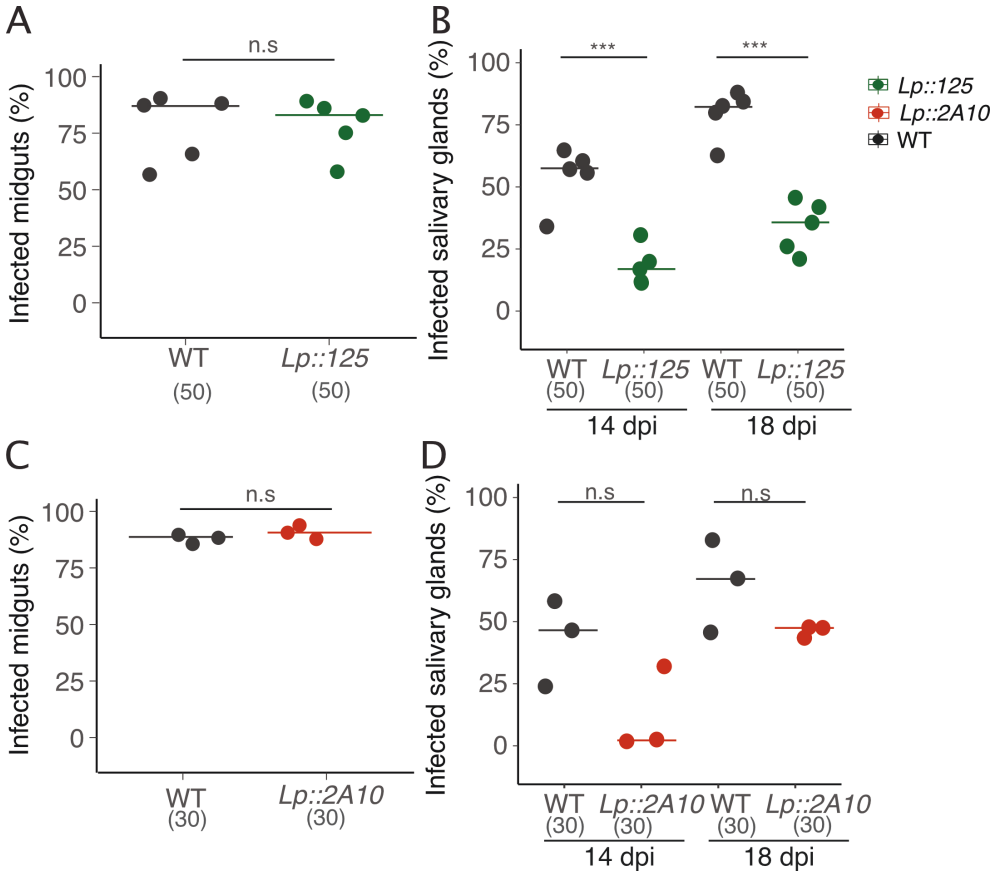

Figure 8. *P. falciparum* prevalence in midgut and salivary gland of wild-type and transgenic mosquitoes expressing scFvs.
Prevalence of *P. falciparum* infected midguts 10 days post infection (dpi) in the wild-type (WT) (**A and C**) heterozygous *Lp::125*(**A**) and *Lp::2A10* (**C**) mosquitoes. Percentage of salivary glands infected with *P. falciparum* at 14 and 18 dpi in the wild-type (N=5, n=10)(**B**) and ($N = 3, n = 10$)(**D**), *Lp::125* ($N = 5, n = 10$)(**B**), and *Lp::2A10* ($N = 3, n = 10$)(**D**). Dots show medians per experiment, and horizontal lines indicate medians of all repeats. Statistical significance was examined by t-test, and p values < 0.0005 are indicated by three asterisks.

4.2.3 *Lp::125* but not *Lp::2A10* mosquitoes are fully resistant to *P. berghei*

Expression of scFv-125 and scFv-2A10 decreased salivary gland sporozoites in infections with *P. falciparum*. A different strain available in the lab was used to analyze further if the sporozoites produced by these mosquitoes are also equally infectious. The murine malaria parasite *P. berghei* is a convenient model for transmission experiments. This species is easy to modify genetically and features a repository of

transgenic lines with gene knockouts [92] [93]. Of particular interest to my project is a replacement strain *Pb-PfCSP* in which *P. bergehi* CSP is replaced by *P. falciparum* CSP. Importantly, *Pb-PfCSP* parasites develop normally in mosquitoes and mice [177]. As within-mosquito development of *P. berghei* takes place at 20°C , the duration of the sporogonic cycle is longer compared to *P. falciparum* which develops at 26°C . Therefore, *Pb-PfCSP* prevalence was examined in the midguts of wild-type and transgenic mosquitoes 14 dpi, and in the salivary glands at 18 and 25 dpi. Similar to infections with *P. falciparum*, 50-60% of wild-type and transgenic mosquitoes featured oocysts in their midguts (Figure 9A and C). The proportion of mosquitoes with infected salivary glands was around 70% for the wild-type at day 18 dpi and 0% for *Lp::125*. At 25 dpi, 70% of wild-type mosquitoes had infected salivary glands, and for *Lp::125* still no infected salivary glands were found (FFigure 9B). After infecting wild-type and *Lp::2A10* mosquitoes with *Pb-PfCSP* 60% of the mosquitoes in both strains were successfully infected, and oocysts were found in the midgut at 14 dpi (Figure 9C). 40% of the wild-type salivary glands were invaded by sporozoites on day 18 dpi, for *Lp::2A10* only 25% of mosquitoes had infected glands. There was a slight increase in infected salivary glands in the wild-type mosquitoes at day 25, reaching 50%. A similar trend was detected in *Lp::2A10* mosquitoes, reaching approximately 30%. The differences between the wild-type and *Lp::2A10* were, however, not significant (Figure 9D).

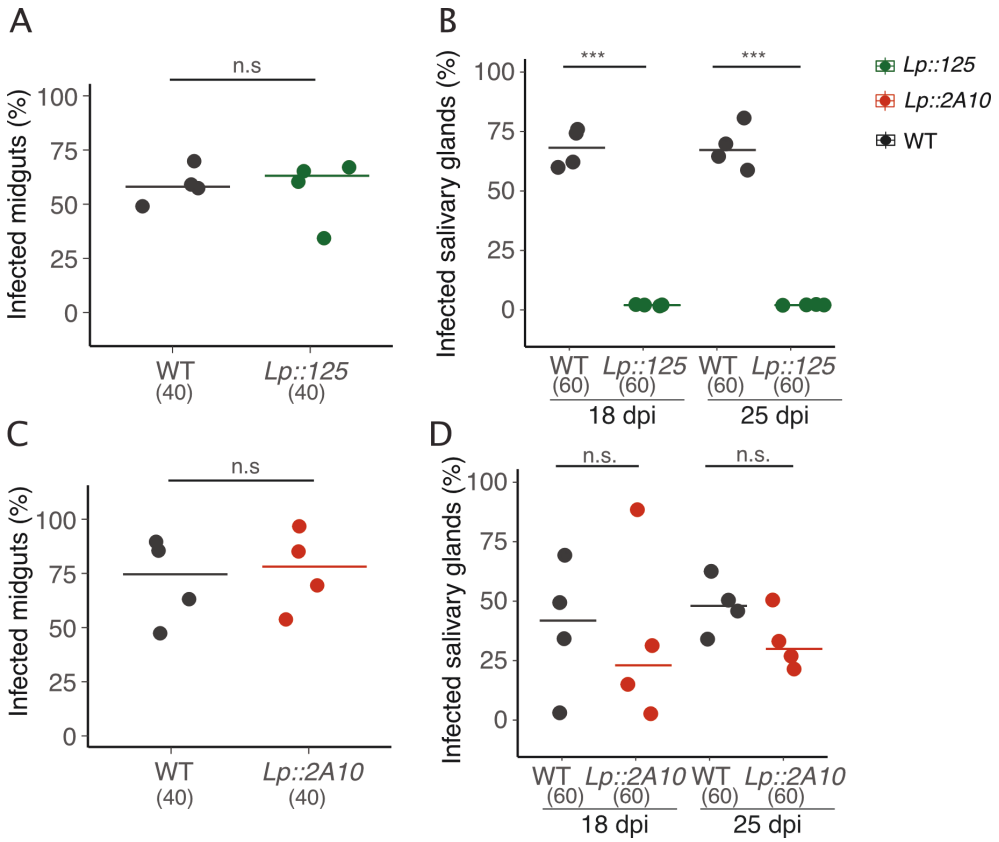

Figure 9. *Pb-PfCSP* prevalence in midgut and salivary gland of wild-type and *Lp::125*. (**A**) Prevalence of *P. berghei* line expressing PfCSP (*Pb-PfCSP*) oocysts 14 dpi. For the wild-type (black) and heterozygous *Lp::125* (+/−) (green). (**B**) Percentage of salivary glands infected with *Pb-PfCSP* at 18 and 25 dpi for wild-type and *Lp::125*. (N=4, n = 15). Dots represent the median per experiment, and lines the median of all experiments. Significance was tested by using the *t*-test. p values are indicated as $* =< 0.05, ** =< 0.005, *** =< 0.0005$. (**C**) Prevalence of *Pb-PfCSP* oocysts 10 dpi. For the wild-type (WT) and homozygous *Lp::2A10* (N=4, n = 10). (**D**) Percentage of salivary glands infected with *Pb-PfCSP* at 18 and 25 dpi for wild-type (WT) and *Lp::2A10* (N=4, n = 15). Dots represent the median per experiment, and horizontal lines indicate the median of all experiments. The *t*-test did not detect statistically significant differences between the groups (n.s.)

Although a lower prevalence of infected salivary glands was observed for both transgenic mosquito strains, the effect was more substantial in scFv-125-expressing mosquitoes than scFv-2A10. As these experiments had an equal number of repeats, these differences may reflect the higher inhibitory potency of scFv-125. Therefore, I had chosen *Lp::125* line for all further analyses.

4.2.4 *Lp::125* scFv binds to CSP in the oocysts

The low prevalence of salivary gland infections could result from scFv binding to the sporozoites in the oocysts or in the hemolymph after oocyst rupture. To identify the sporozoite stage inhibited by the scFv, I compared the numbers of oocysts and sporozoites in the midgut and salivary glands, respectively. I did not observe any

significant difference between the median oocyst numbers in wild-type and *Lp::125* mosquitoes (Figure 10A). In contrast, the mean number of salivary gland sporozoites was higher in wild-type mosquitoes, especially at 14 dpi. Only in one experiment out of 4, I detected sporozoites in the salivary glands (Figure 10B). These results are consistent with the previous experiments, as out of 3 experiments, only in one experiment I observed infected salivary glands (Figure 8B). Moreover, the overall numbers of sporozoites developed in *Lp::125* mosquitoes at the later time point was still 5-fold lower compared to wild-type (Figure 10B).

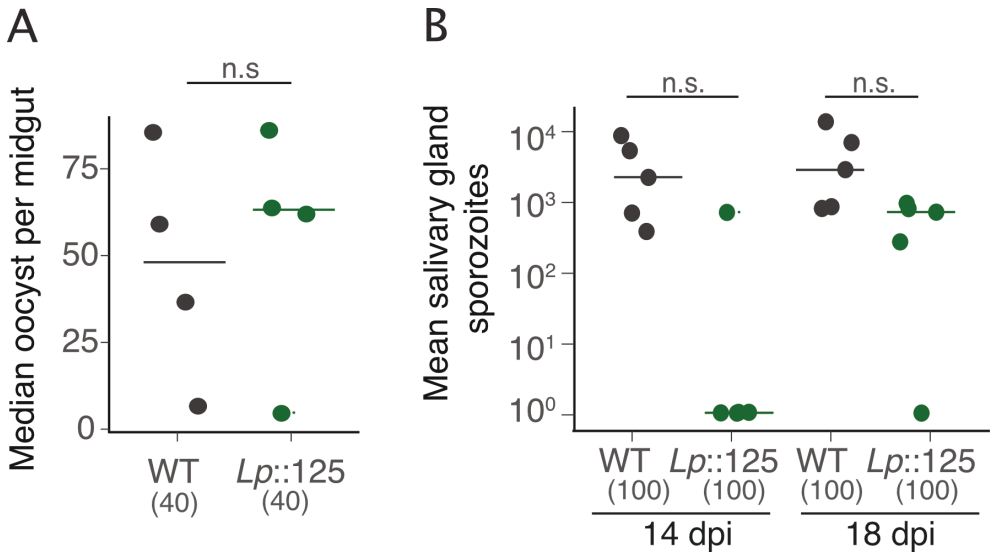

Figure 10. *P. falciparum* loads in wild-type and *Lp::125* mosquitoes
(**A**) Median oocyst numbers in wild-type (WT) and heterozygous *Lp::125* mosquitoes infected with *P. falciparum* at 10 dpi (N=4, n=10) A *t*-test did not reveal a significant difference (**B**) Mean salivary gland sporozoite numbers (log-scale) at 14 and 18 dpi (N=4, n=20). Dots represent the mean per experiment, and horizontal lines indicate the median values of all experiments per condition. A Wilcoxon test did not reveal significant differences (n.s.) between the groups.

To directly examine binding the single-chain antibody to sporozoites, I isolated oocyst sporozoites from infected midguts at 12 dpi and stained them with anti-CSP (2A10) and antic-Myc (9E10) antibodies. Live sporozoites were analyzed by FACS. The sporozoite population isolated from wild-type mosquitoes was 98% positive for anti-CSP antibody staining and negative for anti-c-Myc antibody. Among the sporozoites isolated from the oocysts in *Lp::125* mosquitoes, approximately 21% of sporozoites were negative for the anti-CSP antibody and 15% were double-positive for anti-CSP and anti-c-Myc antibodies (Figure 11A). These results indicated that scFv-125 binds to the sporozoites inside the oocysts. It is currently unclear whether scFv-125 binds to sporozoites after the oocysts rupture or penetrate the oocysts wall. To answer this question, I performed immunofluorescence analyses of the mosquito midguts dissected on day 11 post-infection using the anti-c-Myc antibody and the anti-Cap380 antibodies that label the oocyst wall.

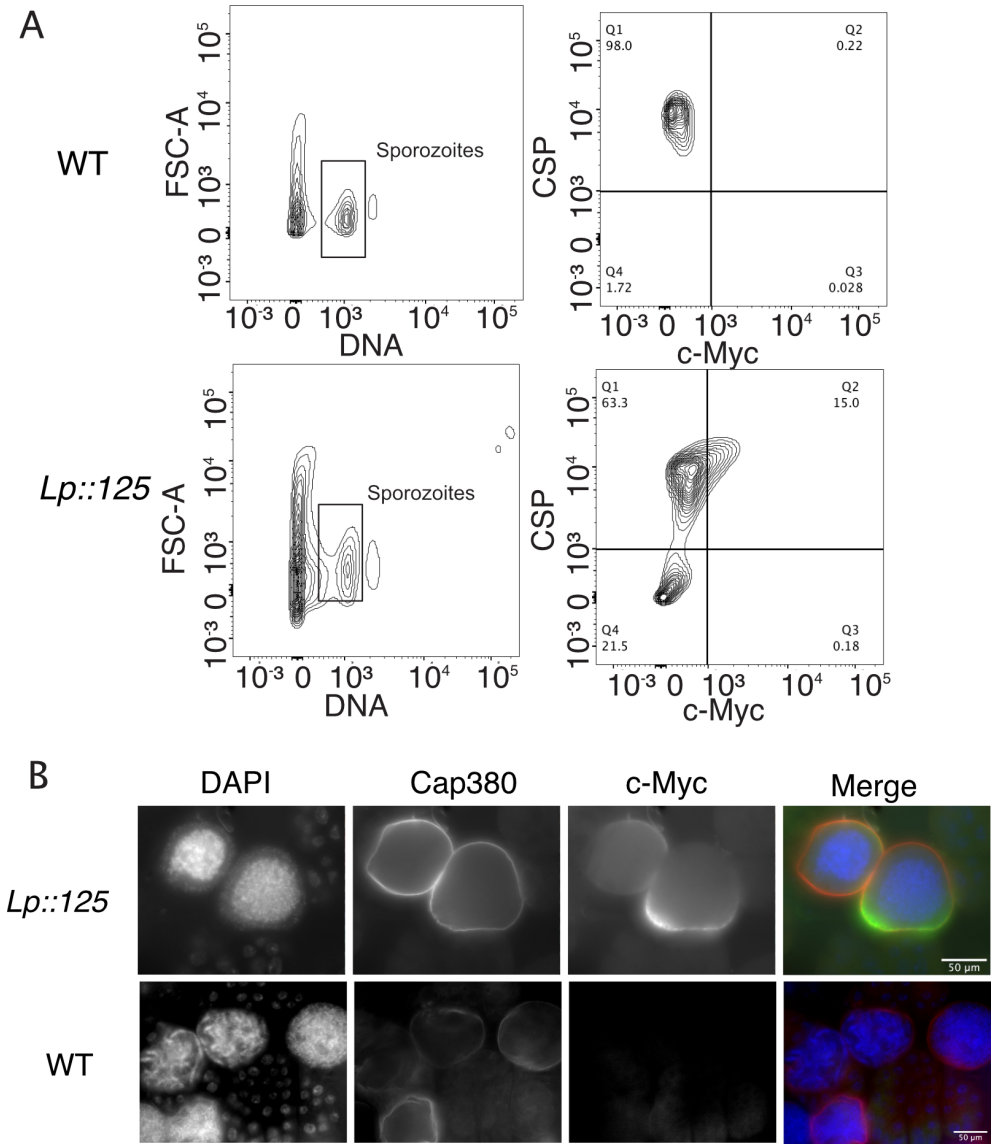

Figure 11. scFv-125 binds to CSP on *P. falciparum* oocyst sporozoites. (**A**) Flow cytometry analyses of the live midgut sporozoites freshly isolated 12 dpi. Sporozoites developed in wild-type mosquitoes (WT) are labeled with anti-CSP antibodies but are not detected by the anti-c-Myc antibody. The majority (63%) of sporozoites isolated from the homozygous *Lp::125* mosquitoes were positive for anti-CSP antibody, while 15% were also recognized by the anti-c-Myc antibody. In addition, 21% of sporozoites were not recognized by either antibody. The graph shows the results of one representative experiment out of three repeats. (**B**) Immunofluorescence analyses of dissected mosquito midguts 11 dpi. Oocysts were stained with anti-PfCap380 (Cap380, in red) and anti-c-Myc (c-Myc, in green) antibodies. Nucleic acids were stained with DAPI (in blue). The image shows the results of one out of three experiments.

At 11 dpi, I observed a normal oocyst development in the midguts of wild-type mosquitoes, including sporoblast formation and budding of new sporozoites evidenced by aligned nuclei. In contrast, the oocysts in transgenic mosquitoes lacked sporoblast formation nor alignment of sporozoite nuclei (Figure 11B). Moreover, a clear c-Myc signal was detected along the Cap380 in the transgenic mosquitoes, suggesting scFv-125 binding to the CSP on oocyst sporozoites.

4.2.5 Expression of scFv-125 in *Lp::125* transgenic mosquitoes inhibits sporogonic development

Transmission electron microscopy was used to analyze further the effects of scFv-125 on oocysts development in *Lp::125* mosquitoes. Three main types of oocysts were observed. (I) Sporulating oocysts featured a formed sporoblast (SB) and retracted plasma membrane. At a later time points, such oocysts contained new sporozoites budding from the sporoblast. (II) Non-sporulating oocysts with the plasma membrane still attached to the oocyst capsule lacking sporoblast formation. (III) Defective oocysts with a retraced plasma membrane, highly vacuolated cytoplasm and no budding sporozoites. At 10 dpi, three-fourths of the oocysts in wild-type mosquitoes were sporulating and featuring a retracted plasma membrane and the first signs of budding sporozoites (spz) from the sporoblast (SB). Instead, three fourths of oocysts isolated from *Lp::125* did not sporulate (Figure 12A). At 12 dpi, the oocysts in wild-type mosquitoes completed sporogony and contained mature sporozoites (Figure 12B). However, the majority of oocysts isolated from *Lp::125*, however, had a defective phenotype displaying retraction of the plasma membrane without sporoblast formation and extensive cytoplasm vacuolization. Only a small percentage of midguts had sporulating oocysts (Figure 12B).

