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Characterisation of a second actin gene: Analysing its function in the *Plasmodium berghei* mosquito midgut stages

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Summary

The causative agent for the debilitating disease malaria is the *Plasmodium* parasite. *Plasmodium spp* is the only genus of Apicomplexa which possesses an additional gene encoding actin besides the major actin isoform actin I. This second actin has been termed actin II. It has been found to be expressed in male gametocytes and ookinetes in the murine model strain *P. berghei*. Disruption resulted in an impairment of the male gametocyte to exflagellate and a 50-fold decrease in ookinete formation, while females remained fertile.

In this study we explored the role of actin II in the mosquito stages. By performing genetic crosses with a knockout strain of *actin II* (*actII(-)*) and the wild type (wt) we determined that heterozygous oocysts were formed, but did not develop further than ~ three days, while the homozygous wt ookinetes developed into mature oocysts. To analyse this defect further, we attempted to alter expression levels of actin II by exchanging its promoter region with promoter regions from proteins known to be expressed in the male gametocyte. Two mutant strains, *cdpk4p-actII* and *setp-actin II* were constructed. While the *cdpk4-actII* parasites did not show any detectable phenotype, the *setp-actII* mutant displayed a growth defect in the asexual stages, even though its driving promoter is exclusively active in the sexual stages. We also studied a pure heterozygous ookinete population with a parasite strain producing only males and the *actII(-)* female and observed that actin II was not expressed in these ookinetes. The resulting oocysts were growth arrested and did not undergo DNA replication. We followed the expression of GFP tagged actin II and showed that the highest protein expression occurs within the first few hours of zygote formation. These results show that actin II is a sex-specific protein required in the male gametogenesis however it is inherited from the female gamete during zygote formation to complete the transmission cycle through the mosquito.

Περίληψη

Ο μολυσματικός παράγοντας της ελονοσίας είναι το παράσιτο που ανήκει στο γένος του πλασμωδίου. Στο φύλο των Apicomplexa to πλασμώδιο είναι το μόνο που έχει ένα επιπλέον γονίδιο που κωδικοποιεί για ένα επιπλέον γονίδιο ακτίνης πέρα της ακτίνης I. Η δεύτερη ακτίνη ονομάζεται Ακτίνη II και εκφράζεται στα αρσενικά γαμετοκύτταρα και ωοκινέτες στο παράσιτο *Plasmodium berghei* των τρωκτικών. Η έλλειψη του γονιδίου είχε ως αποτέλεσμα την ανικανότητα των αρσενικών γαμετοκυττάρων να παράγουνε ενεργά μαστίγια και 50 φορες μειωμένο αριθμό ωοκινετών, ενώ καμιά αλλαγή δεν παρατηρήθηκε στα θηλυκα γαμετοκυτταρα.

Στη μελέτη αυτή διερευνάται ο ρόλος της Ακτίνης II κατα την ανάπτυξη του παρασίτου στο κουνούπι. Πραγματοποιήσαμε γενετικές διασταυρώσεις μεταξύ ενός παρασιτικού στέλεχους στο οποίο λείπει το γονίδιο της ακτίνης II (*actinII(-)*) και του άγριου τύπου(wt) και δείξαμε ότι δημιουργούνται ετερόζυγες ωοκίστες που δεν αναπτύσσονται πέρα απο τις 3 ημέρες σε αντίθεση με τις ομόζυγες ωοκίστες. Για να μελετήσουμε αυτό το φαινότυπο, δοκιμάσαμε να αλλάξουμε τα επίπεδα έκφρασης της ακτίνης II με αντίκατασταση του υποκινητή από υποκινητές γονιδίων που είναι γνωστά οτι εκφράζονται στο αρσενικό γαμετοκύτταρο. Δύο μεταλλαγμένα στελέχη παράχθηκαν το *cdpk4-actinII* kai *setp-actinII*. Τα *cdpk4-actinII* στελέχη δεν παρουσίασαν διαφορετικό φαινότυπο σε σχέση με τον άγριο τύπο ενώ τα *setp-actinII* στελέχη παρουσίασαν πολύ αργό ρυθμό ανάπτυξης στα μη σεξουαλικά στάδια παρόλο που ο υποκινητής αυτός εκφράζεται αποκλειστικά στα σεξουαλικά στάδια. Μελέτήθηκε επίσης ένας ετερόζυγος πληθυσμός οοκινετών που δημιουργήθηκε μεταξύ ενός παρασιτικού στελέχους που παραγει μόνο ενεργά αρσενικά γαμετοκύτταρα με το θηλυκά γαμετοκύτταρά του στελέχους όπου λείπει το γονίδιοτης ακτίνης II. Οι ωοκίστες που παράχθηκαν με αυτή τη διασταύρωση δεν περιείχαν γονιδιακό DNA. Χρησιμοποιώντας ενα στέλεχος όπου η ακτίνη II ειναι συζευγμένη με την πράσινη φθορίζουσα πρωτείνη (GFP) δείξαμε ότι η μεγαλύτερη πρωτεϊνική έκφραση πραγματοποιείται τις πρώτες ώρες μετά το σχηματισμό του ζυγωτού. Τα αποτελέσματα αυτά δείχνουνε ότι η ακτίνη II απαιτείται στα σεξουαλικά στάδια της ζωής του παρασίτου στη