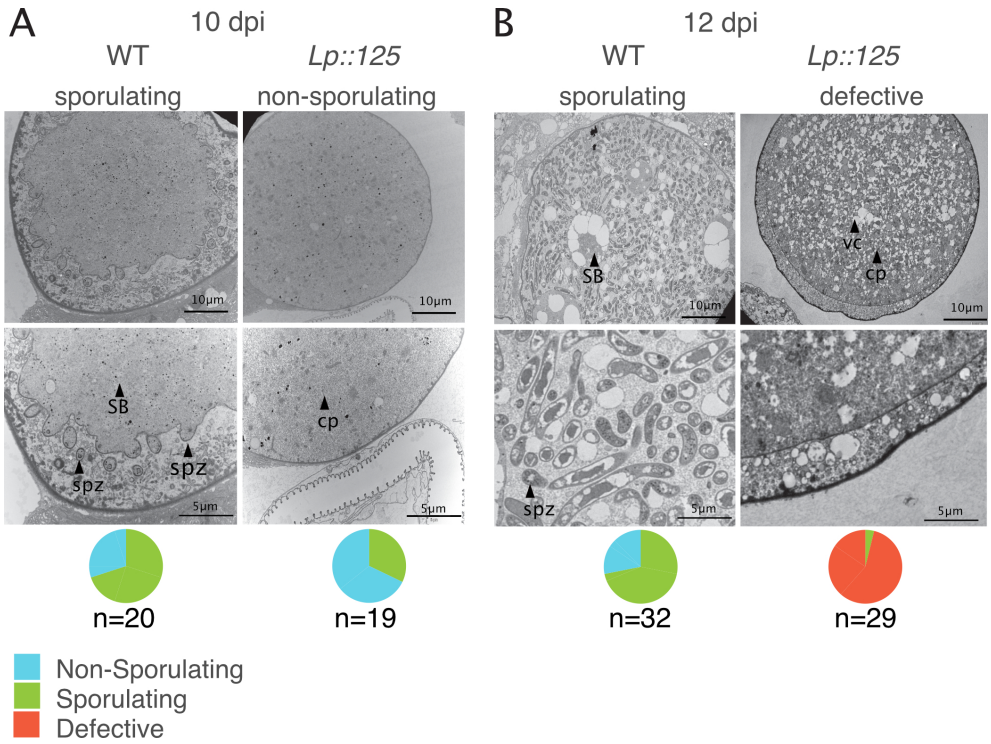

Figure 12. Transmission electron microscopy analyses of *P. falciparum* oocyst development in wild-type and *Lp::125* mosquitoes.
(**A**)At 10 dpi, sporulating oocysts feature sporoblast (SB) formation and budding of sporozoite (spz). The majority of oocysts isolated from *Lp::125* mosquitoes show a sporulation defect and lack sporoblast formation in the cytoplasm (cp).(**B**) At 12 dpi, wild-type (WT) mosquitoes display mature oocysts and freshly budded sporozoites (spz). Note that not all oocysts complete sporulation, and a small proportion of oocysts shows a defective phenotype. The majority of *Lp::125* derived oocysts shows a highly vacuolated (vc) cytoplasm (cp), partial membrane retraction and lack of budding sporozoites. The proportions of sporulating oocysts are shown in green, non-sporulating oocysts - in blue and defective oocysts – in red. N=2, n – total number of examined midguts per condition.

Taken together, the results of microscopic analyses suggest that scFv-125 binds to and inhibits CSP function at the early stages of oocyst sporogony. While this inhibition does not decrease the number of oocysts per midgut, it significantly affects the duration of sporogonic development and compromises the numbers of developed sporozoites.

4.2.6 Threshold for *P.falciparum* sporulation in *Lp::125*

The observed differences in sporulation between homozygous *Lp::125* and wild-type mosquitoes suggested that scFv-125 inhibits the sporogony of some but not all oocysts. I hypothesized that the strength of inhibition might be linked to the infection intensity. To explore this hypothesis, I compared sporulation efficiency and infection intensity of *P. falciparum* in single midguts of wild-type and homozygous *Lp::125* mosquitoes at 11 dpi. Sporulation was examined by nuclear staining.

Only oocysts with developed sporoblasts or fully-formed sporozoites were considered sporulating (Figure 13A). A comparison of sporulating oocysts for both strains revealed a significant difference between wild-type and *Lp::125* (Figure 13B). In accordance with the previous observations (Figure 12). Plotting the proportion of sporulating oocyst against the total number of oocyst in the corresponding midgut shows the contrasting behavior of *Lp::125*. At low parasite densities in wild-type mosquitoes, the oocysts develop normally and the majority of oocysts sporulate. With increasing oocyst densities, the proportion of sporulating oocysts decreases to approximately 75%. In contrasts, oocysts isolated from *Lp::125* mosquitoes do not sporulate at low densities, but rather start sporulation at high densities. The cut-off point for sporulation is around 25 oocysts per midgut. However, the proportion of sporulating oocysts in *Lp::125* mosquitoes never reaches the levels observed in wild-type mosquitoes (Figure 13C).
Separation of infections into low- ($<$ 25 oocysts/midgut) and high-density ($>$ 25 oocysts/midgut) revealed that only in infections with a low infection density, the sporozoites were absent. Infection with a high oocyst density leads to approximately 100-1,000 sporozoites. However, this observed correlation has to be tested further.

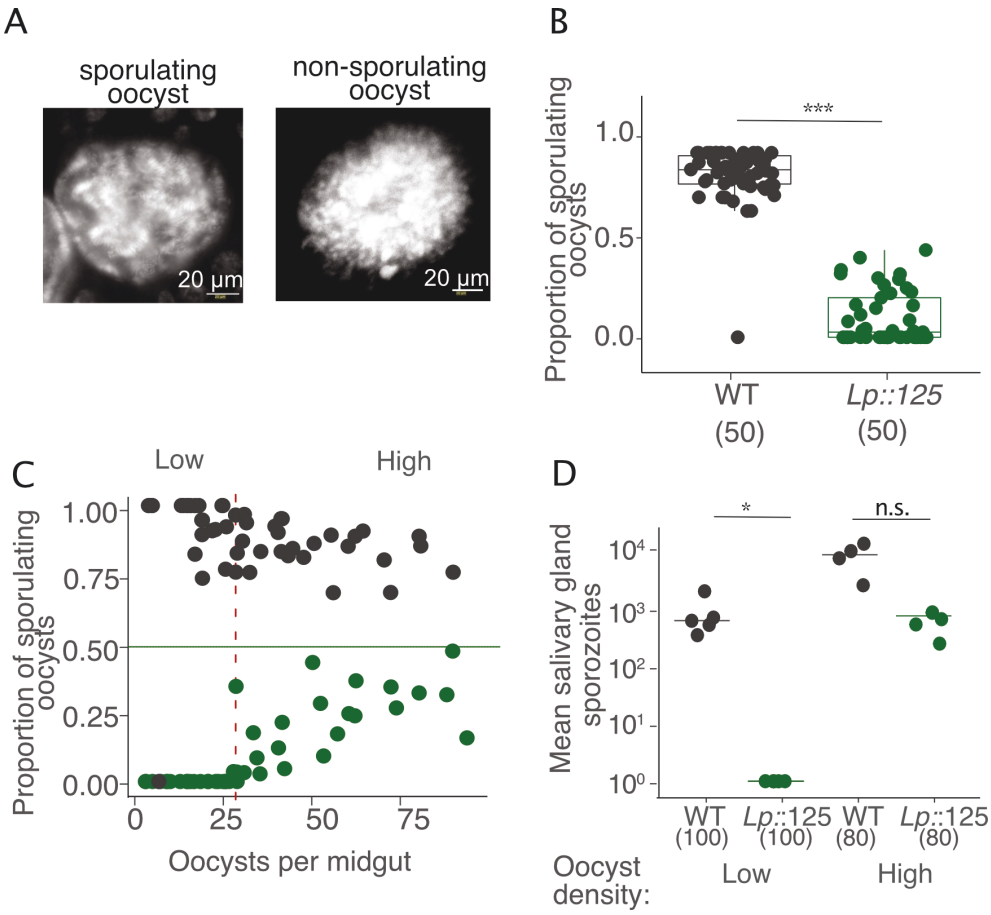

Figure 13. Proportion of sporulating oocysts correlates with infection density.
(**A** Representative images depicting sporulating and non-sporulating oocysts. (**B**) Proportion of sporulating oocysts in the wild-type and *Lp::125* mosquitoes on day 11 post infection (N=5, n=10). (**C**) The proportion of sporulating oocysts plotted against the parasite densities (oocysts per midgut) for wild-type (black) and *Lp::125* (green) mosquitoes. The dashed red line indicates the break point (25 oocysts/midgut) between the oocyst sporulation status and infection density. (**D**) Mean numbers of the salivary gland sporozoites in wild-type (WT) and *Lp::125* mosquitoes in low ($<$ 25 oocysts/midgut) and high ($>$ 25 oocysts/midgut) infection densities (N=4-5, n=20). Dots represent the mean per experiment, and the horizontal lines indicate the median for all experiments. Wilcoxon rank sum test was used to evaluate statistically significant differences (B and D:$* - p < 0.05, * * * p < 0.0005$).

4.2.7 ScFv-125 inhibition of *Plasmodium* sporogony is density-dependent

I have previously observed that infections of *A. coluzzii* Ngousso strain with *P. berghei* parasites showed lower oocyst densities compared to infections with *P. falciparum* (Figure 14). This effect is due to a specific *Anopheles* immune factor called TEP1, which controls infections with *P. berghei* ([24][23]). Therefore, to increase oocyst densities in my experiments, I silenced *TEP1* expression by RNA interference. This approach allowed me to compare low- and high-density infections in the same experiment as in infections with *P. falciparum* (Figure 14B).

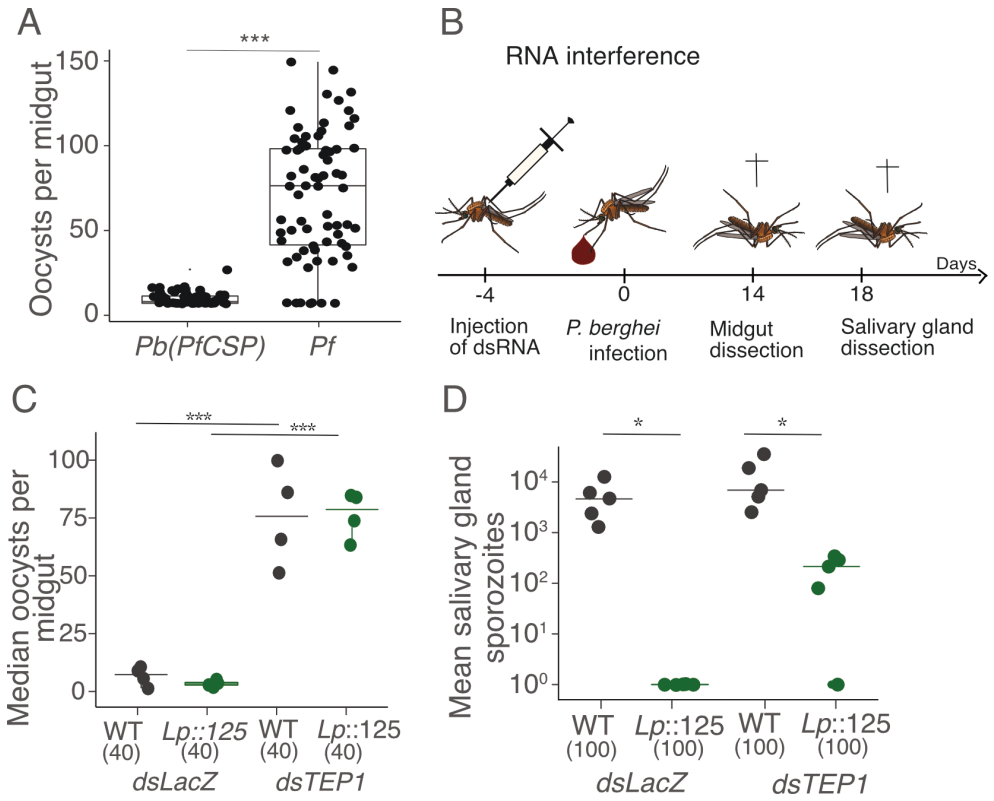

Figure 14. Different infection densities between parasite strains and experimental design for comparing high and low infections
(**A**) Comparison of *Pb-PfCSP* and *P.falciparum* oocyst densities in wild-type mosquitoes (N=10, n=10). (**B**) Schematic description of *TEP1* silencing experiments. Control double-stranded RNA (*dsLacZ*) and *dsTEP1* were injected into female mosquitoes that 4 days later were infected with *P. berghei* expressing P. falciparum CSP (*Pb-PfCSP*). Mosquitoes were dissected 14 and 18 dpi for oocyst and sporozoite enumeration. (**C**) *Pb-PfCSP* oocyst densities in wild-type and homozygous *Lp::125* mosquitoes injected with *dsLacZ* or *dsTEP1* (N=4, n=10). (**D**) *Pb-PfCSP* salivary gland sporozoite densities in wild-type and homozygous *Lp::125* mosquitoes injected with *dsLacZ* or *dsTEP1* (N=5, n=20). Statistical significant differences were evaluated by Wilcoxon test ($*p < 0.05, * * *p < 0.0005$).

TEP1 silencing increased by 6-fold oocyst densities in both wild-type and *Lp::125* mosquitoes compared to the control *dsLacZ*-injected mosquitoes (Figure 14C, Figure 33). While 10^3 - 10^4 sporozoites were detected in wild-type mosquitoes, ten-fold lower numbers of sporozoites were observed in *Lp::125* mosquitoes silenced for *TEP1*, whereas no sporozoites developed in *Lp::125* mosquitoes injected with control *dsLacZ*. These results confirm our previous conclusions on density-dependence of scFv-125 inhibitory potency against *Plasmodium*. Importantly, even in infections with high oocyst densities, scFv-125 expression decreased by ten-fold sporozoite loads in the salivary glands.

4.2.8 Differences in the dynamics of *P. falciparum* and *P. berghei* sporogony

I next analyzed collected data using a regression model to compare dynamics of *P. falciparum* and *P. berghei* sporogony in wild-type and *Lp::125* mosquitoes. The sporulation of *P. falciparum* in wild-type mosquitoes followed a Weibull dose-response function with a positive slope where higher oocyst densities correlated with the decrease in the proportion of sporulating oocysts (Figure 15C). In contrast, *P. falciparum* sporulation in *Lp::125* mosquitoes was characterized by a negative slope Weibull dose-response function. While the proportion of sporulating oocysts positively correlated with the oocyst density, it reached saturation of 30%, around 80 oocysts per midgut. These results suggested a density-dependence of oocyst sporulation, probably regulated by some nutritional constraints. Interestingly, the sporulation dynamics of *P. berghei* in wild-type mosquitoes was clearly different from *P. falciparum* and followed a three-parameter exponential decay mode (Figure 15D), While a similar negative relationship, was observed for sporulation in high oocyst densities, the slope was much more abrupt, leading to a decrease in the proportion of sporulating oocysts from 100 to 25%. The dynamics of *P. berghei* in *Lp::125* mosquitoes was similar to that of *P. falciparum* and followed a negative slope Weibull dose-response, albeit the proportion of sporulating oocysts never reached 25% (Figure 15D). These results indicate some important differences in regulation of sporulation between two *Plasmodium* species that warrant further investigation. Importantly, we interpret similar dynamics of oocyst sporulation in the transgenic strain as the evidence of inhibition imposed by scFv-125 on parasite sporulation. Our results on density-dependence on the efficiency of sporulation inhibition also suggest antibody titration by the CSP molecules that lead to infection breakthrough at high oocyst densities.

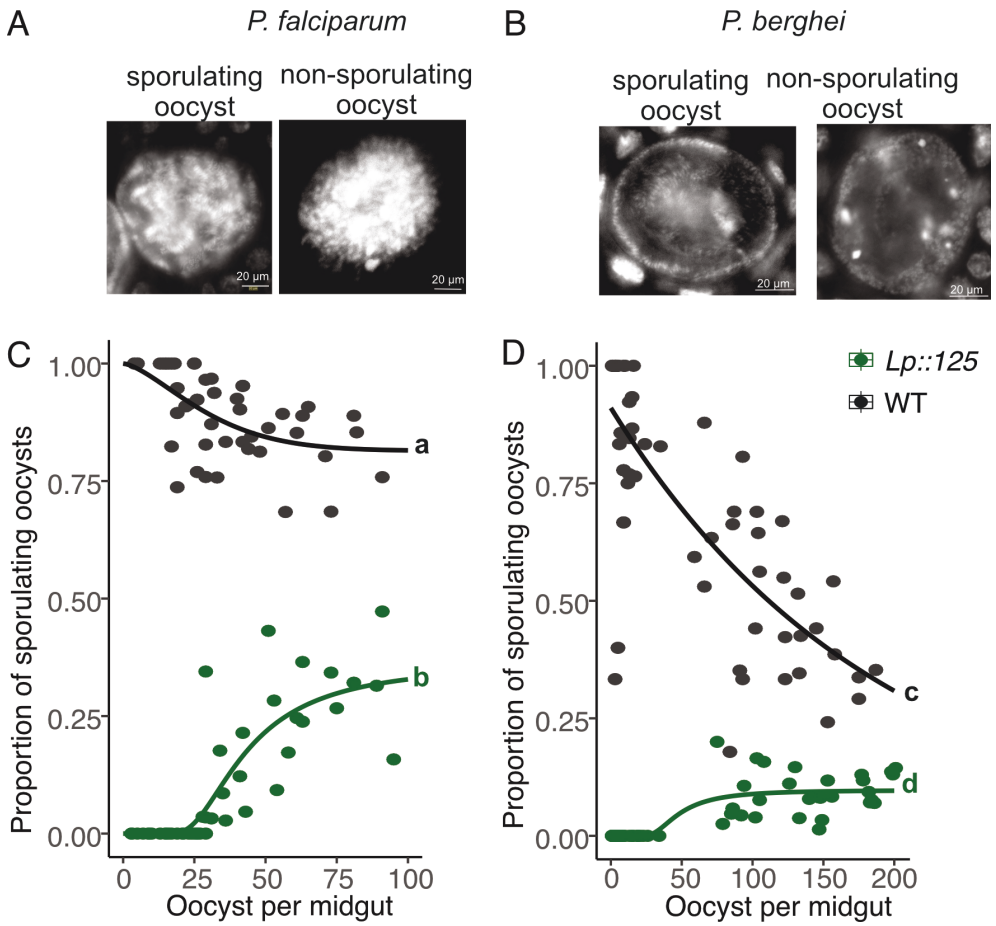

Figure 15. Density dependence in sporulation in *P.falciparum* and *P. berghei* (**A**)Morphology of *P. falciparum* oocysts stained with the nucleic acid dye DAPI at 11 dpi, separated into two distinct groups (sporulating vs. non-sporulating). **B** *P. berghei* oocyst stained with DAPI at 14 dpi show two distinct phenotypes, sporulating and non-sporulating oocysts. **C** *P. falciparum* sporulation dynamics according to the Weibull model with four parameters used to describe the density dependence in sporulation for the wild-type (WT) and *Lp::125* mosquitoes (N=5, n=10). **D** The proportion of sporulating oocyst was plotted against the number of oocysts per midgut. For the wild-type, an exponential decay model with three parameters was used to describe the density-dependent sporulation. For *Lp::125* a Weilbull model with four parameters was used ($N = 4, n = 10$)

.

4.2.9 Low proportion of mosquitoes show infected salivary glands at high infection densities

I next extended my analyses to the salivary gland infection by using the *Pb-PfCSP* infections of *TEP1* silenced mosquitoes as described above. In these experiments, mosquitoes were dissected 25 dpi, and the number of infected lobes of each salivary gland was enumerated. Mosquitoes have two salivary glands, each consisting of three lobes. Similar to the results with oocyst loads, *TEP1* silencing significantly increased the number of infected salivary gland lobes in wild-type mosquitoes (Figure 16A and B). In contrast, no sporozoites were detected in the salivary glands of control *Lp::125*

mosquitoes, whereas *TEP1* silencing increased the proportion of infected salivary glands to 15% (Figure 16B).