γαμετογέννεση του αρσενικού γαμετοκυττάρου ενώ κληροδοτείται στο ζυγωτό μέσω του θηλυκού γαμέτη έτσι ώστε να μπορέσει να ολοκληρώθει ο κύκλος ζωής του παρασίτου μέσα στο κουνούπι.

Abbreviations

AP2	Apetala 2
actII(-)	actin II knockout
bs	base pairs
Cap380	oocyst capsule protein
C-CAP	C-cyclase associated protein
cdc	cell division cycle
cdlk	CDPK-like kinase
cDNA	complementary DNA
cdpk	calcium dependent protein kinase
CelTOS	cell traversal protein for ookinete and sporozoite
CP	capping protein
CTRP	circumsporozoite and TRAP related protein
DHFR	dihydrofolate reductase
DNA	deoxyribonucleic acid
d.p.f.	days post feeding
ECP	egress cysteine protease
EM	erythrocyte membrane
FR	flanking region
gak	G-associated preotein kinase
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GFP	green fluorescent protein
GMEP	Global Malaria Eradication Programme
hrs	hours
i.p.	intraperitonally
kb	kilobase

LAP	LCCL/lectin adhesive-like protein
MAOP	membrane attack ookinete protein
map	mitogen activated protein
mdv-1/peg3	male development-1/protein of early gametocyte3
p.a.	post attachment
PCR	polymerase chain reaction
PbGEST	*Plasmodium berghei* gamete egress and sporozoite traversal
p.i.	post infection
pk	protein kinase
pplp	*Plasmodium* perforin like protein
psop	putative secreted ookinete protein
PV	parasitophorous vacuole
PVM	parasitophorous vacuole membrane
RBC	red blood cell
RNA	ribonucleic acid
ROS	reactive oxygen species
RT	room temperature
RT-PCR	reverse transcription PCR
SOAP	secreted ookinete adhesive protein
UTR	untranslated region
WHO	World Health organisation

Chapter 1

Introduction

1.1. Parasitism

Within the five animal kingdoms parasitism has evolved separately in many different species from different kingdoms. A parasite is defined as an organism that benefits from its host/s, usually at the latter's expense. However, generally the term is used to describe an organism which invades its host and reduces the biological fitness (Combes, 2005). There are many types of parasites that can occupy different compartments of the host. Ectoparasites live on the surface of the host, while endoparasites live inside the host. Smaller parasites have evolved the ability to also invade the host's cells and live intra-cellularly, not just within the cavities. Another distinction between parasite species is their life cycles and whether their life cycle involves a definitive host, which is termed monoxenic, and can be directly transferred from one host to the next. The other option involves the use of a vector and a host, this is called heteroxenic. From an evolutionary standpoint being heteroxenic allows colonisation of many different environments, therefore being able to be transmitted across greater distances and not being limited by low host population densities within a small region (Dobson, 1992). Although this advantage quickly becomes a disadvantage as the host and vector need to overlap regionally in order for transmission to occur.