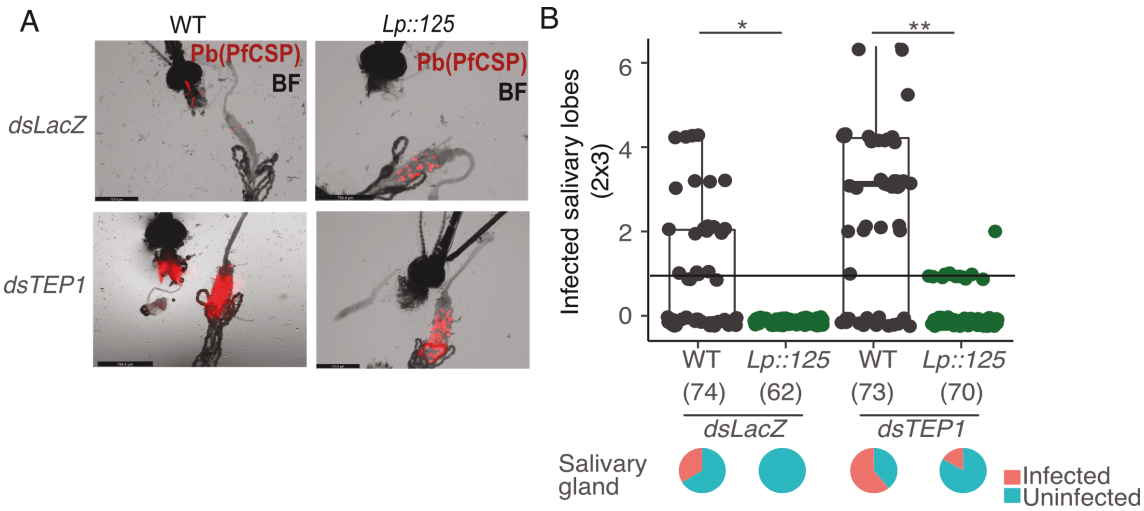

Figure 16. A small proportion of *Lp::125* mosquitoes have infected salivary glands in high-density infections. (**A**) Representative fluorescence microscopy images of dissected mosquito midguts and heads with attached salivary glands in wild-type (WT) and *Lp::125* mosquitoes injected with control (*dsLacZ*) or *dsTEP1* double-stranded RNA. *Pb-PfCSP* oocysts and sporozoites express the red fluorescence protein mCherry. Scale represents 750 μm. (**B**) Numbers of infected salivary gland lobes in the salivary glands of wild-type (WT) and *Lp::125* mosquitoes injected with control (*dsLacZ*) and *dsTEP1* double-stranded RNA. The proportion of infected salivary glands is shown as pie plots (blue–uninfected, red–infected; N=4, n – total number of dissected mosquitoes per group as indicated in the brackets).

4.3 Discussion

Transgenic mosquitoes expressing anti-CSP antibodies have been described in the past, however only using murine derived antibodies. In all published mosquito lines, the salivary gland numbers were reduced in *P. falciparum* infections and the point of attack could so far not be identified. Therefore, a better understanding of anti-CSP antibody effects in transgenic mosquitoes is needed.

Here, I show that human-derived anti-CSP antibodies reduce sporozoite numbers in the salivary glands. The level of inhibition is comparable to previously published strains [86]. In both cases, the anti-CSP antibody was able to prevent invasion of *P. falciparum* sporozoites. Interestingly, the oocyst numbers reported were at least five times lower, the sporozoite numbers in the salivary glands. However, in some experiments comparable. The current hypothesis on the primary target stage was the invasion of salivary glands. There are some indications that CSP can bind to salivary gland cells [190] [156] [100] the role of CSP for infection of glands is not yet fully understood. The domains responsible for this binding were region I and the N-terminus, but not the repeat-region.
We showed that scFv-125 binds to CSP on oocyst and interferes with the essential process of sporogony. The relevance of CSP during sporulation has been shown before by knocking out CSP. A lack of sporulation was detected during early sporulation in *P. berghei* [121]. In *P. falciparum* deletion of CSP lead to the absence of sporozoites in the salivary glands [116] and defective oocysts. In particular, the repeat region plays an essential role, at least in *P. berghei* [59]. I, therefore, conclude that CSP on oocysts is a target of scFv-125. The mechanism by which scFv-125, however, enters the cyst remains unclear. There are no known transporters facilitating protein transport into the oocyst, and I hypothesize due to the size of single-chain antibodies, they enter via osmosis.
Interestingly, this phenotype was neither reported nor did other transgenic lines show similar results. For lines using the murine anti-CSP antibody 2A10 even at a median of 3 oocysts, salivary gland sporozoites could still be detected [86]. In transgenic mosquitoes with multiple effectors targeting ookinetes as well as sporozoites, the sporozoite numbers were decreased, and only around 40% of the mosquitoes showed infected salivary glands [86]. I observed a much lower proportion of infected salivary glands, even at high oocyst densities. Important to note here is that in previous lines, the scFv was fused to an antimicrobial peptide, hence increasing the size of the protein and potentially affecting the binding of the antibody or oocyst penetration [85] [86] [52]. This difference might explain why there was no phenotype on oocyst observed.
I cannot exclude, however, additional effects of antibodies binding to sporozoites in the hemolymph. Since there have been reports of Fc γ-independent cytotoxicity of anti-CSP antibodies [8] towards *P. yoelii P. berghei* and *P. falciparum*, scFv-125 could potentially lead to a removal of the protective CSP coat and make sporozoites vulnerable to pore-forming proteins. The cytotoxicity was shown to be independent of Fc receptor γ; however, there are major differences between full length and single-chain antibodies, so it is not clear if scFvs can cause a similar phenomenon.
Additionally, I confirmed the effect of oocyst crowding in *P. berghei* causing an arrest in development. In *P. berghei* infected mosquitoes, high parasite densities cause

oocyst to enter a dormant state [71]. I showed that in wild-type mosquitoes, the amount of sporulating oocyst decreases with rising infection densities. Additionally, I provided evidence that the same mechanism is true for *P. falciparum*.

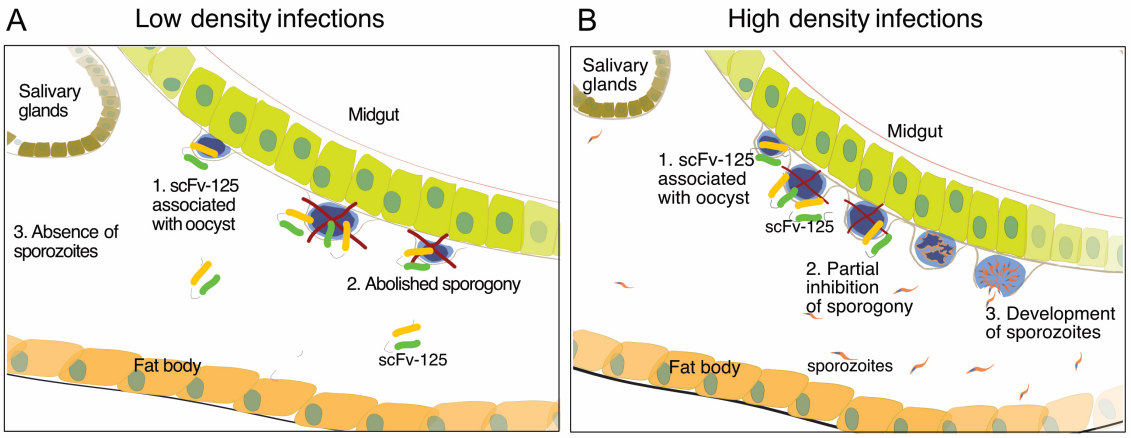

Figure 17. ***Conclusion***
(**A**) In low parasite density infections oocyst sporogony is fully blocked 1. ScFv-125 associates with oocysts and 2. sporogony is abolished in all oocysts. No sporozoites are formed and hence 3. no infection of salivary glands. (**B**) In high parasite density infections 1. scFv-125 associates with oocysts and leads to 2. a partial inhibition of sporogony in oocysts. 3. Some oocysts develop sporozoites and they are able to infect salivary glands, however salivary gland sporozoite numbers are decreased.

Overall I showed several similarities between *P. berghei* and *P. falciparum* however, regarding the ability of scFv-125 to block development, I saw a difference between both parasites. ScFv-125 blocks only approximately 25 *P. falciparum* oocysts, but 50 *P. berghei* oocysts. This observation indicates a similar dose-response pattern. The threshold is, however, different between both parasite strains. *P. berghei* CSP also follows a dose dependency, low CSP doses in *P. berghei* oocysts lead to long and deformed sporozoites [174]. A potential explanation for the differences between *P. falciparum* and *P. berghei* could be that CSP is dose-dependent in *P. berghei* and decreasing CSP availability still inhibits whereas in *P. falciparum* all CSP molecules need to be blocked to arrest sporulation. I observed differences between high and low parasite density infections and showed that the ability of scFv-125 to inhibit sporulation was parasite density-dependent. This result falls in line with previous reports on anti-CSP repeat binders [128][137]. Other anti-repeat binders could protect via passive immunization of mice. The protection was dose-dependent, and with decreasing antibody concentrations, the level of protection weakened in *P. berghei*. We observed a similar dose-dependency. With higher parasite loads, the inhibition was less successful for both *P. falciparum* and *P. berghei*. Hence, I conclude that repeat-binders function in a dose-dependent manner in the mosquito for *P. berghei* and *P. falciparum*. If the same is true for *P. falciparum* in humans remains to be tested.

In conclusion I propose the following model for infections in *Lp::125* (Figure 17).

ScFv-125 binds to CSP on an unexpected target, the oocysts. Here, scFv-125 arrests sporogony. In infections with high oocyst numbers ($>$ 25 oocysts per midgut in *P. falciparum* and $>$ 50 in *P. berghei*), scFv-125 does not block all oocysts, and a small proportion starts sporulating. The few sporozoites formed can invade the salivary glands. At this point, it is not clear if these sporozoites can be transmitted and successfully infect a new host. The recently described correlation between salivary gland sporozoite numbers and infection probability shows that infection probability highly depends on the sporozoite density in the salivary glands [7]. The salivary gland sporozoite numbers needed for a successful infection are around 10,000 sporozoites or more. Looking back at the highest numbers obtained from *Lp::125* mosquitoes were around 1,000 sporozoites maximum and only in infections with very high oocyst densities. Therefore, I propose, that *Lp::125* mosquitoes will not efficiently transmit the parasite. Additionally, in natural infections, the reported infection intensity was rarely above 30 oocysts per gut [25] [122] this indicates that also in a natural setting *Lp::125* could entirely block sporozoite development.

5 Chapter 2: Effect of the transgene on development and survival of mosquitoes expressing single-chain antibodies

5.1 Aim

In the previous chapter, I assessed the capability of a human-derived antibody to interfere with the parasite development. Here I examined the development, survival and fitness of three different transgenic lines expressing antibodies and compared them to the wild-type. Mosquito fitness is vital for the potential application of transgenic mosquitoes expressing single-chain antibodies that should be competitive with wild-type populations.

5.2 Results

5.2.1 Larval development in transgenic and wild-type mosquitoes

The life cycle of mosquitoes takes place in two environments. All stages before metamorphosis are aquatic, whereas adults are airborne. How expression of scFvs affects mosquito fitness is not well studied, and only a few reports analyzed larval development. I used the collection of transgenic lines established in the laboratory to examine the cost of transgene expression on larval development and adult survival. Two of the three transgenic lines expressing antibodies in the fat body *Lp::125* and *Lp::2A10* were used in the previous chapter. The third line *Aapp::125* expresses scFv-125 in the salivary glands. The transmission of sporozoites is impaired in this line. [177].

I measured the development from larvae to pupae by sorting and counting freshly hatched larvae from all groups by a COPAS, an instrument based on flow cytometry of organisms. Each growing pan for each group contained 250 larvae, and 2,5 ml of food were added daily. Pupae were manually counted and removed every day until all larvae pupated or died (Figure 18).

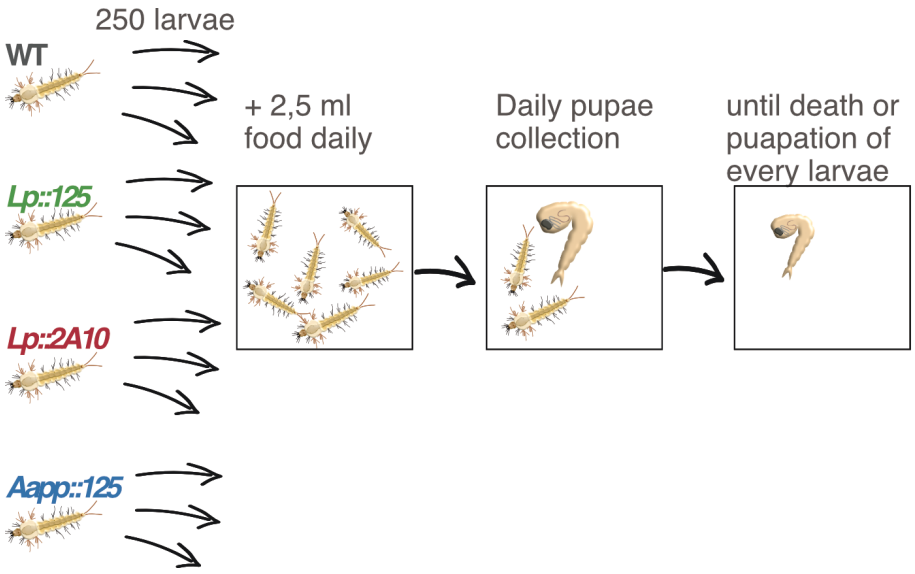

Figure 18. Experimental design for analyzing transgenic larvae development Freshly hatched larvae (first stage L1) from each group were sorted into separate growing pans at the density of 250 larvae per pan. All groups were reared in triplicates and fed daily with 2.5 ml of food during the period of 15 days. From the first day of pupation, all developed pupae were counted daily until the last larvae pupated or died.

A full time series of the pupation dynamics (Figure 31) was obtained. Here only on the onset of pupation and the total percentage of developed pupae are shown (Figure 19). Expression of the transgenes did not affect the onset of pupariation at seven days post eclosion (Figure 19), only for *Aapp::125* there is a trend for earlier pupariation. High inter-experimental variability was observed for the proportion of developed pupae (Figure 19B). While a tendency to lower efficiency of pupal development was observed for *Aapp::125* and *Lp::2A10* lines, these differences were not statistically significant.

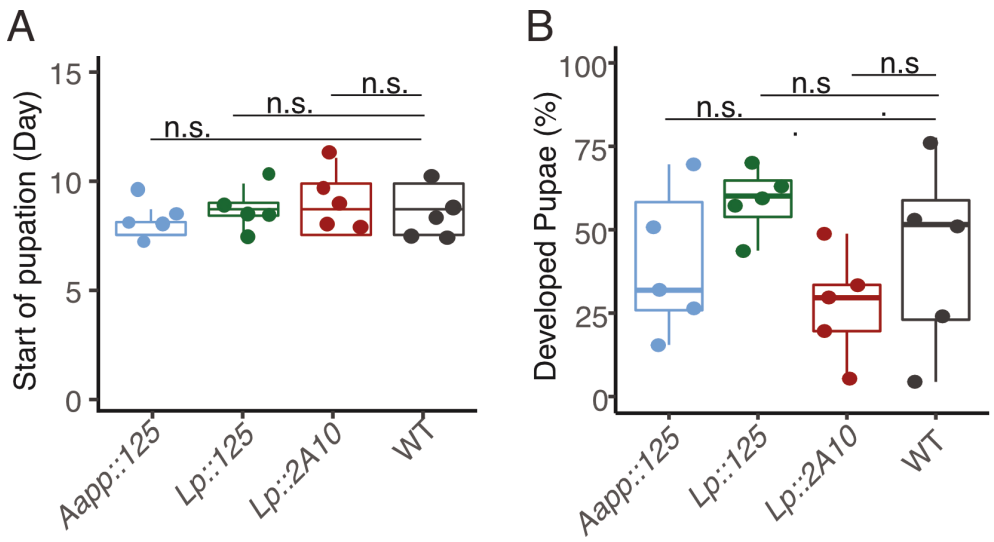

Figure 19. Onset of pupation and the percentage of developed larvae for *Aapp::125*, *Lp::125*, *Lp::2A10* and wild-type mosquitoes
(**A**) Onset of pupation for *Aapp::125*(blue), *Lp::125* (green), *Lp::2A10* (red) and wild-type mosquitoes. (**B**) The proportion of developed pupae per experiment for *Aapp::125*, *Lp::125*, *Lp::2A10* and wild-type mosquitoes. Boxplots represent the upper and lower quantile and the median, whiskers the 95 CI. The Wilcoxon rank-sum test did not reveal any statistically-significant differences (n.s.; N=5 , n=3).

As the high variability in the development data complicated the analysis, I used a model recently developed in the laboratory (Estupiñán et al., in preparation). A model was fitted to the time-series data for each mosquito line. The developmental parameters such as the death rate, growth rate and the pupation day were estimated with this approach. The death rate was the limiting factor for pupariation efficiency for *Lp::2A10* (Figure 20A). In contrast, *Aapp::125* showed a higher death rate but faster pupation onset compared to wild-type mosquitoes (Figure 20 A and B). Growth rate, death rate, and the onset of pupation in the *Lp::125* line was similar to the wild-type.

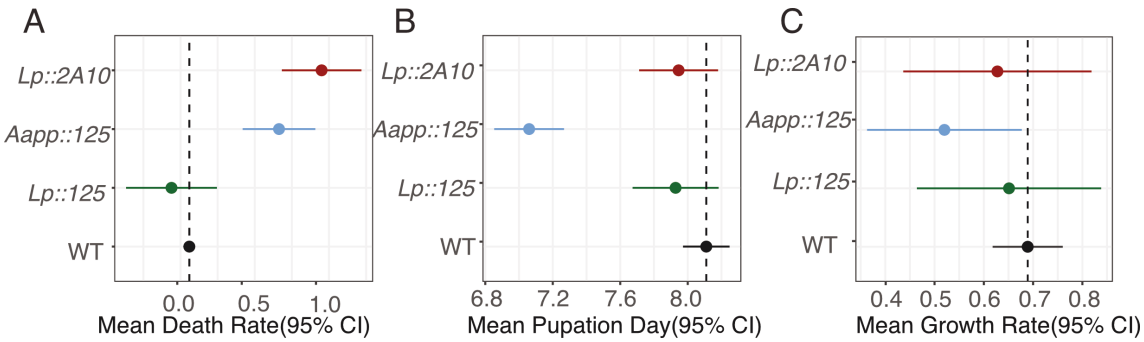

Figure 20. Parameter estimates for the developmental variables
(**A**) Mean death rate for all three transgenic strains in comparison to the wild-type (WT) (in black). (**B**) Mean pupation day for all three transgenic strains in comparison to the wild-type (WT). (**C**) Mean growth rate for all three transgenic strains in comparison to the wild-type (WT). A recently established mathematical model for population parameter estimation (Estupiñán et al. in preparation) was used to estimate the different parameters. The dots represent the median estimated value and the lines the standard deviation.

5.2.2 Survival rates of adult transgenic and wild-type mosquitoes

I next examined how transgene expression affected survival, size and fertility of transgenic and wild-type adults. Since adult fitness is affected by larval environment conditions, the larvae were reared in the same standard conditions as described above (Figure 18). Pupae were collected only on one day, allowing age matching for all groups. Six days after emergence, the adults were blood fed, and three days later, the females were allowed to depose eggs into an egg dish. Dead mosquitoes were daily counted and removed. The wing length was measured on dead mosquitoes.

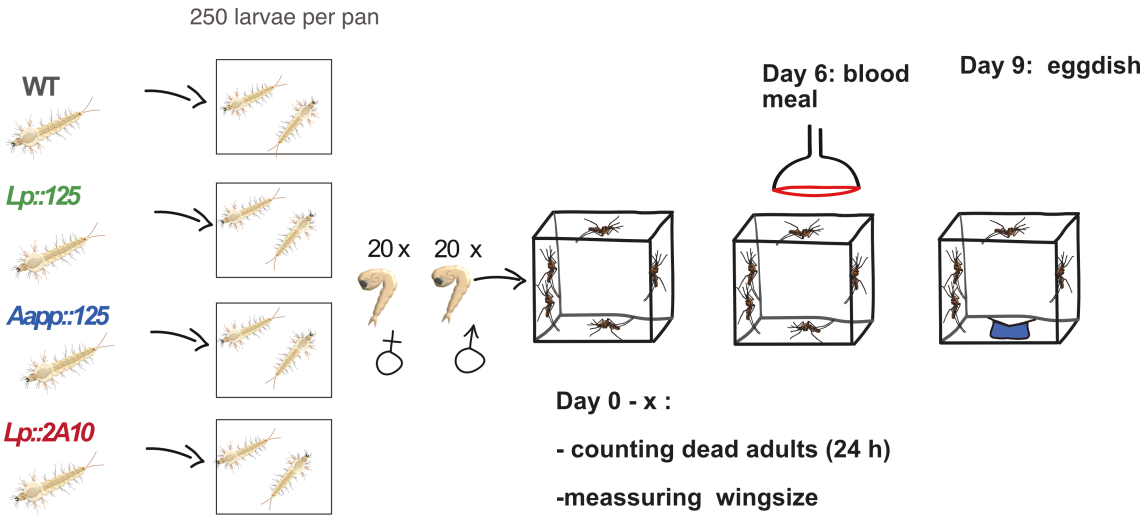

Figure 21. Experimental design for survival and fitness of transgenic mosquitoes

Larvae were sorted into 250 larvae per pan as described in Figure 18. 20 female and 20 male pupae were merged into a small cage, and survival was monitored trough out the whole experiment every 24 h. Six days after emergence, a blood meal was offered. Three days after the blood meal, a wet filter paper was inserted into the cage giving blood-fed females an oviposition site. The wings of dead adults were removed and measured.

The survival rates were very similar between transgenic and wild-type male and female mosquitoes (Figure 22) with an average life span of 29-30 days. There was a non-statistically significant trend in early mosquito mortality for *Lp::2A10* line at 3-4 days after emergence, and for *Aapp::125* line at 10-12 days after emergence, but the biological significance of these observations is unclear.

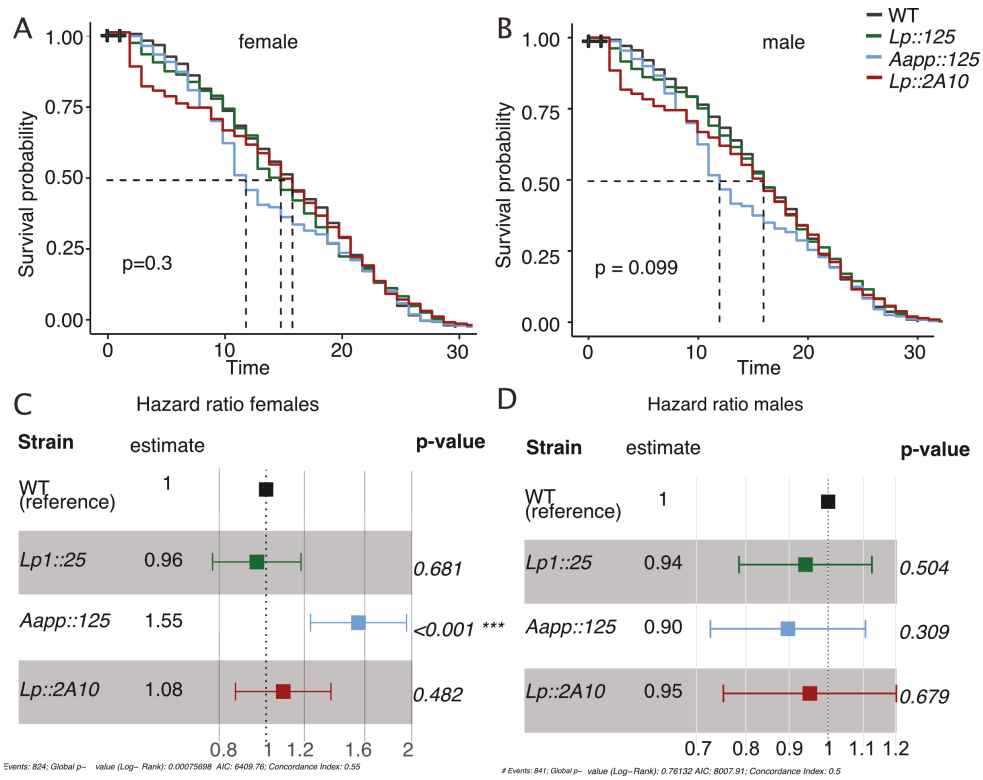

Figure 22. Survival of transgenic lines
(**A**) Kaplan-Meier curves for *Lp::125* (in green), *Lp::2A10* (in red) and *Aapp::125*(in blue) female adults in comparison with wild-type(in black) adult females. The dotted line represents the median survival time. (**B**) Kaplan-Meier curves for *Lp::125* (in green), *Lp::2A10* (in red) and *Aapp::125*(in blue) female adults in comparison with wild-type(in black) adult males. A Log-rank test comparing the median survival time was used to comparing the transgenic lines and the wild-type. The overall difference was not significant.(**C**) Hazard ratio for female mosquitoes computed by a Cox-proportional hazards model.(**D**) Hazard ratio for male mosquitoes. The median is represented by the square and the line represents the 95% CI (N=5, n=20).

To reveal the differences in the survival rates, I used a well-established model for survival analysis - the Cox-proportional hazards model. This model evaluates the influence (Hazard) of a specific parameter (in this case, the transgenic mosquito) on the survival rate. The hazard ratios of female *Lp::125* and *Lp::2A10* mosquitoes were similar to the wild-type around 0.9 to 1.2, whereas it was slightly increased to 1.6 for *Aapp::125* females (Figure 22C). No differences in hazard ratios were detected for male mosquitoes (Figure 22D). These results suggested that genetic modifications did not significantly affect the life span of adults, except for *Aapp::125* where the transgene had a modest but significant impact on female survival.

I next examined the effects of genetic manipulation on mosquito size by measuring the wing length of dead mosquitoes. The wing length of individual mosquitoes varied between 2.5 and 3.5 mm, with the mean length of 3 mm for all groups (Figure 23A). Therefore I concluded that genetic modification did not impact mosquito size. Finally, the fecundity was analyzed on a population level by

measuring the average number of eggs laid by blood-fed females. While wild-type females laid around 30 eggs per female, 15-20 eggs per female were observed for all three transgenic lines. However, these differences were not statistically significant (Figure 23B).

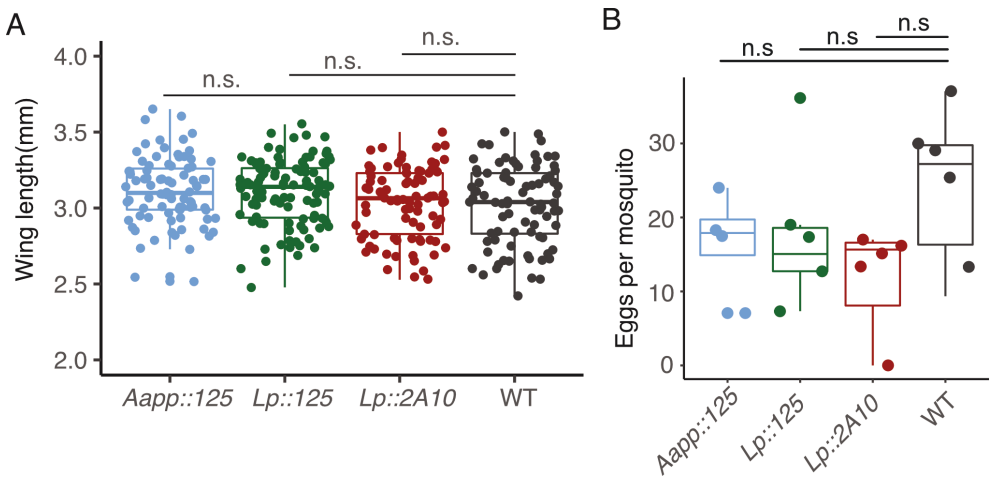

Figure 23. Wing length and fecundity of transgenic lines
(**A**) Wing length of all groups. Individual wings from different time points were plotted. (**B**) Eggs per blood-fed female mosquito (N=5, n=20). Boxplots represent the upper and lower quantile and the median, whiskers the 95 CI. Data follows a non-parametric distribution, and a Wilcoxon rank-sum test was used to test for significance.

5.2.3 Nutritional stress in wild-type and transgenic mosquito lines

Previous experiments were performed under standard rearing conditions supplying the mosquitoes with the optimum of nutrients. However, this is often not the case in natural environments.

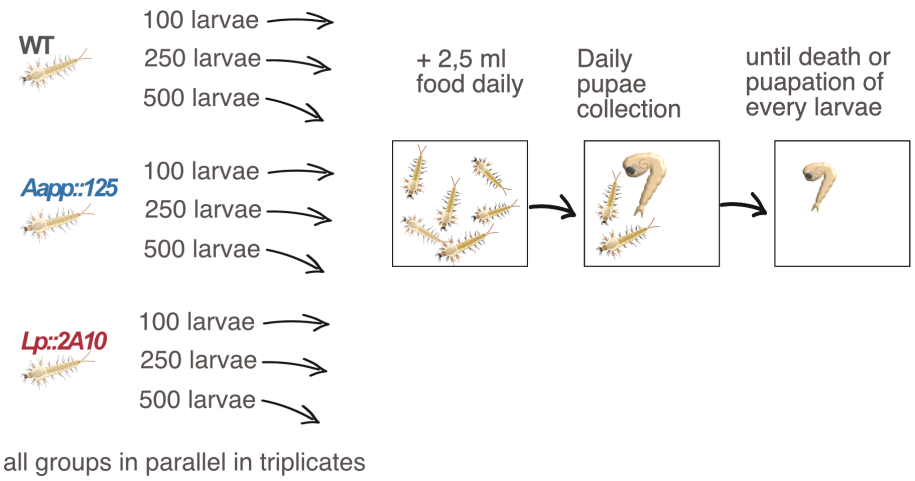

Figure 24. Experimental set up testing larval development under nutritional stress

Aapp::125 Lp::2A10 and wild-type (WT) mosquitoes were reared in three different larval densities per rearing pan (100, 200 and 500 larvae per pan). Food supply was the same for each setting and the food per larvae different. From day 7 on pupae were collected and counted daily until the last larva either pupated or died. Experiments were performed in triplicates.

To examine if nutritional stress aggravated the observed trends in transgenic mosquitoes, I reared larvae at three densities (100, 250 and 500 larvae per pan) but kept the food regiment. Therefore, higher and lower amounts of food were available at 100 larvae and 500 larvae densities compared to the control 250 larvae per pan. The entire time series is shown in (Figure 32). I focused, similar to the previous approach, only on the start of pupation and the total percentage of developed pupae. Density-induced nutritional stress did not significantly affect the timing of the pupariation onset or the proportion of pupae regardless of the mosquito genetic background (Figure 25 A). Instead, I observed a modest increase in pupae development in the *Aapp::125* line compared to wild-type at low and medium larval densities (Figure 25 B), however, this trend was not statistically significant. Interestingly, the most conserved but low efficiency of pupal development across tested conditions was observed for *Lp::2A10* line, which also performed poorly under optimal conditions Figure 19. At low food conditions (500 larvae) both lines *Aapp::125* and *Lp::2A10* tend to perform worse than the wild-type. The high variability in the results combined with the low number of biological replicates, however, makes it challenging to draw a reliable conclusion.

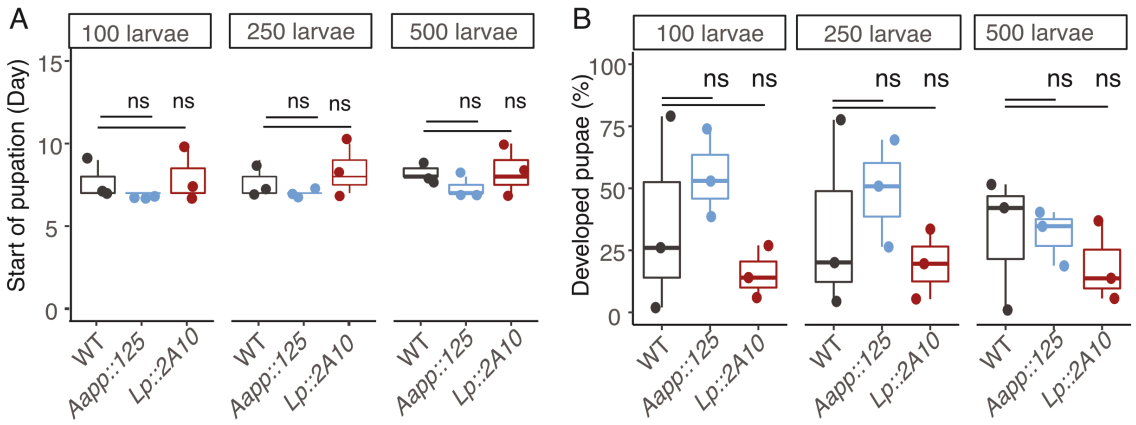

Figure 25. Start of pupation and the total percentage of developed pupae for two transgenic strains under nutritional stress A Start of pupation for different nutritional conditions for larvae reared at low (100 larvae) and high (500 larvae) densities compared to the control density (250 larvae) for the two transgenic strains *Aapp::125*(blue), *Lp::2A10* (red) and the wild-type (black).**B** Percentage of developed pupae for *Aapp::125*(blue) and *Lp::2A10* (red) compared to the wild-type (black) reared at three larval densites (100, 250, 500). Boxplots represent the upper and lower quantile and the median, whiskers the 95 CI. Data follows a non-parametric distribution, and a Wilcoxon rank-sum test was used to test for significance.

5.3 Discussion

Mosquito early-stage development is highly controlled by environmental factors, for example, temperature and nutritional availability [154][113]. Also knock out of genes or insertion of foreign genes can cause developmental defects and affect mosquito fitness [53] [84] [32] [125] [201] [1]. Therefore, it is crucial to assess the fitness of genetically manipulated lines, especially for potential application.

I showed that development, survival and mosquito size approximated by the wing length is not significantly affected in *Lp::125* mosquitoes. This result falls in line with reports from previously published strains. Antibody expressing mosquito lines did not survive differently than the corresponding docking line. In comparison with their wild-type, the transgenic lines did however show a significantly shorter survival probability [86] [52]. I observed a trend in all transgenic lines laying slightly fewer eggs than the wild-type, even though it was not significant. This trend was already previously observed, but only when comparing a docking line with wild-type mosquitoes, and was not caused by a specific transgene [86].
Lp::2A10 larvae developed poorly in all tested settings, and a maximum of 25% of larvae developed into pupae. The parameter estimations revealed this effect was mainly due to the increased death rate of larvae. *Lp::2A10* adults, however, survived comparable to the wild-type. So far, there have been no reports of antibody expression in *Anopheles* mosquitoes increasing larval death rates, since there is a lack of in-depth analysis of transgenic mosquito development. Other lines expressing a 2A10 fusion protein in the fat body developed similar to their wild-type [85][52].

Only for *Ae. aegypti* expressing anti-dengue antibodies, a longer larvae to pupae development time was reported [28]. Since the insertion locus of the transgene could not be confirmed in the *Lp::2A10* line, the observed phenotype could be explained by a disruption of regions in the genome or off-target effects of the scFv. In conclusion, the *Lp::2A10* genetic background negatively affected larval development but had no impact on survival.
Interestingly, in *Aapp::125* both larval development and survival were slightly affected. The model revealed earlier onset of pupation and higher a death rate compared to the wild-type. In contrast to other transgenic lines, I also found an effect on the survival of adult females. *Aapp::125* females had a higher risk of dying. Whether the fast larval development or the high death rate cause the higher risk of dying as adults remains to be tested. The trends I observed here are different to previous mosquito lines expressing the murine derived anti-CSP antibody 2A10 under the same promote. For these transgenic lines the survival of adults was tested in one experiment where no difference to the wild-type was observed [168]. Higher fitness cost in particular regarding the survival probability of transgenic mosquitoes was reported in mosquitoes expressing the bee venom phospholipase A2, which is a hydrolytic enzyme and effects on mosquito physiology are to be expected [126] [125]. The insertion locus of the transgene in *Aapp::125* was the same as in *Lp::125* (Figure 29) hence, the genomic locus of the transgene did not impair development or survival. Another hypothesis explaining the defect observed here is that off-target effects, are only manifested when the transgene is expressed under the *Aapp* promoter.

In summary, I showed that the expression of transgenes in *Aapp::125* and *Lp::2A10* affected development and survival differently (Figure 26). In *Lp::2A10* larvae, the death rate is increased during larval development but does not affect adult survival. Whereas for *Aapp::125* the onset of pupation is decreased and larval death rate higher, additionally the risk of dying during adult stages increases. At this point, I can only hypothesize that the defects seen in *Aapp::125* are caused by off-target effects of the single-chain antibody. In *Lp::2A10* it could also be due to disruptions in the mosquito genome at the insertion site.

One limitation of this study is that it was performed in artificial laboratory settings. Therefore, how these transgenic mosquitoes will perform in semi-field or field settings remains to be tested. However, the recent development of gene drive technologies may compensate for the observed effects in the transgenic lines [4] [29]. Gene drives allow a rapid super-mendelian spread of transgene through a population and may compensate for minor defects in competitiveness [73].

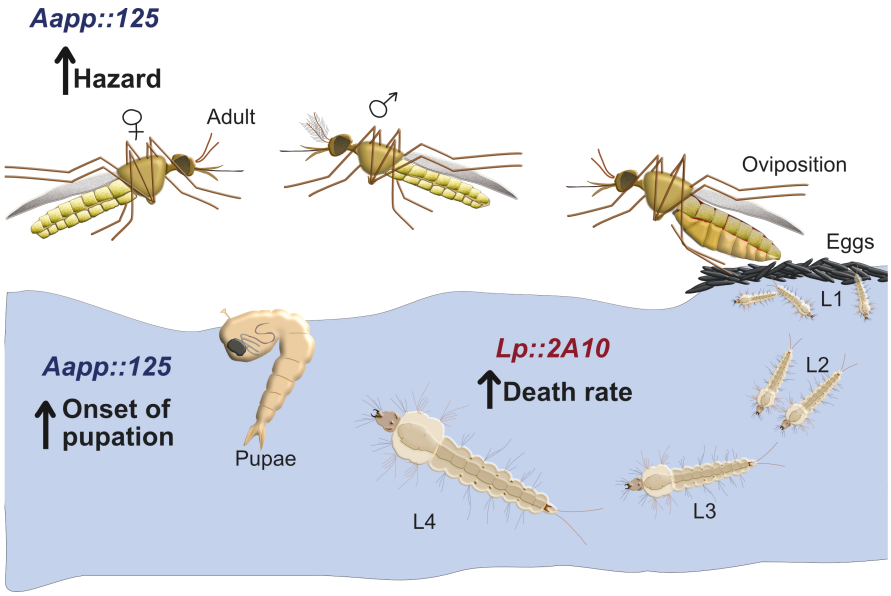

Figure 26. Life cycle of transgenic mosquitoes showing the factors affected in *Lp::2A10* and *Aapp::125* Oviposition occurs at a normal range in all lines. During the larval development from early stages to pupae, there is an increase in death for *Lp::2A10* mosquitoes. For *Aapp::125* the onset of pupation is delayed. During the later stages as adult mosquitoes, *Aapp::125* females show an increased hazard in their survival, causing them to die faster.

6 Chapter 3: Screening for sporogony inhibitory antibodies by passive immunization of mosquitoes

6.1 Aim

In chapters 1 and 2, I examined the effect of transgenically-expressed human single-chain antibodies on *Plasmodium* development in a mosquito. Importantly, I observed that some antibodies (e.g. scFv-125) were more potent in inhibiting parasite sporogony than others (e.g. scFv-2A10). With the increasing numbers of reports describing new potent anti-CSP antibodies, there is a need for a faster evaluation method to compare the antibody efficiency to inhibit *Plasmodium* sporogony. In this chapter, I present a new method of passive mosquito immunization that I have developed to compare the potency of antibodies to inhibit parasite sporogony in the mosquito. In these experiments, I used monoclonal antibodies targeting three different domains of CSP. Monoclonal antibody 1710 was described as having a high affinity towards the CSP C-terminus (C-CSP) [152]. 5D5 is a murine monoclonal antibody that recognizes an N-terminal CSP (N-CSP) epitope [57], whereas mAb1210 binds to the CSP repeat region [82].

6.2 Results

6.2.1 Kinetics of antibodies injected into mosquito hemolymph

I first examined the persistence of the injected full-length mAb2A10 antibody in the mosquito hemolymph. In these experiments, freshly emerged *A. coluzzii* females were injected with 100 nl of a 1 mg/ml antibody solution. The concentration of mAb2A10 in the mosquito was measured by ELISA on days 1, 5, 10 and 15 after injection (Figure 27A). I observed a progressive decrease in the mAb2A10 concentration that was undetectable 15 days post-injection (Figure 27B). Notably, the results described in Chapter 1 on my thesis suggested that anti-CSP antibodies interfere with *Plasmodium* sporogony one week after infection, the time point that will be equivalent to 10 days after mAb injection. Although a substantial reduction in the antibody concentration was observed at this time point, the mAb was still detectable, suggesting that it could still inhibit parasite development.