Transmission for parasites using faecal-oral or predator-prey ingestion is a sufficient life cycle progression for intercellular parasites. But for parasites living in the blood stream or inside blood cells, transmission would prove very difficult indeed by the just mentioned approaches. Therefore different blood parasites have evolved separately the adoption of haematophagous arthropods as their vectors to switch between hosts (Wiser, 2011). Examples of such heteroxenic parasites are the African trypanosomes and their vector *Tse Tse*, *Leishmania* and sand flies, and *Plasmodium* and mosquitoes*.* Their hosts are in each case mammalian species, including humans. In addition, some *Plasmodium* species are able to infect birds and reptiles. In each of these parasites sexual replication takes place within the vector while the disease manifests itself within the host. These

diseases have a devastating impact on human health and in extension on the ecology and economy of the affected areas.

1.2. Malaria

Malaria is caused by the *Plasmodium* parasite. The 2011 Malaria report of the World Health Organisation (WHO) states that malaria is endemic in 106 countries in the world. 3.3 billion people were at risk in 2010, with 216 million official incidences of the disease, which sadly resulted in 665.000 deaths (www.who.int/malaria). However, incident rates and mortality rates have been declining during the last decade by 17% and 26% respectively. This is due to several factors: the use of insecticide-treated bed-nets, indoor residual spraying, increased access to rapid diagnosis tests and artimisinin-based combination therapies, as well as increased vector control.

There are five species of *Plasmodium* which are capable of infecting humans, *P. vivax*, *P. ovale*, *P. malariae*, *P. knowlesi*, a simian parasite which has only recently been found to infect humans, and *P. falciparum*, with the latter being the most deadly form (Cox-Singh et al., 2008, Cox-Singh and Singh, 2008). The human form of the disease malaria is caused by the asexual replication within the host. This gives rise to the symptoms associated with this devastating illness, such as recurring fever cycles, anaemia and organ damage. In non-immune patients infected with *P. falciparum* the disease can eventually lead to the patient falling into a coma and/or dying. Sadly, specifically pronounced on the sub-Saharan African continent, the majority of people dying from this tropical disease are children under the age of five and pregnant women. Most people living in endemic areas are bitten several times in their lives by *Plasmodium* infected mosquitoes and develop over time an immune resistance. However, this only offers mild protection against the severity of the symptoms and not the infection itself.

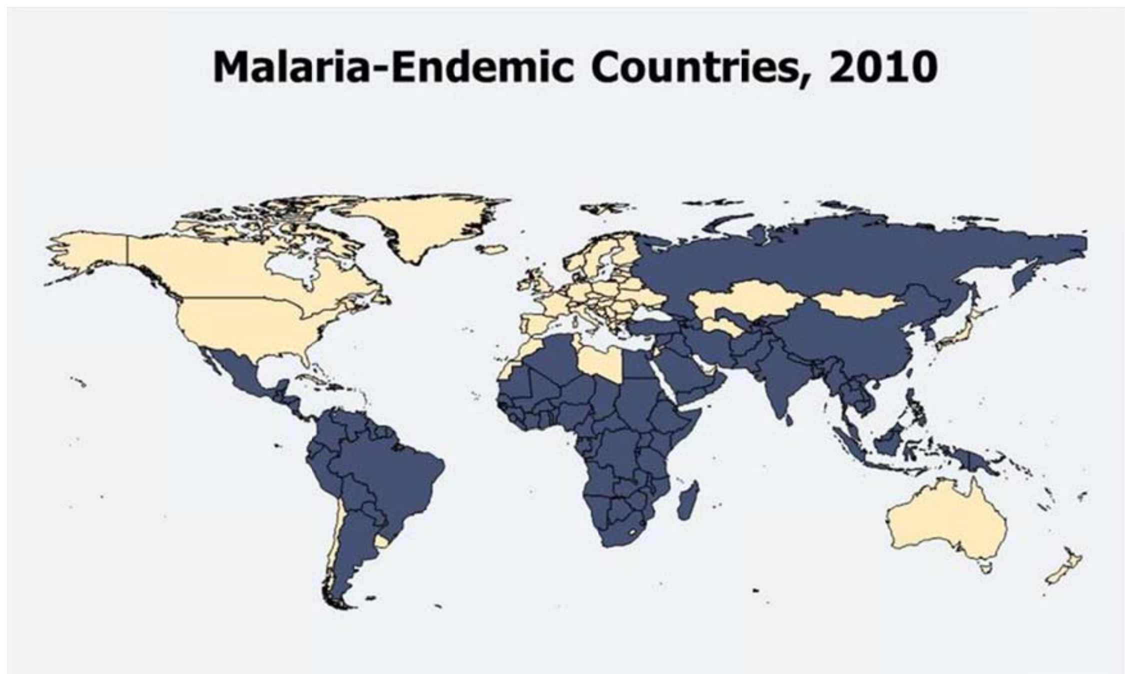


Figure 1.1. World map of the malaria endemic countries in purple

Morocco and Turkmenistan became certified malaria free that year. This map does not show Armenia as malaria free, which occurred as of October 2011.