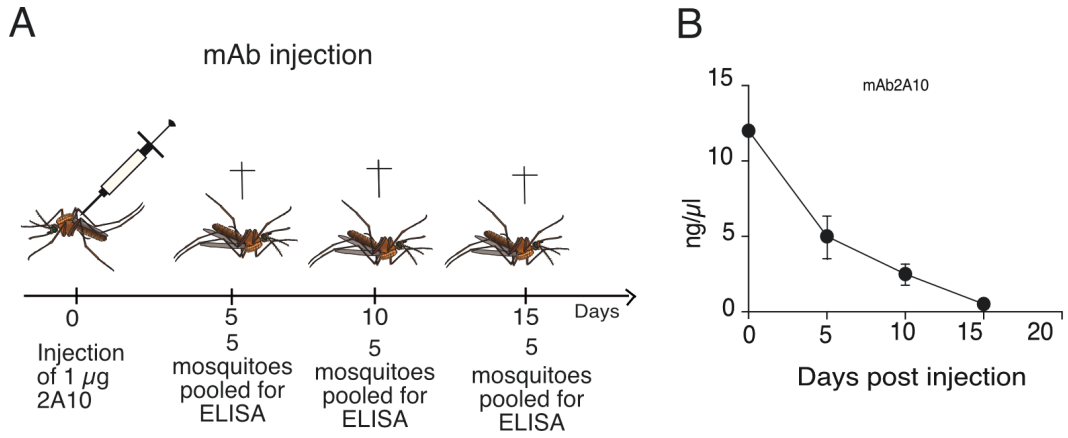

Figure 27. Injection of full-length anti-CSP antibodies into mosquito hemolymph
(**A**) Mosquitoes were injected with 1 μg of full-length mAb2A10 and the antibody concentration in the mosquitoes was measured by ELISA.(**B**) Kinetics of the full-length mAb2A10 concentration in the mosquito hemolymph injected into mosquito hemolymph during 15 days after injection (N=2, n=15). Dots show mean concentrations and vertical lines indicate standard deviation of three independent experiments

6.2.2 Passive immunization of mosquitoes with antibodies targeting N-terminal, repeat and C-terminal CSP domains.

I next injected scFv-1710, scFv-1210 and scFv-5D5 into freshly-emerged females, as well as PBS as an injection control. Three days later, females were fed with *P. falciparum* gametocyte cultures, and were either dissected 10 days later for evaluation of oocyst development or 14 days later for sporozoite counting in the salivary glands (Figure 28A). In line with my previous observations, I did not observe any differences in oocyst numbers between the tested groups (Figure 28B). As large variability between the mosquitoes was observed in the mean number of the salivary gland sporozoites in all groups (Figure 28C), I expressed the results relative to the

injection control (PBS). A statistically-significant two-fold decrease was detected in the sporozoite numbers in the mosquitoes injected with scFv-1210 (CSP repeat binder) but not in mosquitoes injected with scFv-1710 (C-CSP binder) or scFv-5D5 (N-CSP binder) (Figure 28D).

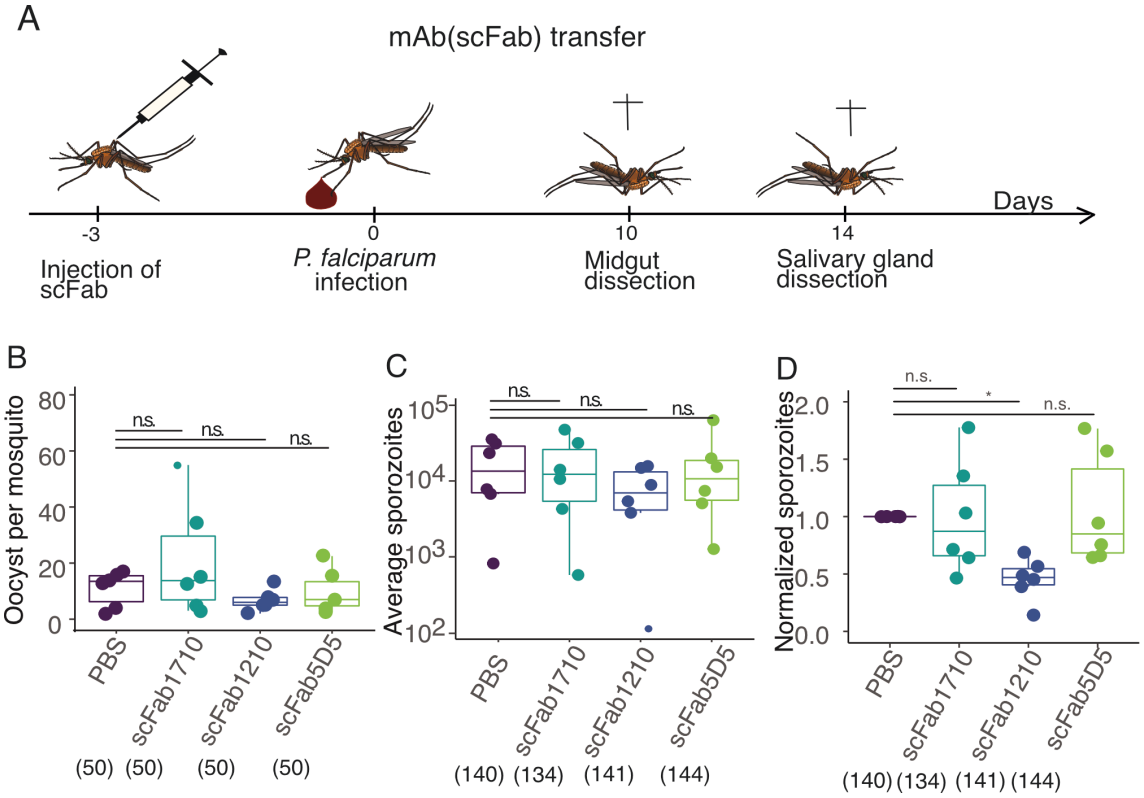

Figure 28. Passive immunisation of mosquitoes with anti-CSP scFabs targeting distinct domains.
(**A**) Experimental scheme of passive immunization experiments. Mosquitoes were injected with 386 nl of either scFabs or PBS and infected with *P. falciparum* 3 days post-injection.(**B**) 10 days post-injection, the median oocyst per midgut were counted in a pool of 10 mosquitoes per group. (**C**) The mean sporozoite loads per mosquito were measured at 14 days post-infection. The data shown were obtained from 6 independent experiments, and the median for each group was plotted. Boxplots represent the upper and lower quantile and the median, whiskers the 95 CI. Data follows a non-parametric distribution, and a Wilcoxon rank-sum test was used to test for significance.**D** Normalized salivary gland sporozoite numbers in mosquitoes injected with scFabs. The data were normalized to the injection control (PBS). The data shown were obtained from 6 independent experiments. The median for each group was plotted. Box plots represent the upper and lower quantile and the median, whiskers the 95 CI. Statistically significant differences were tested by the Wilcoxon rank-sum test (N=6, n= indicated in brackets, p values: $* < 0.05$

6.3 Discussion

I could show that antibodies injected into the mosquito hemolymph are stable and remain reactive to CSP until ten days post-injection. I made a comparison of the different antibodies and their ability to block parasite development in parallel. In previous work, the injection of anti-CSP antibodies decreased salivary gland sporozoite loads. However, the antibody used was a full-length antibody and injection was done after sporulation of oocysts. Additionally, an artificial system was used by infecting *Ae. aegypti* with *P. gallinaceum* [191]. Here, we optimized the approach in a natural model using *A. coluzzii* and *P. falciparum* and by using the smaller scFabs instead of a full-length antibody. The infection experiments showed that only one antibody (1210) could decrease salivary gland sporozoite loads. This falls in line with observations *in vivo* and *in vitro* as discussed in chapter 1.
Antibodies targeting different regions such as 5D5 (N-terminus) and 1710 (C-terminus) did not decrease sporozoite loads compared to the negative control. Although, all used antibodies were shown to bind to recombinant CSP and sporozoites [152] [57] [82]. 5D5 did not show any decrease in salivary gland sporozoites; this lack of inhibition falls in line with recent results showing that hepatocyte traversal cannot be inhibited by 5D5 [173]. It was, however, proposed that the N-terminus plays a role during salivary gland invasion [100] [43]. The inability of 1710 to inhibit *Plasmodium* development was expected, since the antibody showed no inhibition capabilities in hepatocyte traversal assays [152]. Based on the previous results in chapter 1 showing that scFvs interfere with sporogony, I propose that injected antibodies can have a similar effect. Interestingly, it has been proposed that exposure of the C-terminus is important during oocyst development [43].
Therefore, I conclude that using anti-repeat region antibodies is the most promising approach to interfere with parasite development. The effect observed was not very strong and did not entirely abolish parasite development. This can be explained by the fading concentration of the injected antibodies. It was not possible to inject the concentration used for the antibody kinetics; therefore, concentrations during the passive immunization experiments were much lower.

This new tool can be used to rapidly test new antibodies in the mosquito context and potentially lead to new and better transgenic mosquitoes.

7 Conclusions

New vector control strategies are needed, and the use of transgenic mosquitoes has been proposed [90] [37] [197] [187]. Therefore, the release of resistant mosquitoes is a potential application in the future. Several approaches have been tested in the lab so far to establish resistant mosquito lines using antiparasitic enzymes or anti-*Plasmodium* antibodies, both with limited success. Here I show that sporogony is limited in *A. coluzzii* equipped with a human-derived anti-CSP antibody. This limitation is dose-dependent, and in low-density infections, results in complete arrest of sporogony. At this point, I hypothesize that these transgenic mosquitoes would not contribute to infections in a natural setting. Mosquitoes collected from the field usually have less than 30 oocysts per midgut [25] and the antibody blocks up to 30 oocysts in our laboratory setting.

The lack of information on genetically modified mosquito development and survival shows a need for appropriate analysis strategies considering the development and survival dynamics. The analysis pipeline I described allowed the identification of potential fitness defects for two mosquito lines. Interestingly, scFv125 expression in the fat body did not impair mosquito survival, only parasite development. This observation makes the line even more interesting for future applications.

Up to this point, only a few mosquito lines expressing single-chain antibodies were described, and the lack of knowledge on the functionality of antibodies in this context limits the approach. There is a need for alternative testing strategies, particularly for the high amount of human-derived antibodies available. Here, I established a testing pipeline for faster screening of potentially interesting antibodies in the mosquito context. Of the three antibodies tested, only repeat binding antibodies decreased the parasite load in the mosquito glands. This finding aligns with the results from the first chapter and highlights the importance of the repeat region during mosquito stages.

8 References

References

1. Abraham, E. G. *et al.* Driving midgut-specific expression and secretion of a foreign protein in transgenic mosquitoes with AgAper1 regulatory elements. *Insect Molecular Biology* **14,** 271–279. ISSN: 09621075 (2005).
2. Adini, A. & Warburg, A. Interaction of Plasmodium gallinaceum ookinetes and oocysts with extracellular matrix proteins. *Parasitology* **119,** 331–336. ISSN: 00311820 (1999).
3. Adolfi, A., Pondeville, E., Lynd, A., Bourgouin, C. & Lycett, G. J. Multi-tissue GAL4-mediated gene expression in all Anopheles gambiae life stages using an endogenous polyubiquitin promoter. *Insect Biochemistry and Molecular Biology* **96,** 1–9. ISSN: 0965-1748. `https://www.sciencedirect.com/science/article/pii/S0965174818300663` (2018).
4. Adolfi, A. *et al.* Efficient population modification gene-drive rescue system in the malaria mosquito Anopheles stephensi. *Nature Communications* **11.** ISSN: 20411723 (2020).
5. Agnandji, S. T., Fernandes, J. F., Bache, E. B. & Ramharter, M. Clinical development of RTS,S/AS malaria vaccine: A systematic review of clinical Phase I-III trials. *Future Microbiology* **10,** 1553–1578. ISSN: 17460921 (2015).
6. Akaki, M. & Dvorak, J. A. A chemotactic response facilitates mosquito salivary gland infection by malaria sporozoites. *Journal of Experimental Biology* **208,** 3211–3218. ISSN: 00220949 (2005).
7. Aleshnick, M., Ganusov, V. V., Nasir, G., Yenokyan, G. & Sinnis, P. Experimental determination of the force of malaria infection reveals a non-linear relationship to mosquito sporozoite loads. *PLoS Pathogens* **16,** 1–23. ISSN: 15537374. `http://dx.doi.org/10.1371/journal.ppat.1008181` (2020).
8. Aliprandini, E. *et al.* Cytotoxic anti-circumsporozoite antibodies target malaria sporozoites in the host skin. *Nature Microbiology* **3,** 1224–1233. ISSN: 20585276. `http://dx.doi.org/10.1038/s41564-018-0254-z` (2018).
9. Allen, M. L., O'Brochta, D. A., Atkinson, P. W. & Levesque, C. S. Stable, Germ-Line Transformation of Culex quinquefasciatus (Diptera: Culicidae). *Journal of Medical Entomology* **38,** 701–710. ISSN: 0022-2585. eprint: `https://academic.oup.com/jme/article-pdf/38/5/701/18204989/jmedent38-0701.pdf`. `https://doi.org/10.1603/0022-2585-38.5.701` (Sept. 2001).
10. Aly, A. S. & Matuschewski, K. A malarial cysteine protease is necessary for Plasmodium sporozoite egress from oocysts. *Journal of Experimental Medicine* **202,** 225–230. ISSN: 00221007 (2005).
11. Aly, A. S., Vaughan, A. M. & Kappe, S. H. Malaria Parasite Development in the Mosquito and Infection of the Mammalian Host. *Annual Review of Microbiology* **63,** 195–221. ISSN: 0066-4227 (2009).

12. Ancsin, J. B. & Kisilevsky, R. A binding site for highly sulfated heparan sulfate is identified in the N terminus of the circumsporozoite protein: Significance for malarial sporozoite attachment to hepatocytes. *Journal of Biological Chemistry* **279,** 21824–21832. ISSN: 00219258 (2004).

13. Atella, G. C., Silva-Neto, M. A. C., Golodne, D. M., Arefin, S. & Shahabuddin, M. Anopheles gambiae lipophorin: Characterization and role in lipid transport to developing oocyte. *Insect Biochemistry and Molecular Biology* **36,** 375–386. ISSN: 0965-1748. https://www.sciencedirect.com/science/article/pii/S0965174806000300 (2006).

14. Athni, T. S. *et al.* ecological mechanisms. **24,** 829–846 (2021).

15. Barreaux, A. M., Stone, C. M., Barreaux, P. & Koella, J. C. The relationship between size and longevity of the malaria vector Anopheles gambiae (s.s.) depends on the larval environment. *Parasites and Vectors* **11,** 1–9. ISSN: 17563305 (2018).

16. Bartoloni, A. & Zammarchi, L. Clinical aspects of uncomplicated and severe malaria. *Mediterranean Journal of Hematology and Infectious Diseases* **4.** ISSN: 20353006 (2012).

17. Basu, S. *et al.* Silencing of end-joining repair for efficient site-specific gene insertion after TALEN/CRISPR mutagenesis in Aedes aegypti. *Proceedings of the National Academy of Sciences* **112,** 4038–4043. ISSN: 0027-8424. eprint: https://www.pnas.org/content/112/13/4038.full.pdf. https://www.pnas.org/content/112/13/4038 (2015).

18. Baum, J. *et al.* Reticulocyte-binding protein homologue 5–an essential adhesin involved in invasion of human erythrocytes by Plasmodium falciparum. *International journal for parasitology* **39,** 371–380 (2009).

19. Behet, M. C. *et al.* The Complement System Contributes to Functional Antibody-Mediated Responses Induced by Immunization with. *Infection and Immunity* **86,** 1–15 (2018).

20. Benedict, M. Q. & Robinson, A. S. The first releases of transgenic mosquitoes: an argument for the sterile insect technique. *Trends in Parasitology* **19,** 349–355. ISSN: 1471-4922. https://www.sciencedirect.com/science/article/pii/S1471492203001442 (2003).

21. Billker, O. *et al.* Identifcation of xanthurenic acid as the putative inducer of malaria development in the mosquito. *Nature* **392,** 289–292 (1998).

22. Bischof, J., Maeda, R. K., Hediger, M., Karch, F. & Basler, K. An optimized transgenesis system for Drosophila using germ-line-specific phiC31 integrases. *Proceedings of the National Academy of Sciences* **104,** 3312–3317. ISSN: 0027-8424. eprint: https://www.pnas.org/content/104/9/3312.full.pdf. https://www.pnas.org/content/104/9/3312 (2007).

23. Blandin, S. *et al.* Complement-like protein TEP1 is a determinant of vectorial capacity in the malaria vector Anopheles gambiae. *Cell* **116,** 661–670. ISSN: 00928674 (2004).

24. Blandin, S. *et al.* Reverse genetics in the mosquito Anopheles gambiae: Targeted disruption of the Defensin gene. *EMBO Reports* **3,** 852–856. ISSN: 1469221X (2002).

25. Bompard, A. *et al.* High Plasmodium infection intensity in naturally infected malaria vectors in Africa. *International Journal for Parasitology* **50,** 985–996. ISSN: 18790135. https://doi.org/10.1016/j.ijpara.2020.05.012 (2020).

26. Bosch, J. *et al.* Aldolase provides an unusual binding site for thrombospondin-related anonymous protein in the invasion machinery of the malaria parasite. *Proceedings of the National Academy of Sciences of the United States of America* **104,** 7015–7020. ISSN: 00278424 (2007).

27. Bruce, M. C., Alano, P., Duthie, S. & Carter, R. Commitment of the malaria parasite Plasmodium falciparum to sexual and asexual development. *Parasitology* **100,** 191–200 (1990).

28. Buchman, A. *et al.* Broad dengue neutralization in mosquitoes expressing an engineered antibody. *PLoS pathogens* **16,** e1008103. ISSN: 15537374 (2020).

29. Carballar-Lejarazú, R. *et al.* Next-generation gene drive for population modification of the malaria vector mosquito, Anopheles gambiae. *Proceedings of the National Academy of Sciences of the United States of America* **117,** 22805–22814. ISSN: 10916490 (2020).

30. Carter, R. & Mendis, K. N. Evolutionary and historical aspects of the burden of malaria. *Clinical Microbiology Reviews* **15,** 564–594. ISSN: 08938512 (2002).

31. Carvalho, D. O. *et al.* Suppression of a field population of Aedes aegypti in Brazil by sustained release of transgenic male mosquitoes. *PLoS Neglected Tropical Diseases* **9,** 1–15. ISSN: 19352735 (2015).

32. Catteruccia, F., Godfray, J. C. H. & Crisanti, A. Impact of genetic manipulation on the fitness of Anopheles stephensi mosquitoes. *Science* **299,** 1225–1227. ISSN: 00368075 (2003).

33. Catteruccia, F. *et al.* Stable germline transformation of the malaria mosquito Anopheles stephensi. *Nature* **405,** 959–962. ISSN: 00280836 (2000).

34. Cerami, C. *et al.* The basolateral domain of the hepatocyte plasma membrane bears receptors for the circumsporozoite protein of plasmodium falciparum sporozoites. *Cell* **70,** 1021–1033. ISSN: 0092-8674. https://www.sciencedirect.com/science/article/pii/0092867492902517 (1992).

35. Chaudhury, S. *et al.* Breadth of humoral immune responses to the C-terminus of the circumsporozoite protein is associated with protective efficacy induced by the RTS,S malaria vaccine. *Vaccine* **39,** 968–975. ISSN: 18732518. https://doi.org/10.1016/j.vaccine.2020.12.055 (2021).

36. Chen, X.-G., Marinotti, O., Whitman, L., Jasinskiene, N. & James, A. A. The anopheles gambiae vitellogenin gene(VGT2) promoter directs persistent accumulation of a reporter gene product in transgenic Anopheles Stephensi following multiple bloodmeals. *The American Journal of Tropical Medicine and Hygiene Am J Trop Med Hyg* **76,** 1118–1124. https://www.ajtmh.org/view/journals/tpmd/76/6/article-p1118.xml (1Jun. 2007).

37. Christophides, G. K. Transgenic mosquitoes and malaria transmission. *Cellular Microbiology* **7,** 325–333. ISSN: 14625814 (2005).

38. Clyde, D. F., McCarthy, V. C., Miller, R. M. & Woodward, W. E. Immunization of Man against Falciparum and Vivax Malaria by Use of Attenuated Sporozoites. *The American Journal of Tropical Medicine and Hygiene* **24,** 397–401. https://www.ajtmh.org/view/journals/tpmd/24/3/article-p397.xml (1May. 1975).

39. Cohen, S., McGregor, I. A. & Carrington, S. Gamma-globulin and aquired immunity to human malaria. *Nature,* 733–734 (1961).

40. Coon, K. L., Vogel, K. J., Brown, M. R. & Strand, M. R. Mosquitoes rely on their gut microbiota for development. *Molecular Ecology* **23,** 2727–2739. eprint: https://onlinelibrary.wiley.com/doi/pdf/10.1111/mec.12771. https://onlinelibrary.wiley.com/doi/abs/10.1111/mec.12771 (2014).

41. Coppi, A., Pinzon-Ortiz, C., Hutter, C. & Sinnis, P. The Plasmodium circumsporozoite protein is proteolytically processed during cell invasion. *Journal of Experimental Medicine* **201,** 27–33. ISSN: 00221007 (2005).

42. Coppi, A. *et al.* Heparan Sulfate Proteoglycans Provide a Signal to Plasmodium Sporozoites to Stop Migrating and Productively Invade Host Cells. *Cell Host and Microbe* **2,** 316–327. ISSN: 19313128 (2007).

43. Coppi, A. *et al.* The malaria circumsporozoite protein has two functional domains, each with distinct roles as sporozoites journey from mosquito to mammalian host. *Journal of Experimental Medicine* **208,** 341–356. ISSN: 00221007 (2011).

44. Costa, G. *et al.* Non-competitive resource exploitation within mosquito shapes within-host malaria infectivity and virulence. *Nature Communications* **9,** 1–11. ISSN: 20411723. http://dx.doi.org/10.1038/s41467-018-05893-z (2018).

45. Dame, J. B. *et al.* Structure of the gene encoding the immunodominant surface antigen on the sporozoite of the human malaria parasite plasmodium falciparum. *Science* **225,** 587–593. ISSN: 00368075 (1984).

46. Datoo, M. S. *et al.* Efficacy of a low-dose candidate malaria vaccine, R21 in adjuvant Matrix-M, with seasonal administration to children in Burkina Faso: a randomised controlled trial. *The Lancet* **397,** 1809–1818. ISSN: 1474547X (2021).

47. Davidson, G. *Genetic control of insect pests* (Elsevier, 2012).

48. De Lara Capurro, M. *et al.* Virus-expressed, recombinant single-chain antibody blocks sporozoite infection of salivary glands in Plasmodium gallinaceum-infected Aedes aegypti. *American Journal of Tropical Medicine and Hygiene* **62,** 427–433. ISSN: 00029637 (2000).

49. Delemarre, B. & Van Der Kaay, H. Falciparum malaria contracted in the natural way in The Netherlands. *Nederlands Tijdschrift voor Geneeskunde* **123.** cited By 48, 1981–1982 (1979).

50. Dimmock, N. Update on the neutralisation of animal viruses. *Reviews in Medical Virology* **5,** 165–179 (1995).

51. Dixit, R. *et al.* Salivary gland transcriptome analysis during Plasmodium infection in malaria vector Anopheles stephensi. *International Journal of Infectious Diseases* **13,** 636–646. ISSN: 12019712 (2009).

52. Dong, Y., Simões, M. L. & Dimopoulos, G. Versatile transgenic multistage effector-gene combinations for Plasmodium falciparum suppression in Anopheles. *Science Advances* **6,** 1–12. ISSN: 23752548 (2020).

53. Dong, Y., Simões, M. L., Marois, E. & Dimopoulos, G. CRISPR/Cas9 - mediated gene knockout of Anopheles gambiae FREP1 suppresses malaria parasite infection. *PLoS Pathogens* **14,** 1–16. ISSN: 15537374 (2018).

54. Douglas, A. D. *et al.* The blood-stage malaria antigen PfRH5 is susceptible to vaccine-inducible cross-strain neutralizing antibody. *Nature communications* **2,** 1–9 (2011).

55. Dundas, K. *et al.* Alpha-v–containing integrins are host receptors for the Plasmodium falciparum sporozoite surface protein, TRAP. *Proceedings of the National Academy of Sciences of the United States of America* **115,** 4477–4482. ISSN: 10916490 (2018).

56. Ejigiri, I. *et al.* Shedding of TRAP by a rhomboid protease from the malaria sporozoite surface is essential for gliding motility and sporozoite infectivity. *PLoS Pathogens* **8,** 7. ISSN: 15537366 (2012).

57. Espinosa, D. A. *et al.* Proteolytic Cleavage of the Plasmodium falciparum Circumsporozoite Protein Is a Target of Protective Antibodies. *Journal of Infectious Diseases* **212,** 1111–1119. ISSN: 15376613 (2015).

58. Feng, G. *et al.* Mechanisms and targets of Fcγ-receptor mediated immunity to malaria sporozoites. *Nature Communications* **12.** ISSN: 20411723. http://dx.doi.org/10.1038/s41467-021-21998-4 (2021).

59. Ferguson, D. J. *et al.* The repeat region of the circumsporozoite protein is critical for sporozoite formation and maturation in Plasmodium. *PLoS ONE* **9,** 1–25. ISSN: 19326203 (2014).

60. Fontenille, D. & Simard, F. Unravelling complexities in human malaria transmission dynamics in Africa through a comprehensive knowledge of vector populations. *Comparative Immunology, Microbiology and Infectious Diseases* **27.** Advances on some vector-borne diseases, 357–375. ISSN: 0147-9571. https://www.sciencedirect.com/science/article/pii/S0147957104000244 (2004).

61. Frevert, U. *et al.* Malaria circumsporozoite protein binds to heparan sulfate proteoglycans associated with the surface membrane of hepatocytes. *Journal of Experimental Medicine* **177,** 1287–1298. ISSN: 0022-1007. eprint: https://rupress.org/jem/article-pdf/177/5/1287/1103917/1287.pdf. https://doi.org/10.1084/jem.177.5.1287 (May 1993).

62. Friedman-Klabanoff, D. J. *et al.* Low dose recombinant full-length circumsporozoite protein-based Plasmodium falciparum vaccine is well-tolerated and highly immunogenic in phase 1 first-in-human clinical testing. *Vaccine* **39,** 1195–1200. ISSN: 0264-410X. https://www.sciencedirect.com/science/article/pii/S0264410X20315905 (2021).

63. Fu, G. *et al.* Female-specific flightless phenotype for mosquito control. *Proceedings of the National Academy of Sciences* **107,** 4550–4554. ISSN: 0027-8424. eprint: https://www.pnas.org/content/107/10/4550.full.pdf. https://www.pnas.org/content/107/10/4550 (2010).

64. Gato, R. *et al.* Sterile Insect Technique: Successful Suppression of an Aedes aegypti Field Population in Cuba. *Insects* **12.** ISSN: 2075-4450. https://www.mdpi.com/2075-4450/12/5/469 (2021).

65. Gaudinski, M. R. *et al.* A Monoclonal Antibody for Malaria Prevention. *New England Journal of Medicine* **0,** null. eprint: https://doi.org/10.1056/NEJMoa2034031. https://doi.org/10.1056/NEJMoa2034031 (0).

66. Gerald, N., Mahajan, B. & Kumar, S. Mitosis in the human malaria parasite plasmodium falciparum. *Eukaryotic Cell* **10,** 474–482. ISSN: 15359778 (2011).

67. Ghosh, A. K., Dinglasan, R. R., Ikadai, H. & Jacobs-Lorena, M. An improved method for the in vitro differentiation of Plasmodium falciparum gametocytes into ookinetes. *Malaria Journal* **9,** 194. ISSN: 1475-2875. https://doi.org/10.1186/1475-2875-9-194 (2010).

68. Gloor, G., Nassif, N., Johnson-Schlitz, D., Preston, C. & Engels, W. Targeted gene replacement in Drosophila via P element-induced gap repair. *Science* **253,** 1110–1117. ISSN: 0036-8075. eprint: https://science.sciencemag.org/content/253/5024/1110.full.pdf. https://science.sciencemag.org/content/253/5024/1110 (1991).

69. Golenda, C. F., Starkweather, W. H. & Wirtz, R. A. The distribution of circumsporozoite protein (CS) in Anopheles stephensi mosquitoes infected with Plasmodium falciparum malaria. *Journal of Histochemistry and Cytochemistry* **38,** 475–481. ISSN: 00221554 (1990).

70. Grossman, G. *et al.* Germline transformation of the malaria vector, Anopheles gambiae, with the piggyBac transposable element. *Insect molecular biology* **10,** 597–604 (2001).

71. Habtewold, T. *et al.* Plasmodium oocysts respond with dormancy to crowding and nutritional stress. *Scientific Reports* **11,** 1–10. ISSN: 20452322. https://doi.org/10.1038/s41598-021-81574-0 (2021).

72. Hamilton, A. J., Davies, C. S. & Sinden, R. E. Expression of circumsporozoite proteins revealed in situ in the mosquito stages of Plasmodium berghei by the Lowicryl-immunogold technique. *Parasitology* **96,** 273–280. ISSN: 14698161 (1988).

73. Hammond, A. *et al.* A CRISPR-Cas9 gene drive system targeting female reproduction in the malaria mosquito vector Anopheles gambiae. *Nature biotechnology* **34,** 78–83 (2016).

74. Harris, A. F. *et al.* Field performance of engineered male mosquitoes. *Nature Biotechnology* **29,** 1034–1037. ISSN: 10870156 (2011).

75. Harris, C. *et al.* Polymorphisms in Anopheles gambiae immune genes associated with natural resistance to plasmodium falciparum. *PLoS Pathogens* **6.** ISSN: 15537366 (2010).

76. Hawking, F., Worms, M. J. & Gammage, K. 24- and 48-hour cycles of malaria parasites in the blood; their purpose, production and control. *Transactions of The Royal Society of Tropical Medicine and Hygiene* **62,** 731–760. ISSN: 0035-9203. eprint: https://academic.oup.com/trstmh/article-pdf/62/6/731/5252324/62-6-731.pdf. https://doi.org/10.1016/0035-9203(68)90001-1 (Jan. 1968).

77. Hillyer, J. F., Barreau, C. & Vernick, K. D. Efficiency of salivary gland invasion by malaria sporozoites is controlled by rapid sporozoite destruction in the mosquito haemocoel. *International Journal for Parasitology* **37,** 673–681. ISSN: 0020-7519. https://www.sciencedirect.com/science/article/pii/S0020751906004401 (2007).

78. Hiran Dutta, M. & Ashok Dutt, K. Malarial ecology: A global perspective. *Social Science and Medicine. Part D: Medical Geography* **12,** 69–84. ISSN: 0160-8002. https://www.sciencedirect.com/science/article/pii/0160800278900102 (1978).

79. Hoffman, S. L. *et al.* Immunity to Malaria and Naturally Acquired Antibodies to the Circumsporozoite Protein of Plasmodium falciparum. *New England Journal of Medicine* **315.** PMID: 3526148, 601–606. eprint: https://doi.org/10.1056/NEJM198609043151001. https://doi.org/10.1056/NEJM198609043151001 (1986).

80. Hoffman, S. L. *et al.* Naturally acquired antibodies to sporozoites do not prevent malaria: Vaccine development implications. *Science* **237,** 639–642. ISSN: 00368075 (1987).

81. Hviid, L. & Jensen, A. T. PfEMP1–A parasite protein family of key importance in Plasmodium falciparum malaria immunity and pathogenesis. *Advances in parasitology* **88,** 51–84 (2015).

82. Imkeller, K. *et al.* Antihomotypic affinity maturation improves human B cell responses against a repetitive epitope. *Science* **360.** ISSN: 10959203 (2018).

83. Impoinvil, D. E., Cardenas, G. A., Gihture, J. I., Mbogo, C. M. & Beier, J. C. Constant Temperature and Time Period Effects on Anopheles Gambiae. *J Am Mosq Control Assoc* **23,** 124–130. ISSN: 6176321972. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3624763/pdf/nihms412728.pdf (2007).

84. Inbar, E. *et al.* Knockout of Anopheles stephensi immune gene LRIM1 by CRISPR-Cas9 reveals its unexpected role in reproduction and vector competence. *PLoS Pathogens* **17,** 1–23. ISSN: 15537374. http://dx.doi.org/10.1371/journal.ppat.1009770 (2021).

85. Isaacs, A. T. *et al.* Engineered resistance to Plasmodium falciparum development in transgenic anopheles stephensi. *PLoS Pathogens* **7.** ISSN: 15537366 (2011).

86. Isaacs, A. T. *et al.* Transgenic Anopheles stephensi coexpressing single-chain antibodies resist Plasmodium falciparum development. *Proceedings of the National Academy of Sciences of the United States of America* **109.** ISSN: 00278424 (2012).

87. Ishino, T., Yano, K., Chinzei, Y. & Yuda, M. Cell-passage activity is required for the malarial parasite to cross the liver sinusoidal cell layer. *PLoS Biology* **2,** 77–84. ISSN: 15449173 (2004).

88. Itsara, L. S. *et al.* PfCap380 as a marker for Plasmodium falciparum oocyst development in vivo and in vitro. *Malaria Journal* **17,** 1–13. ISSN: 14752875 (2018).

89. Jacobs-Lorena, M. Interrupting malaria transmission by genetic manipulation of anopheline mosquitoes. *Journal of Vector Borne Diseases* **40,** 73–77. ISSN: 09729062 (2003).

90. James, A. A. *et al.* Controlling malaria transmission with genetically-engineered, Plasmodium-resistant mosquitoes: Milestones in a model system. *Parassitologia* **41,** 461–471. ISSN: 00482951 (1999).

91. James, A. A. Blocking malaria parasite invasion of mosquito salivary glands. *Journal of Experimental Biology* **206,** 3817–3821. ISSN: 00220949 (2003).

92. Janse, C. J., Ramesar, J. & Waters, A. P. High-efficiency transfection and drug selection of genetically transformed blood stages of the rodent malaria parasite Plasmodium berghei. *Nature Protocols* **1,** 346–356. ISSN: 17542189 (2006).

93. Janse, C. J. *et al.* A genotype and phenotype database of genetically modified malaria-parasites. *Trends in parasitology* **27,** 31–39 (2011).

94. Jimah, J. R. *et al.* Malaria parasite CelTOS targets the inner leaflet of cell membranes for pore-dependent disruption. *eLife* **5** (ed Levashina, E.) e20621. ISSN: 2050-084X. https://doi.org/10.7554/eLife.20621 (2016).

95. Joice, R. *et al.* Plasmodium falciparum transmission stages accumulate in the human bone marrow. *Sci Transl Med.* **6** (2014).

96. Kariu, T., Ishino, T., Yano, K., Chinzei, Y. & Yuda, M. CelTOS, a novel malarial protein that mediates transmission to mosquito and vertebrate hosts. *Molecular microbiology* **59,** 1369–1379 (2006).

97. Kemp, D. J., Coppel, R. L. & Anders, R. F. Repetitive Proteins and Genes of Malaria. *Annual Review of Microbiology* **41.** PMID: 3318667, 181–181. eprint: https://doi.org/10.1146/annurev.mi.41.100187.001145. https://doi.org/10.1146/annurev.mi.41.100187.001145 (1987).

98. Khater, E. I., Sinden, R. E. & Dessens, J. T. A malaria membrane skeletal protein is essential for normal morphogenesis, motility, and infectivity of sporozoites. *Journal of Cell Biology* **167,** 425–432. ISSN: 00219525 (2004).

99. Kisalu, N. K. *et al.* A human monoclonal antibody prevents malaria infection by targeting a new site of vulnerability on the parasite. *Nature Medicine* **24.** ISSN: 1546170X (2018).

100. Kojin, B. B. *et al.* Endogenously-expressed NH2-terminus of circumsporozoite protein interferes with sporozoite invasion of mosquito salivary glands. *Malaria Journal* **15,** 1–11. ISSN: 14752875 (2016).

101. Kokoza, V. *et al.* Blocking of Plasmodium transmission by cooperative action of Cecropin A and Defensin A in transgenic Aedes aegypti mosquitoes. *Proceedings of the National Academy of Sciences* **107,** 8111–8116. ISSN: 0027-8424. eprint: https://www.pnas.org/content/107/18/8111.full.pdf. https://www.pnas.org/content/107/18/8111 (2010).

102. Kriu, T., Ishino, T., Yano, K., Chinzei, Y. & Yuda, M. CelTOS, a novel malaria protein that mediates transmission to mosquito and vertebrate hosts. *Molecular Microbiology* **56,** 1369–1379 (2006).

103. Kurtovic, L. *et al.* Human antibodies activate complement against Plasmodium falciparum sporozoites, and are associated with protection against malaria in children. *BMC Medicine* **16.** ISSN: 17417015 (2018).

104. Labbé, G. M. C., Scaife, S., Morgan, S. A., Curtis, Z. H. & Alphey, L. Female-Specific Flightless (fsRIDL) Phenotype for Control of Aedes albopictus. *PLOS Neglected Tropical Diseases* **6,** 1–8. https://doi.org/10.1371/journal.pntd.0001724 (July 2012).

105. Langreth, S., Jensen, J., Reese, R. & Trager, W. *Fine structure of human malariain vitro* (1978).

106. Lasonder, E. *et al.* Proteomic profiling of Plasmodium sporozoite maturation identifies new proteins essential for parasite development and infectivity. *PLoS Pathogens* **4.** ISSN: 15537366 (2008).

107. Lazarus, M. D., Schneider, T. G. & Taraschi, T. F. A new model for hemoglobin ingestion and transport by the human malaria parasite Plasmodium falciparum. *Journal of cell science* **121,** 1937–1949 (2008).

108. Lensen, A. *et al.* Measurement by membrane feeding of reduction in Plasmodium falciparum transmission induced by endemic sera. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **90,** 20–22. ISSN: 00359203 (1996).

109. Lensen, A. *et al.* Plasmodium falciparum:Infectivity of Cultured, Synchronized Gametocytes to Mosquitoes. *Experimental Parasitology* **91,** 101–103. ISSN: 0014-4894. https://www.sciencedirect.com/science/article/pii/S0014489498943545 (1999).

110. Levashina, E. A. *et al.* Conserved role of a complement-like protein in phagocytosis revealed by dsRNA knockout in cultured cells of the mosquito, Anopheles gambiae. *Cell* **104,** 709–718. ISSN: 00928674 (2001).

111. Li, M., Akbari, O. S. & White, B. J. Highly Efficient Site-Specific Mutagenesis in Malaria Mosquitoes Using CRISPR. *G3 Genes—Genomes—Genetics* **8,** 653–658. ISSN: 2160-1836. eprint: https://academic.oup.com/g3journal/article-pdf/8/2/653/37183804/g3journal0653.pdf. https://doi.org/10.1534/g3.117.1134 (Feb. 2018).

112. Lindner, S. E. *et al.* Transcriptomics and proteomics reveal two waves of translational repression during the maturation of malaria parasite sporozoites. *Nature Communications* **10,** 1–13. ISSN: 20411723. http://dx.doi.org/10.1038/s41467-019-12936-6 (2019).

113. Lyimo, E. O., Takken, W. & Koella, J. C. Effect of rearing temperature and larval density on larval survival, age at pupation and adult size of Anopheles gambiae. *Entomologia Experimentalis et Applicata* **63,** 265–271. ISSN: 15707458 (1992).

114. MacDonald, N. J. *et al.* Structural and immunological characterization of recombinant 6-cysteine domains of the Plasmodium falciparum sexual stage protein Pfs230. *Journal of Biological Chemistry* **291,** 19913–19922 (2016).

115. Manzoni, G. *et al.* Plasmodium P36 determines host cell receptor usage during sporozoite invasion. *eLife* **6,** 1–24. ISSN: 2050084X (2017).

116. Marin-Mogollon, C. *et al.* Chimeric Plasmodium falciparum parasites expressing Plasmodium vivax circumsporozoite protein fail to produce salivary gland sporozoites. *Malaria Journal* **17,** 1–16. ISSN: 14752875. https://doi.org/10.1186/s12936-018-2431-1 (2018).

117. Marinotti, O. *et al.* Genome-wide analysis of gene expression in adult Anopheles gambiae. *Insect Molecular Biology* **15,** 1–12. ISSN: 09621075 (2006).

118. Marti, M., Good, R. T., Rug, M., Knuepfer, E. & Cowman, A. F. Targeting Malaria Virulence and Remodeling Proteins to the Host Erythrocyte. *Science* **306,** 1930–1933. ISSN: 0036-8075. eprint: https://science.sciencemag.org/content/306/5703/1930.full.pdf. https://science.sciencemag.org/content/306/5703/1930 (2004).

119. Matuschewski, K. Murine infection models forvaccine development: The malaria example. *Human Vaccines and Immunotherapeutics* **9,** 450–456. ISSN: 2164554X (2013).

120. Meis, J. F., Wismans, P. G., Jap, P. H., Lensen, A. H. & Ponnudurai, T. A scanning electron microscopic study of the sporogonic development of Plasmodium falciparum in Anopheles stephensi. *Acta Tropica* **50,** 227–236. ISSN: 0001706X (1992).

121. Ménard, R. *et al. Circumsporozoite protein is required for development of malaria sporozoites in mosquitoes* 1997.

122. Mendes, A. M. *et al.* Conserved mosquito/parasite interactions affect development of Plasmodium falciparum in Africa. *PLoS Pathogens* **4.** ISSN: 15537366 (2008).

123. Mesoely, C. A., Erbe, E. F., Beaudoin, L. M. & Beaudoin, A. L. Sporozoite development in the early oocyst. *American Journal of Tropical Medicine and Hygiene* **41** (1989).