Focusing on vector control has a larger impact on disease control overall than trying to just cure the infected individual. As it is the sexual stages that are responsible for transmission by the mosquito, a detailed understanding of these stages of the *Plasmodium* parasite would contribute to the development of novel therapeutic or prevention methods. Such transmission-blocking methods are an essential part of schemes to eradicate malaria (see reviews in MalERA (2011e, 2011d, 2011a, 2011b, 2011c).

Vector control has had its greatest impact with the Global Malaria Eradication Programme (GMEP) which ended in 1969. The programme eradicated malaria in Europe and led to a temporary reduction of the disease in Africa and Asia (Tanner and de Savigny, 2008). However, soon there was a resurgence of the parasite in these areas. Recently, increased funding and novel strategies have led to a steady decrease in the malaria burden.

1.3. The *Plasmodium* life cycle

Infection with mammalian *Plasmodium* occurs after a bite of a female *Anopheles* mosquito carrying the parasite. The injected forms of the parasite, called sporozoites, travel from the injection site to the liver of the host and invade a hepatocyte. Within several days or weeks each invading parasite multiplies into thousands of merozoites, which upon rupture of the hepatocytes, are then released into the bloodstream (Prudencio et al., 2006). They next invade the erythrocytes, as the parasite cannot survive for long outside of the red blood cells (RBC). Immediately upon invasion a parasite-specific compartment is formed in the erythrocyte cytoplasm called the parasitophorous vacuole (PV). The PV membrane (PVM) plays an important role in nutrient acquisition from the RBC ((de Koning-Ward et al., 2009) for review see (Lingelbach and Joiner, 1998)). From here on the cycle splits into the asexual replicative cycle and the development of gametocytes; in both cases the parasite is intra-erythrocytic. During asexual replication the intracellular merozoites develop into the so called ring stages, to trophozoites and finally schizogony leads to the release of new merozoites. They next reinvade the red blood cells thus repeating the asexual cycle. In the human forms of the parasite the rupture of the RBC's is synchronised and responsible for the recurring fever cycles in the disease.

The sexual cycle is differentiated and after the trophozoite stage, male or female gametocytes develop (for recent reviews see (Kuehn and Pradel, 2010, Liu et al., 2011)). After maturation these parasite forms circulate in an inactive state in the bloodstream until taken up in a mosquito blood meal. The sexual stages become activated and male and female gametes mature, which then fuse to form a zygote. The zygote develops into a banana-shaped ookinete, which traverses the midgut and attaches itself to the outside of the epithelium. It then develops into an oocyst, in which during the next two weeks several thousand sporozoites are formed. Upon rupture of the oocyst the sporozoites migrate through the haemocoel to the salivary glands and from there get injected into a new host with the next blood meal.

1.4. The sexual stages of *Plasmodium*

The vast majority of research was previously focused mainly on the asexual cycle of the parasite. Research into the sexual stages of *Plasmodium* has only gained momentum in the last 10 - 15 years, although many of the processes involved are still poorly understood. However, recently these stages have attracted a lot of interest. There is progress in the understanding of these processes and a number of proteins have been studied that are specifically required for the sexual stages and the transmission stages of *Plasmodium* in the mosquito life cycle (see review (Liu et al., 2011)).

The sexual stages of *Plasmodium* species are named gametocytes, gametes and zygotes. Their development is not yet completely understood. It is known that there are important differences between different *Plasmodium* species in certain aspects of the formation of the sexual stages. Examples are the time of commitment to develop into gametocytes, the length of the maturation and the morphology of the gametocytes. Comparing the most deadly human parasite strain *P. falciparum* with the mouse model parasite strain *P. berghei*, which this study will be employing, shows important differences between the two species.