124. Mishra, S., Nussenzweig, R. S. & Nussenzweig, V. Antibodies to Plasmodium circumsporozoite protein (CSP) inhibit sporozoite's cell traversal activity. *Journal of Immunological Methods* **377,** 47–52. ISSN: 00221759. http://dx.doi.org/10.1016/j.jim.2012.01.009 (2012).

125. Moreira, L. A., Wang, J., Collins, F. H. & Jacobs-Lorena, M. Fitness of Anopheline Mosquitoes Expressing Transgenes That Inhibit Plasmodium Development. *Genetics* **166,** 1337–1341. ISSN: 00166731 (2004).

126. Moreira, L. A. *et al.* Bee venom phospholipase inhibits malaria parasite development in transgenic mosquitoes. *Journal of Biological Chemistry* **277,** 40839–40843. ISSN: 00219258 (2002).

127. Murugan, R. *et al.* Clonal selection drives protective memory B cell responses in controlled human malaria infection. *Science Immunology* **3.** ISSN: 24709468 (2018).

128. Murugan, R. *et al.* Evolution of protective human antibodies against Plasmodium falciparum circumsporozoite protein repeat motifs. *Nature Medicine* **26,** 1135–1145. ISSN: 1546170X. http://dx.doi.org/10.1038/s41591-020-0881-9 (2020).

129. Myung, J. M., Marshall, P. & Sinnis, P. The Plasmodium circumsporozoite protein is involved in mosquito salivary gland invasion by sporozoites. *Molecular and Biochemical Parasitology* **133,** 53–59. ISSN: 0166-6851. https://www.sciencedirect.com/science/article/pii/S0166685103002573 (2004).

130. Ng'habi, K. R. *et al.* Effect of larval crowding on mating competitiveness of Anopheles gambiae mosquitoes. *Malaria Journal* **4,** 1–9. ISSN: 14752875 (2005).

131. Nirmala, X. *et al.* Functional characterization of the promoter of the vitellogenin gene, AsVg1, of the malaria vector, Anopheles stephensi. *Insect Biochemistry and Molecular Biology* **36,** 694–700. ISSN: 09651748 (2006).

132. NUSSENZWEIG, R. S., VANDERBERG, J., MOST, H. & ORTON, C. Protective Immunity produced by the Injection of X-irradiated Sporozoites of Plasmodium berghei. *Nature* **216,** 160–162. ISSN: 1476-4687. https://doi.org/10.1038/216160a0 (1967).

133. Nussenzweig, V. & Nussenzweig, R. S. Circumsporozoite proteins of malaria parasites. *Bulletin et memoires de l"Academie royale de medecine de Belgique* **144,** 493–504. ISSN: 03778231 (1989).

134. Olotu, A., Fegan, G. & Wambua, J. Seven-Year Efficacy of RTS , S / AS01 Malaria Vaccine among Young African Europe PMC Funders Group Seven-Year Efficacy of RTS , S / AS01 Malaria Vaccine among Young African Children. *New England Journal of Medicine* **374,** 2519–2529 (2016).

135. Orfano, A. S. *et al.* Species-specific escape of Plasmodium sporozoites from oocysts of avian, rodent, and human malarial parasites. *Malaria Journal* **15,** 1–13. ISSN: 14752875 (2016).

136. Oyen, D. *et al.* Cryo-EM structure of P. falciparum circumsporozoite protein with a vaccine-elicited antibody is stabilized by somatically mutated inter-Fab contacts. *Science Advances* **4.** ISSN: 23752548 (2018).

137. Oyen, D. *et al.* Structure and mechanism of monoclonal antibody binding to the junctional epitope of Plasmodium falciparum circumsporozoite protein. *PLoS pathogens* **16,** e1008373. ISSN: 15537374 (2020).

138. Pholcharee, T. *et al.* Structural and biophysical correlation of anti-NANP antibodies with in vivo protection against P. falciparum. *Nature Communications* **12.** ISSN: 20411723. http://dx.doi.org/10.1038/s41467-021-21221-4 (2021).

139. Pimenta, P. F., Tourat, M. & Miller, L. The Journey of Malaria Sporozoites in the Mosquito Salivary Gland. *Journal of Eukaryotic Microbiology* **41,** 608–624. ISSN: 15507408 (1994).

140. Pondeville, E. *et al.* Hemocyte-targeted gene expression in the female malaria mosquito using the hemolectin promoter from Drosophila. *Insect Biochemistry and Molecular Biology* **120,** 103339. ISSN: 0965-1748. https://www.sciencedirect.com/science/article/pii/S096517482030028X (2020).

141. Posthuma, G. *et al.* Immunogold localization of circumsporozoite protein of the malaria parasite Plasmodium falciparum during sporogony in Anopheles stephensi midguts. *European journal of cell biology* **46,** 18–24. ISSN: 0171-9335. http://europepmc.org/abstract/MED/3294006 (1988).

142. Potocnjak, P., Yoshida, N., Nussenzweig, R. & Nussenzweig, V. Monovalent fragments (Fab) of monoclonal antibodies to a sporozoite surface antigen (Pb44) protect mice against malarial infection. *Journal of Experimental Medicine* **151,** 1504–1513. http://www.embase.com/search/results?subaction=viewrecord&from=export&id=L100629460Ahttp://dx.doi.org/10.1084/jem.151.6.1504 (1980).

143. Pradel, G. *et al.* A multidomain adhesion protein family expressed in Plasmodium falciparum is essential for transmission to the mosquito. *Journal of Experimental Medicine* **199,** 1533–1544. ISSN: 00221007 (2004).

144. Quinn, C., Anthousi, A., Wondji, C. & Nolan, T. CRISPR-mediated knock-in of transgenes into the malaria vector Anopheles funestus. *G3: Genes, Genomes, Genetics* **11.** ISSN: 21601836 (2021).

145. R Core Team. *R: A Language and Environment for Statistical Computing* R Foundation for Statistical Computing (Vienna, Austria, 2021). https://www.R-project.org/.

146. Ranson, H. & Lissenden, N. Insecticide Resistance in African Anopheles Mosquitoes: A Worsening Situation that Needs Urgent Action to Maintain Malaria Control. *Trends in Parasitology* **32,** 187–196. ISSN: 14715007. http://dx.doi.org/10.1016/j.pt.2015.11.010 (2016).

147. Ranson, H. *et al.* Pyrethroid resistance in African anopheline mosquitoes: What are the implications for malaria control? *Trends in Parasitology* **27,** 91–98. ISSN: 14714922. http://dx.doi.org/10.1016/j.pt.2010.08.004 (2011).

148. Rathore, D. *et al.* An Immunologically Cryptic Epitope of Plasmodium falciparum Circumsporozoite Protein Facilitates Liver Cell Recognition and Induces Protective Antibodies That Block Liver Cell Invasion*. *Journal of Biological Chemistry* **280,** 20524–20529. ISSN: 0021-9258. https://www.sciencedirect.com/science/article/pii/S0021925820640534 (2005).

149. Ribeiro, J. M., Mans, B. J. & Arcà, B. An insight into the sialome of blood-feeding Nematocera. *Insect Biochemistry and Molecular Biology* **40,** 767–784. ISSN: 0965-1748. https://www.sciencedirect.com/science/article/pii/S0965174810001700 (2010).

150. Risco-Castillo, V. *et al.* Malaria sporozoites traverse host cells within transient vacuoles. *Cell host & microbe* **18,** 593–603 (2015).

151. Rossignol, P. A., Ribeiro, J. M. C. & Spielman, A. Increased Intradermal Probing Time in Sporozoite-Infected Mosquitoes. *The American Journal of Tropical Medicine and Hygiene* **33,** 17–20. https://www.ajtmh.org/view/journals/tpmd/33/1/article-p17.xml (1984).

152. Scally, S. W. *et al.* Rare PfCSP C-terminal antibodies induced by live sporozoite vaccination are ineffective against malaria infection. *Journal of Experimental Medicine* **215,** 63–75. ISSN: 15409538 (2018).

153. Schneider, P., Takken, W. & McCall, P. Interspecific competition between sibling species larvae of Anopheles arabiensis and An. gambiae. *Medical and Veterinary Entomology* **14,** 165–170 (2000).

154. Shapiro, L. L., Murdock, C. C., Jacobs, G. R., Thomas, R. J. & Thomas, M. B. Larval food quantity affects the capacity of adult mosquitoes to transmit human malaria. *Proceedings of the Royal Society B: Biological Sciences* **283.** ISSN: 14712954 (2016).

155. Siciliano, G. *et al.* Critical Steps of Plasmodium falciparum Ookinete Maturation. *Frontiers in Microbiology* **11,** 1–9. ISSN: 1664302X (2020).

156. Sidjanski, S. P., Vanderberg, J. P. & Sinnis, P. Anopheles stephensi salivary glands bear receptors for region I of the circumsporozoite protein of Plasmodium falciparum. *Molecular and Biochemical Parasitology* **90,** 33–41. ISSN: 01666851 (1997).

157. Silvie, O. *et al.* Hepatocyte CD81 is required for Plasmodium falciparum and Plasmodium yoelii sporozoite infectivity. *Nature Medicine* **9,** 93–96. ISSN: 10788956 (2003).

158. Sinden, R. E. & Strong, K. An ultrastructural study of the sporogonic development of plasmodium falciparum in anopheles gambiae. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **72,** 477–491. ISSN: 18783503 (1978).

159. Singh, K. *et al.* Structure and function of a malaria transmission blocking vaccine targeting Pfs230 and Pfs230-Pfs48/45 proteins. *Communications Biology* **3,** 395. ISSN: 2399-3642. https://doi.org/10.1038/s42003-020-01123-9 (2020).

160. Smalley, M., Brown, J. & Bassett, N. M. The rate of production of Plasmodium falciparum gametocytes during natural infections. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **75,** 318–319. ISSN: 0035-9203 (1981).

161. Smidler, A. L., Terenzi, O., Soichot, J., Levashina, E. A. & Marois, E. Targeted Mutagenesis in the Malaria Mosquito Using TALE Nucleases. *PLoS ONE* **8,** 1–9. ISSN: 19326203 (2013).

162. Smith, J. D., Rowe, J. A., Higgins, M. K. & Lavstsen, T. Malaria's deadly grip: cytoadhesion of P lasmodium falciparum-infected erythrocytes. *Cellular microbiology* **15,** 1976–1983 (2013).

163. St Johnston, D. Using mutants, knockdowns, and transgenesis to investigate gene function in Drosophila. *WIREs Developmental Biology* **2,** 587–613. eprint: https://wires.onlinelibrary.wiley.com/doi/pdf/10.1002/wdev.101. https://wires.onlinelibrary.wiley.com/doi/abs/10.1002/wdev.101 (2013).

164. Steward, M. J. & Vanderberg, J. P. Malaria Sporozoites Leave Behind Trails of Circumsporozoite Protein During Gliding Motility1. *The Journal of Protozoology* **35,** 389–393. eprint: https://onlinelibrary.wiley.com/doi/pdf/10.1111/j.1550-7408.1988.tb04115.x. https://onlinelibrary.wiley.com/doi/abs/10.1111/j.1550-7408.1988.tb04115.x (1988).

165. Stewart, M. J. & Vanderberg, P. J. Malaria Sporozoites Release Circumsporozoite Protein from Their. *The Journal of protozoology* **38,** 411–421 (1991).

166. Sultan, A. A. *et al.* TRAP Is Necessary for Gliding Motility and Infectivity of Plasmodium Sporozoites. *Cell* **90,** 511–522. ISSN: 0092-8674. https://www.sciencedirect.com/science/article/pii/S0092867400805115 (1997).

167. Sulyok, Z. *et al.* Heterologous protection against malaria by a simple chemoattenuated PfSPZ vaccine regimen in a randomized trial. *Nature Communications* **12,** 1–10. ISSN: 20411723. http://dx.doi.org/10.1038/s41467-021-22740-w (2021).

168. Sumitani, M. *et al.* Reduction of malaria transmission by transgenic mosquitoes expressing an antisporozoite antibody in their salivary glands. *Insect Molecular Biology* **22,** 41–51. ISSN: 09621075 (2013).

169. Talaat, K. R. *et al.* Safety and Immunogenicity of Pfs25-EPA/Alhydrogel®, a Transmission Blocking Vaccine against Plasmodium falciparum: An Open Label Study in Malaria Naïve Adults. *PLOS ONE* **11,** 1–17. https://doi.org/10.1371/journal.pone.0163144 (Oct. 2016).

170. Tan, J. *et al.* A public antibody lineage that potently inhibits malaria infection through dual binding to the circumsporozoite protein. *Nature Medicine* **24,** 401–407. ISSN: 1546170X (2018).

171. Terzakis, J. A., Sprinz, H. & Ward, R. A. The transformation of the plasmodium gallinaceum oocyst in Aedes aegypti mosquitoes. *Journal of Cell Biology* **34,** 311–325. ISSN: 00393746 (1967).

172. Tewari, R., Spaccapelo, R., Bistoni, F., Holder, A. A. & Crisanti, A. Function of region I and II adhesive motifs of Plasmodium falciparum circumsporozoite protein in sporozoite motility and infectivity. *Journal of Biological Chemistry* **277,** 47613–47618. ISSN: 00219258 (2002).

173. Thai, E. *et al.* A high-affinity antibody against the CSP N-terminal domain lacks Plasmodium falciparum inhibitory activity. *Journal of Experimental Medicine* **217** (2020).

174. Thathy, V. *et al.* Levels of circumsporozoite protein in the Plasmodium oocyst determine sporozoite morphology. *EMBO Journal* **21,** 1586–1596. ISSN: 02614189 (2002).

175. Thiévent, K., Zilio, G., Hauser, G. & Koella, J. C. Malaria load affects the activity of mosquito salivary apyrase. *Journal of Insect Physiology* **116,** 10–16. ISSN: 0022-1910. https://www.sciencedirect.com/science/article/pii/S0022191018304141 (2019).

176. Timmermann, S. E. & Briegel, H. Larval growth and biosynthesis of reserves in mosquitoes. *Journal of Insect Physiology* **45,** 461–470. ISSN: 00221910 (1999).

177. Triller, G. *et al.* Natural Parasite Exposure Induces Protective Human Anti-Malarial Antibodies. *Immunity* **47,** 1197–1209.e10. ISSN: 10974180 (2017).

178. Tse, E. G., Korsik, M. & Todd, M. H. The past, present and future of anti-malarial medicines. *Malaria Journal* **18,** 1–21. ISSN: 14752875. https://doi.org/10.1186/s12936-019-2724-z (2019).

179. Tufail, M. & Takeda, M. Molecular characteristics of insect vitellogenins. *Journal of Insect Physiology* **54,** 1447–1458. ISSN: 0022-1910. https://www.sciencedirect.com/science/article/pii/S0022191008001698 (2008).

180. Uwimana, A. *et al.* Association of ¡em¿Plasmodium falciparum kelch13¡/em¿ R561H genotypes with delayed parasite clearance in Rwanda: an open-label, single-arm, multicentre, therapeutic efficacy study. *The Lancet Infectious Diseases* **21,** 1120–1128. ISSN: 1473-3099. https://doi.org/10.1016/S1473-3099(21)00142-0 (2021).

181. Van Schaijk, B. C. *et al.* Type II fatty acid biosynthesis is essential for Plasmodium falciparum sporozoite development in the midgut of anopheles mosquitoes. *Eukaryotic Cell* **13,** 550–559. ISSN: 15359778 (2014).

182. Vanderberg, J. P. . Development of Infectivity by the Plasmodium berghei Sporozoite. *The American Society of Parasitologists* **61,** 43–50 (1975).

183. Vanderberg, J. P. & Frevert, U. Intravital microscopy demonstrating antibody-mediated immobilisation of Plasmodium berghei sporozoites injected into skin by mosquitoes. *International Journal for Parasitology* **34,** 991–996. ISSN: 00207519 (2004).

184. Venugopal, K., Hentzschel, F., Valkiūnas, G. & Marti, M. Plasmodium asexual growth and sexual development in the haematopoietic niche of the host. *Nature Reviews Microbiology* **18,** 177–189. ISSN: 17401534 (2020).

185. Volohonsky, G. *et al.* Tools for anopheles gambiae transgenesis. *G3: Genes, Genomes, Genetics* **5,** 1151–1163. ISSN: 21601836 (2015).

186. Volohonsky, G. *et al.* Transgenic Expression of the Anti-parasitic Factor TEP1 in the Malaria Mosquito Anopheles gambiae. *PLoS Pathogens* **13,** 1–26. ISSN: 15537374 (2017).

187. Wang, G. H. *et al.* Combating mosquito-borne diseases using genetic control technologies. *Nature Communications* **12,** 1–12. ISSN: 20411723. http://dx.doi.org/10.1038/s41467-021-24654-z (2021).

188. Wang, Q., Fujioka, H. & Nussenzweig, V. Exit of plasmodium sporozoites from oocysts is an active process that involves the circumsporozoite protein. *PLoS Pathogens* **1,** 0072–0079. ISSN: 15537366 (2005).

189. Wang, Q., Fujioka, H. & Nussenzweig, V. Mutational analysis of the GPI-anchor addition sequence from the circumsporazoite protein of Plasmodium. *Cellular Microbiology* **7,** 1616–1626. ISSN: 14625814 (2005).

190. Wang, S. & Jacobs-Lorena, M. Genetic approaches to interfere with malaria transmission by vector mosquitoes. *Trends Biotechnology* **31,** 185–193. ISSN: 15378276. arXiv: NIHMS150003 (2013).

191. Warburg, A., Touray, M., Krettli, A. U. & Miller, L. H. Plasmodium gallinaceum: Antibodies to circumsporozoite protein prevent sporozoites from invading the salivary glands of Aedes aegypti. *Experimental Parasitology* **75,** 303–307. ISSN: 10902449 (1992).

192. Warrell, D. A. in *Essential malariology* 191–205 (CRC Press, 2017).

193. Wells, M. B. & Andrew, J. Sporozoite Availability for Transmission. *mBio* **10,** 1–17 (2019).

194. White, G. Anopheles gambiae complex and disease transmission in Africa. *Transactions of The Royal Society of Tropical Medicine and Hygiene* **68,** 278–298. ISSN: 0035-9203. eprint: https://academic.oup.com/trstmh/article-pdf/68/4/278/5408055/68-4-278.pdf. https://doi.org/10.1016/0035-9203(74)90035-2 (Jan. 1974).

195. WHO. *World malaria report 2020- WHO* ISBN: 9789240015791 (2020).

196. WHO. *WHO recommends groundbreaking malaria vaccine for children at risk* Oct. 2021. https://www.who.int/news/item/06-10-2021-who-recommends-groundbreaking-malaria-vaccine-for-children-at-risk.

197. Wilke, A. B., Beier, J. C. & Benelli, G. Transgenic Mosquitoes – Fact or Fiction? *Trends in Parasitology* **34,** 456–465. ISSN: 14715007 (2018).

198. Wotton, R. S., Chaloner, D. T., Yardley, C. A. & Merritt, R. W. Growth of Anopheles mosquito larvae on dietary microbiota in aquatic surface microlayers. *Medical and Veterinary Entomology* **11,** 65–70. eprint: https://onlinelibrary.wiley.com/doi/pdf/10.1111/j.1365-2915.1997.tb00291.x. https://onlinelibrary.wiley.com/doi/abs/10.1111/j.1365-2915.1997.tb00291.x (1997).

199. Wu, Y. *et al.* Phase 1 trial of malaria transmission blocking vaccine candidates Pfs25 and Pvs25 formulated with montanide ISA 51. *PloS one* **3,** e2636 (2008).

200. Yahouédo, G. A. *et al.* Effect of three larval diets on larval development and male sexual performance of Anopheles gambiae s.s. *Acta Tropica* **132,** S96–S101. ISSN: 18736254. http://dx.doi.org/10.1016/j.actatropica.2013.11.014 (2014).

201. Yang, J. *et al.* Disruption of mosGILT in Anopheles gambiae impairs ovarian development and Plasmodium infection. *Journal of Experimental Medicine* **217.** e20190682. ISSN: 0022-1007. eprint: https://rupress.org/jem/article-pdf/217/1/e20190682/874886/jem_20190682.pdf. https://doi.org/10.1084/jem.20190682 (Oct. 2019).

202. Yoshida, N., Nussenzweig, R. S. & Nussenzweig, V. Hybridome Produces Antibodies Directed Against the Sporozoite Stage of Malaria Parasite. *Science* **207,** 71–73 (1980).

203. Yoshida, S. & Watanabe, H. Robust salivary gland-specific transgene expression in Anopheles stephensi mosquito. *Insect Molecular Biology* **15,** 403–410. ISSN: 09621075 (2006).

204. Zavala, F., Cochrane, A. H., Nardin, E. H., Nussenzweig, R. S. & Nussenzweig, V. Circumsporozoite proteins of malaria parasites contain a singleimmu- nodominant region with two or more identical epitopes. *Journal of Experimental Medicine* **157,** 1947–1957 (1983).