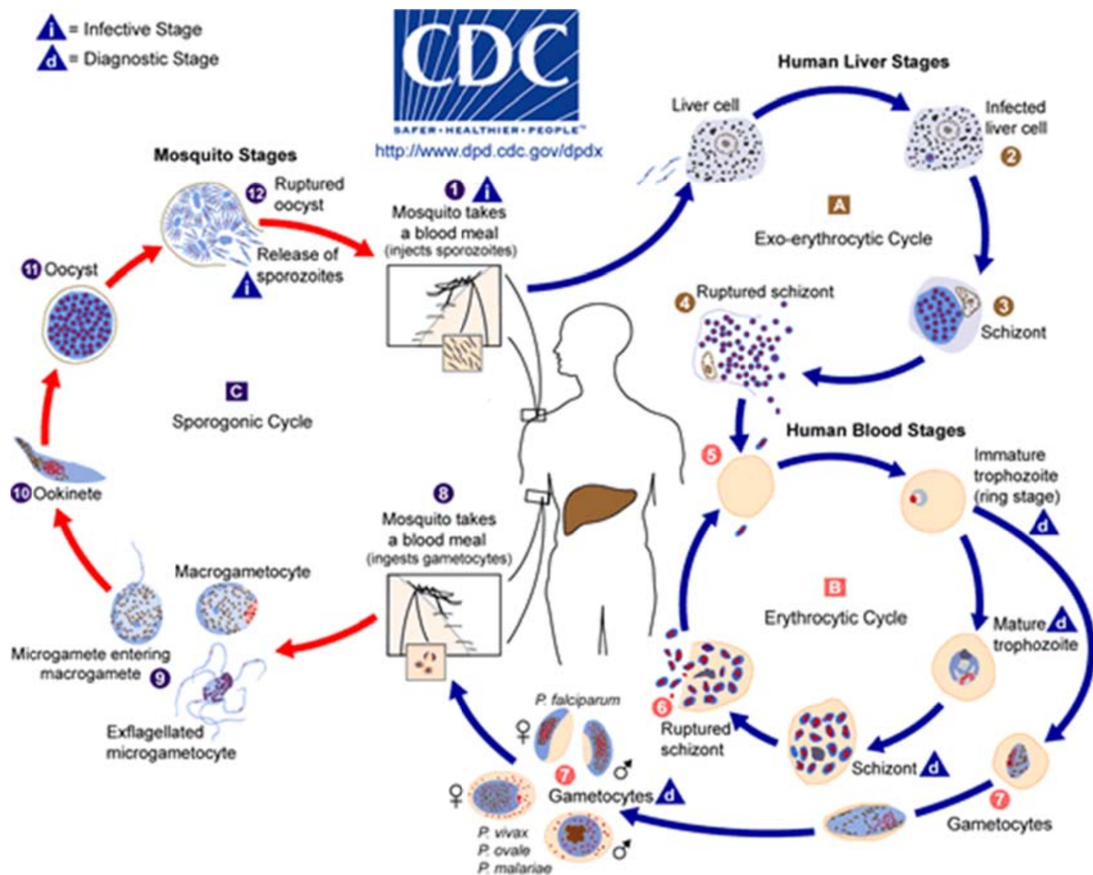


Figure 1.2. The human Plasmodium life cycle

The disease is asymptomatic in the liver stages, with onset in the blood of the patient. The mosquito is not affected by any symptoms upon infection with the parasite. But it is known that high parasite ingestion can lead to vast gut epithelial cell damage due to simultaneous traversal of higher numbers of formed ookinetes (Vlachou et al., 2004) (Taken from: http://dpd.cdc.gov/dpdx/html/Malaria.htm)

1.4.1. Differences between *P. falciparum* and *P. berghei* gametocytes

In general the fraction of sexual stages compared to the asexual stages is very small, in the order of a few percent. In infected humans it has been seen that gametocytaemia of *P. falciparum* peaks just after the immunological response to the asexual replication and release of malarial toxins, as this produces a 'stressful environment' in the patient's blood (Smalley and Brown, 1981). *In vitro* culturing of this parasite results in gametocyte formation if stress is triggered in the culture such as a lowered haematocrit or addition of host hormones (Carter and Miller, 1979, Lingnau et al., 1993). In the rodent *P. berghei* gametocytes are produced alongside the asexual stages, although the number of gametocytes has been suggested to be increased by inducing anaemia in the mouse using the drug phenylhydrazine (Landau, 1978). This leads to an increased erythropoiesis, which favours the development of gametocytes due to the preference of their precursors for invasion of reticulocytes. In general several reports have shown a preference of both *P. falciparum* and *P. berghei* for invasion into reticulocytes (Cromer et al., 2006, McNally et al., 1992, Trager and Gill, 1992, Trager et al., 1999). The asexual forms of the parasite commit to develop into gametocytes at some point in the blood cycle. In *P. falciparum* this probably occurs within the trophozoite from the previous cycle, as reports suggested it to be prior to schizont maturation (Silvestrini et al., 2000). Therefore the invading merozoite is already committed to developing into a gametocyte. This is supported by the fact that all merozoites developing from the committed schizonts will be of the same sex (Silvestrini et al., 2000, Smith et al., 2000, Talman et al., 2004). In *P. berghei*, this commitment is less well understood, although it has been suggested that it occurs after invasion of the erythrocyte in which the gametocyte will develop. Probably a switch from the asexual to the sexual cycle occurs during the trophozoite stage (Mons, 1986). The time of maturation varies greatly, with 8 - 12 days in the human parasite, while in the rodent parasite it takes about 26 - 30 hours (hrs). This time difference is reflected in the life span of those gametocytes. *P. falciparum* gametocytes circulate in the blood stream of their host on average for two to four days, although there

have been reports detecting their presence in the bloodstream for several weeks (Smalley and Sinden, 1977). A shorter lifespan is found for the gametocytes of *P. berghei*, which degenerate around 24 - 30 hrs after maturation. The morphology of mature gametocytes differs between the two species, being crescent shaped in *P. falciparum* and round to oval in *P. berghei.* Sexual dimorphism is apparent in both species just before full maturation.

1.4.2. Gametogenesis

Plasmodium gametocytes in the mammalian host are blocked in their cell cycle and remain inactive circulating in the blood stream or sequestering in the bone marrow and spleen in the case of *P. falciparum* (Tiburcio et al., 2012). Activation leads to the formation of mature gametes and occurs once they are taken up by the mosquito vector. Activation is due to mosquito factors such as xanthurenic acid and a drop in temperature from the warm vertebrate host to the ectotherm insect midgut (Billker et al., 1998). Gametogenesis can be initiated *in vitro* by changing the pH, decreasing the temperature and adding xanthurenic acid to the medium (Billker et al., 1998, Billker et al., 1997, Billker et al., 2000). Activation induces reprogramming of key cellular functions as well as egress of the parasite from the RBC and its PV. Egress is the escape of the parasite from its surrounding membranes that have previously protected the parasite from detection by the hosts' immune system as well as providing nutrients (see review (Blackman, 2008)). First, rupture of the PVM takes places within the first minute after activation by rupture (Sologub et al., 2011) (Deligianni and Morgan et al., 2013). The erythrocyte membrane (EM) is disrupted some minutes after. The female is now competent to be fertilised. The male on the other hand, undergoes a rapid series of events leading to the so called exflagellation. During the 10 minutes following activation, eight axonemes are assembled; there are three rounds of DNA replication, resulting in the formation of eight flagellated gametes (for review see (Sinden et al., 2010)) (Janse et al., 1986a, Sinden et al., 1976). The resulting flagellar gametes are highly motile and exit the residual gametocyte. Upon

formation of the haploid, mature gametes in the mosquito blood meal, the male and female gametes fuse to form a diploid zygote (Billker et al., 2004). These processes are still poorly understood on the molecular level.

1.4.3. Proteins regulating gametogenesis

A number of proteins have been implicated in playing a role in gametogenesis in *P. berghei*. The function of these proteins is usually assessed by generating knock-out mutants and analysing their phenotype.