205. Zhao, J., Bhanot, P., Hu, J. & Wang, Q. A comprehensive analysis of Plasmodium circumsporozoite protein binding to hepatocytes. *PLoS ONE* **11,** 1–13. ISSN: 19326203 (2016).

9 Supplemental

Figure	**Shapiro test**	**Test used**	**exact value**	**Post-hoc test**
Figure 8 A	0.2643	t-test	0.8413	
Figure 8 B 14 dpi	0.1931	t-test	0.0004934	
Figure 8 B 18 dpi	0.1931	t-test	0.0001063	
Figure 8 C	0.9845	t-test	0.2746	
Figure 8 D 14 dpi	0.5319	t-test	0.09411	
Figure 8 D 18 dpi	0.5319	t-test	0.1564	
Figure 9 A	0.2123	t-test	0.8657	
Figure 9 B 18 dpi	0.0587	t-test	3.914e-06	
Figure 9 B 25 dpi	0.0587	t-test	7.202e-06	
Figure 9 C	0.3635	t-test	0.7486	
Figure 9 D 18 dpi	0.7684	t-test	0.7486	
Figure 9 D 25 dpi	0.7684	t-test	0.127	
Figure 10A	0.1734	t-test	0.6623	
Figure 10B 14 dpi	1.539e-05	Wilcoxon	0.093	Benjamin Hochberg
Figure 10B 18 dpi	1.539e-05	Wilcoxon	0.093	Benjamin Hochberg
Figure 13B	3.131e-05	Wilcoxon	2e-16	Benjamin Hochberg
Figure 13D Low	9.523e-06	Wilcoxon	0.045	Benjamin Hochberg
Figure 13D High	9.523e-06	Wilcoxon	0.057	Benjamin Hochberg
Figure 14 A	0.08837	Wilcoxon	2.2e-16	
Figure 14 C WT	0.0001234	Wilcoxon	2.3e-11	Benjamin Hochberg
Figure 14 C Lp	1.356e-07	Wilcoxon	9.2e-14	Benjamin Hochberg
Figure 14 D *LacZ*	0.002224	Wilcoxon	0.0075	Benjamin Hochberg
Figure 14 D *TEP1*	0.0005301	Wilcoxon	0.0079	Benjamin Hochberg
Figure 16B *LacZ*	2.2e-16	Wilcoxon	0.043	Benjamin Hochberg

Figure 16B *TEP1*	2.2e-16	Wilcoxon	1.6e-10	Benjamin Hochberg
Figure 22 A	-	Log-Rank test	0.3	-
Figure 22 B	-	Log-Rank test	0.099	-
Figure 28 A	2.2e-16	Wilcoxon	0.9969	Benjamin Hochberg
Figure 28 B scFab1210	0.001391	Wilcoxon	0.017	Benjamin Hochberg
Figure 28 B scFab1710	0.001391	Wilcoxon	1	Benjamin Hochberg
Figure 28 B scFab5D5	0.001391	Wilcoxon	1	Benjamin Hochberg

Table 17. Exact p-values for all test performed

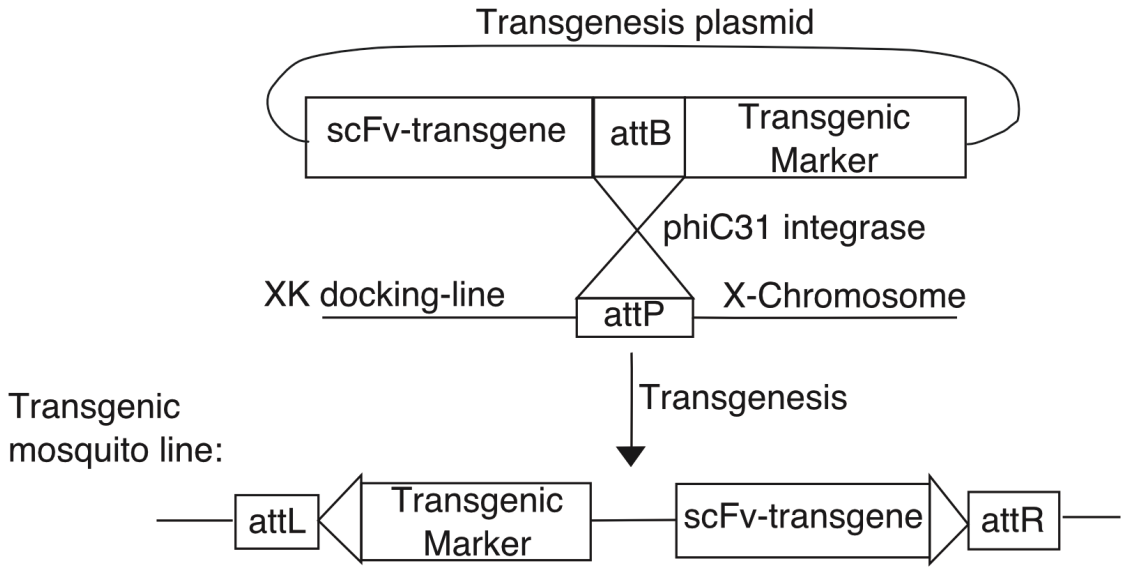

Figure 29. Design of Transgenic lines A The transgenic mosquito lines *Aapp::125*, *Lp::125* and *Lp::2A10* were designed and developed by Dr. Eric Marois and Maria Pissarev. The transgenesis plasmid containing the scFv-transgene, an attB site and the transgenic marker was inserted using a phage $\phi C31$ mediated integrase system into the docking line XK containing the attP insertion sequence on the X-chromosome.

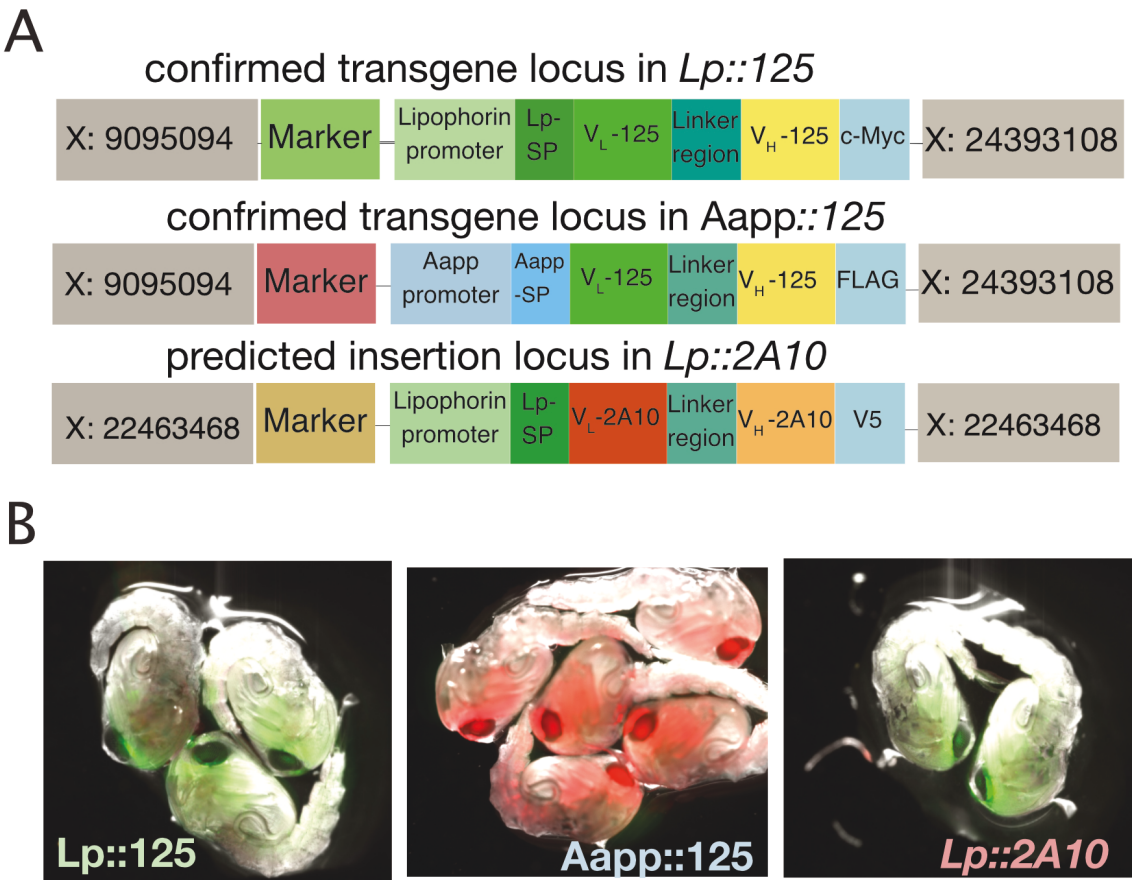

Figure 30. Insertion locus and Transgenesis Marker
The transgene locus was confirmed after loss of homozygous lethality for *Lp::125* and *Aapp::125*. Inverse PCR followed by sequencing showed the upstream and downstream genomic sequence of the inserted gene. The transgene is located on the X chromosome. The insertion locus of *Lp::2A10* could not be confirmed after loss of homozygous lethality. The original insertion locus was also located on the X chromosome. **B**Pupae from three transgenic lines. *Lp::125* expresses GFP in the nervous system and eyes. *Aapp::125* expresses RFP in eyes and nervous system. *Lp::2A10* expresses YFP in eyes and nervous system.

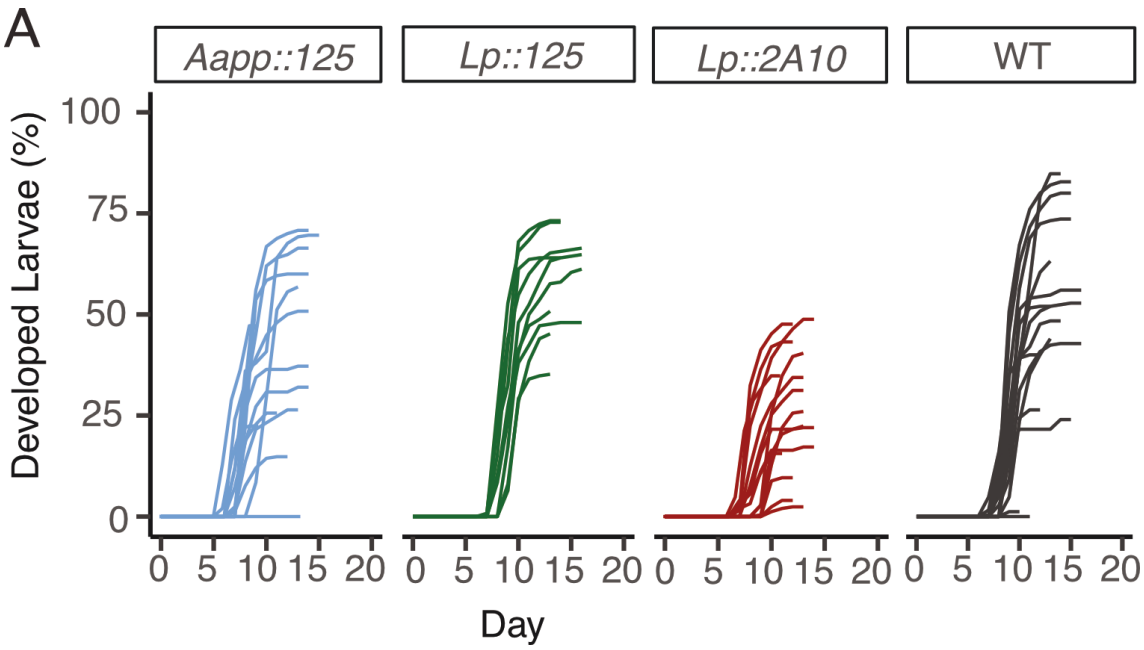

Figure 31. Cumulative pupae time series
Full time series of larval development for transgenic lines and wild-type. The cumulative proportion of developed pupae is shown over the time course of 20 days. The lines represent the pupation dynamics of every single experimental pan. The line end when all larvae either pupated or died.

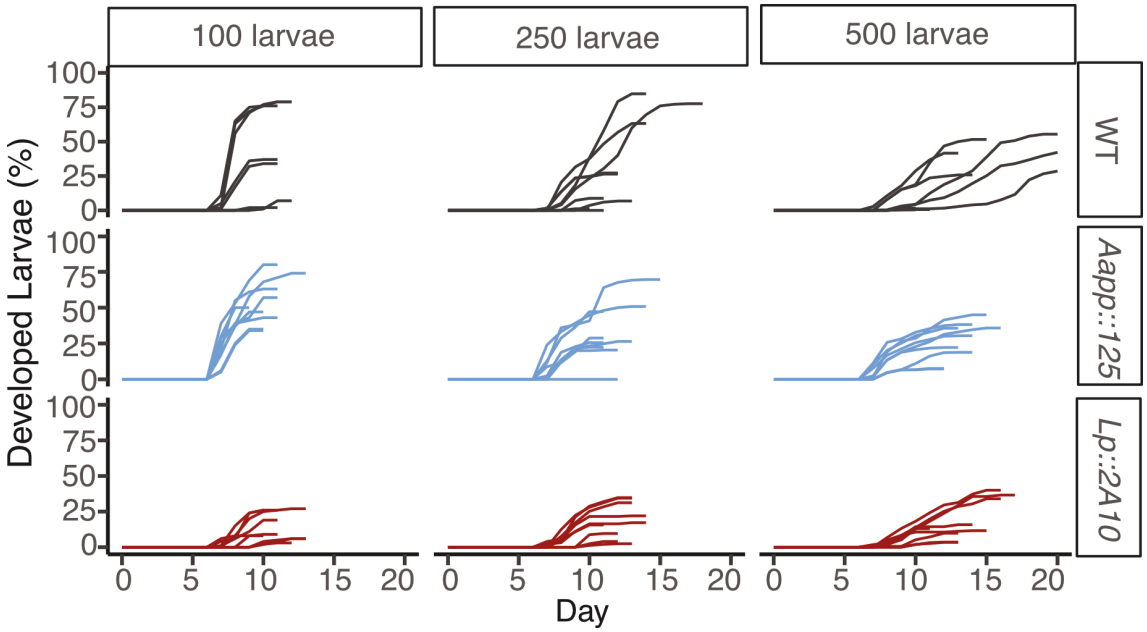

Figure 32. Cumulative pupae time series for nutritional stress settings
Full time series of larval development for transgenic lines and wild-type using different larval densities . The cumulative proportion of developed pupae is shown over the time course of 20 days. The lines represent the pupation dynamics of every single experimental pan. The line ends when all larvae either pupated or died.

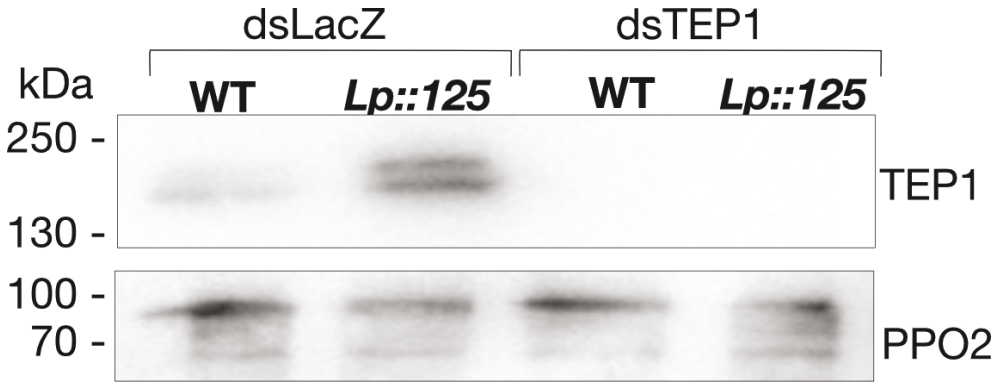

Figure 33. Western Blot depicting the knock down efficiency for TEP1
Pooled hemolymph samples from wild-type (WT) and *Lp::125* after control injections(*dsLacZ*) or injection of *dsTEP1*. The lower panel shows PPO2 expression as loading control.

10 Acknowledgments

Throughout the four years I have been working at the MPIIB, I encountered many people helping me through the process and nurturing my professional and personal development and growth.
Firstly, I want to thank my supervisor Elena Levashina for allowing me to work in her group and on this project. I am grateful for your feedback and supervision, and I enjoyed working with mosquitoes, even though it is exhausting sometimes. I want to thank Giulia for her patience in teaching me to work with parasites and mosquitoes, and for helping me become more organized and a better scientist. Thank you for building the foundation of the project together with Maria.
I want to thank Prof. Dr Arturo Zychlinsky and Prof. Dr Hedda Wardemann for their feedback and discussions during meetings and for taking the time to evaluate my thesis. I am also deeply grateful to all MPIIB employers who supported me with their expertise, particularly the MPIIB microscopy core facility and the DRFZ flow cytometry core facility. I want to thank Paola for the great collaboration, the helpful comments and energizing discussions. Paula, Juan, Charly and Marta, for a great team effort on the development project, I enjoyed working with all of you. I am thankful for the last 4.5 years working in the Vector Biology department, particularly for Manuela, Conny, and Daniel helping with the mouse and mosquito work. Pablo, Galo, Suzana, Robin and the rest of the lab for the discussions and fun times outside the work.
I am very thankful for the IMPRS coordinators, who supported me and helped with the bureaucracy. I want to thank Uli, French and Verena for their support over the last 30 years. I hope I can give you the same level of support.
Thank you to Martin, Felix, Lorenz and Leona for the mental and scientific support.
Last but not least, thank you, Bob, for your support and for not giving up on me.
In memory of Luna, Franziska and Anna, I miss you.

Declarations

Eidesstattliche Erklärung

1. Bei der eingereichten Dissertation mit dem Titel "Blocking *Plasmodium* development in the mosquitoes by human antibodies" handelt es sich um mein eigenständig erstelltes Werk, das den Regeln guter wissenschaftlicher Praxis entspricht.
   The submitted doctoral dissertation on the subject 'Blocking Plasmodium development in the mosquitoes by human antibodies' is my own work and to the rules of proper scientific conduct.

2. Ich habe nur die angegebenen Quellen und Hilfsmittel benutzt und mich keiner unzulässigen Hilfe Dritter bedient. Insbesondere habe ich wörtliche und nicht wörtliche Zitate aus anderen Werken als solche kenntlich gemacht.
   I did not seek unauthorized assistance of a third party and I have employed no other sources or means except the ones listed. I clearly marked any direct and indirect quotations derived from the works of others.

3. Die Arbeit oder Teile davon habe ich wie folgt/bislang nicht an einer Hochschule des In- oder Auslands als Bestandteil einer Prüfungs- oder Qualifikationsleistung vorgelegt.
   I did not yet present this doctoral dissertation or parts of it at any other higher education institution in Germany or abroad.

4. Die Richtigkeit der vorstehenden Erklärung bestätige ich.
   I hereby confirm the accuracy of the affirmation above.

5. Die vorliegende Arbeit wurde am Max-Planck-Institut für Infektionsbiologie unter Leitung von Prof. Dr. Elena Levashina durchgeführt.
   This work was conducted at the Max-Planck-Institute for Infection Biology under the supervision of Prof. Dr. Elena A. Levashina

Ich bin damit einverstanden, dass die Arbeit zum Zwecke des Plagiatsabgleichs in elektronischer Form versendet, gespeichert und verarbeitet wird. *I agree that for the purpose of assessing plagiarism the dissertation may be electronically forwarded, stored and processed.*

Vorname:Anna Maria Nachname: Weyrich Matrikelnummer: 619337

Ort, Datum Unterschrift:

Erweiterte eidesstattliche Erklärung

Hiermit versichere ich, Anna Maria Weyrich, dass folgende Publikationen:

"A high-affinity antibody against the CSP N-terminal domain lacks Plasmodium falciparum inhibitory activity "
E. Thai, G. Costa, **A. Weyrich**, R. Murugan, D. Oyen, Y. Flores-Garcia, K. Prieto, A. Bosch, A. Valleriani, N.C. Wu, T. Pholcharee, S. W. Scally, I. A. Wilson, H. Wardemann, J.P. Julien, E. A. Levashina. J Exp Med. 2020 Nov2;217

in den revelanten Teilen von mir verfasst wurden. Mögliche Übereinstimmungen mit Textpassagen aus meiner Dissertation "Blocking *Plasmodium* development in the mosquitoes by human antibodies" stellen somit keinen Plagiatsfall dar.
Dies wird bei Bedarf durch die Betreuerin der Dissertation und Co-Autorin der aufgeführten Publikation Prof. Dr. Elena Levashina bestätigt.

Berlin, Anna Maria Weyrich