MDV-1/PEG3 (male developmet-1/protein of early gametocyte3) is a protein found in both male and female gametocytes, although more abundant in the latter. It is associated with osmiophillic bodies, which are electron dense granules predominantly found in females (Ponnudurai et al., 1986, Sinden, 1982). The protein has been shown to be important for the egress of gametocytes. Mutants of both sexes lacking this protein are blocked in normal egress as both the erythrocyte membrane and the PVM persist (Ponzi et al., 2009). In the males flagellated gametes could be seen beating inside the host cell, thus the flagella were not affected in their motility. Egress in the female was also severely diminished. Zygote formation was not completely abolished as ookinetes were still observed, albeit with an 86% reduction (Lal et al., 2009). Oocyst numbers were reduced to 10% of that of the wt, but the mutants were transmitted normally to naïve mice. This suggests that there is no function of this protein in the later mosquito stages. Gametocytes devoid of the ortholog in *P. falciparum*, *Pfmdv-1/peg3*, were also arrested in their development (Furuya et al., 2005, Silvestrini et al., 2005).

A similar phenotype in egress was observed upon disruption of the PbGEST protein (gamete egress and sporozoite traversal) (Talman et al., 2011). Mutant gametocytes of both sexes were unable to egress from the RBC and the PV. Exflagellation of the male gametes took place within the erythrocyte, but

differently from the *mdv-1/peg3* mutant, the individual male gametes could not be distinguished from each other and appeared to be bundled together. Furthermore,

a second phenotype during the sporozoite stage was observed. The mutant sporozoites had a defect in traversing the host's skin cells after a mosquito bite. This resulted in a delay of the prepatency period, i.e. the time for an infection to be established in the mouse.

Another protein implicated in gametocyte egress is pplp2, *Plasmodium* perforin-like protein (Deligianni and Morgan et al., 2013). This perforin-like protein displayed a similar phenotype to *ΔPbGEST* during exflagellation. Although motile flagellated gametes were formed, they were not properly separated and were seen beating together in a bundle. While the *ΔPbGEST* mutants were trapped within the PVM, the Δ*pplp2* mutants were blocked in the rupture of the RBC membrane. In the Δ*pplp2* mutant ookinete and oocyst numbers were reduced by more than 75% compared to the wild-type. The mosquitoes infected with the mutant were fully capable of transmitting the parasites back into naïve mice. Taken together, the data indicate that pplp2 only functions during male exflagellation.

cdpk4 is a calcium-dependent protein kinase which has been implicated in gametogenesis (Billker et al., 2004). It has a function downstream of a calcium signal induced by xanthurenic acid during gametogenesis. It has been found to be an important regulator of cell-cycle progression in the male gametocyte. In the deletion mutant there was a block in nuclear division of the male gametocyte and no axonemes were assembled. Female gametocytes were not affected.

Two other proteins essential for the formation of male gametes are cell-division cycle protein 20, cdc20, and mitogen-activated protein kinase 2, map2. Mutants lacking either of these proteins have a similar phenotype (Guttery et al., 2012a, Tewari et al., 2005). In contrast to *Δcdpk4*, which has a defect in DNA replication within male gametocytes, DNA replication in these two mutants is not affected suggesting *cdc20* and *map2* act downstream of *cdpk4*. In these two mutants exflagellation does not occur due to defects in axoneme motility, chromatin

condensation and a lack of cytokinesis. Again, the function of these proteins is restricted to the males.

A protein that has been implicated in playing a vital role in male gamete formation is α-tubulin II (Kooij et al., 2005). α-tubulin II is an essential component of the microtubules of the axonemes of the male gametes. The transcription of the gene is strongly upregulated during gametogenesis in the male. However, it is also found in the asexual stages, hence disruption experiments were unsuccessful.

Another gene that has been implicated in gamete development is encoding the SET protein, suggested to be involved in chromatin dynamics (Pace et al., 2006). This protein is also found in the asexual stages of the parasite, but the expression of the gene product in the sexual and asexual stages is regulated by different promoters. The distal male gametocyte-specific promoter and the proximal asexual promoter are partly located on separate genomic regions after one another, but also share 200 bp (base pairs) directly upstream of the coding region. During male gametocyte formation, the distal promoter is active, leading to the formation of an mRNA with an extension in the 5'-UTR (untranslated region) compared to the asexual mRNA; however, the encoded protein is identical in the two cases. These two promoters are the only well-studied promoter regions in *P. berghei* (Pace et al., 1998). Differently sized transcripts were also found in the *P. falciparum* homolog, but whether this is due to similar promoter regulation remains to be elucidated.

1.5. The mosquito stages

1.5.1 The invasive ookinete

Once the parasite is taken up by the mosquito in its blood meal, it experiences a completely different environment than in the warm-blooded mammal. The parasite therefore needs to adapt quickly, which only the sexual stages are capable of. The asexual stages perish with the digestion of the blood bolus. When the

diploid zygote has been formed within the mosquito blood meal, it undergoes meiosis and develops within the next 17 - 24 hrs into the tetraploid, banana-shaped ookinete (Janse et al., 1986b, Sinden and Hartley, 1985) (Figure 1.3). Ookinete size is between 10 and 12 μm in length and about 2 μm in width (Canning and Sinden, 1973). They are motile and move at a speed of about 5 μm min^{-1} with three different forms of movement, stationary rotation, translocational spiralling and straight-segment motility (Vlachou et al., 2004, Moon et al., 2009). Once the ookinete has developed, it glides to reach the midgut epithelium and starts its invasion process. It traverses the midgut epithelial cells causing apoptosis (Vlachou et al., 2004, Rodrigues et al., 2012). Once the ookinete has successfully traversed the midgut, it attaches itself to the basal lamina of the midgut epithelium and starts rounding up to form an oocyst.

Various gene deletions have been described resulting in decreased numbers of ookinetes, although these are still competent to form healthy oocysts in most cases. In some mutant parasites a defect in ookinetes however, results in no oocysts, suggesting a defect in motility or cell traversal of the midgut. One mutant is *ΔPbsdha* that has the flavoprotein subunit of complex II deleted, which is important for succinate dehydrogenase activity in the mitochondria (Hino et al., 2012). The phenotype is represented by a decreased number of ookinetes and a transmission block at the oocyst stage, as no oocyst were ever detected, suggesting that mitochondrial oxidative phosphorylation is required for ATP metabolism during oocyst formation. It seems that the glycolysis energy metabolism in the mammal switches oxidative phosphorylation in the insect vector. Mutants of the perforin-like protein pplp5, expressed only in the ookinete stages, and MAOP, membrane attack ookinete protein, form normal ookinetes, but they are unable to traverse the midgut as they cannot invade the epithelial cells, resulting in a transmission block in the mosquito (Ecker et al., 2007) (Kadota et al., 2004). These ookinetes are most likely unable to destabilise the apical membrane of the epithelial cells. If invasion of the epithelium is circumvented by experimental injection of these ookinetes into the haemocoel, oocysts are formed normally.

Several proteins which have a role in motility, have been shown to be essential for invasion of the midgut epithelium. When the gene encoding *cdpk3* (calcium dependent protein kinase 3) was deleted, ookinetes were formed, but their motility was severely impaired (Siden-Kiamos et al., 2006). This resulted in very few oocysts being formed. Myosin A is an unconventional myosin, which is essential in the parasite (Siden-Kiamos et al., 2011). A conditional knock-out of the gene encoding the protein resulted in ookinetes being completely blocked in motility, and as a result no oocysts were formed. Another protein believed to play a direct role in motility is the circumsporozoite and TRAP-related protein (*CTRP*) (Dessens et al., 1999, 2003, Kadota et al., 2004). The deletion of *CTRP* also leads to a defect in ookinete motility resulting in the ookinete not being able to invade the midgut epithelium *in vivo*, and this leads to a complete block in transmission. In each of these cases oocyst formation could be rescued by injecting the mutant ookinetes directly into the haemocoel, proving that these proteins only function during the invasion of the epithelium by the ookinete.

Another protein that has been shown to have a role in ookinete cell traversal is SOAP (secreted ookinete adhesive protein) ((Dessens et al., 1999, Dessens et al., 2003, Kadota et al., 2004). The function of SOAP is not clear, but the protein has been shown to be associated with the laminin of *Anopheles*, possibly aiding the ability to invade the midgut epithelium (Mahairaki et al., 2005). However, the mutant is able to form oocysts in an *in vitro* system, which suggests the view that its role is limited to the invasion of the epithelium (Nacer et al., 2008a).

Another protein important for midgut invasion is cell – traversal protein for ookinetes and sporozoites (CelTOS) (Kariu et al., 2006). Mutants lacking this protein have a 200-fold reduction of the number of oocysts formed. The mutant ookinetes are motile and able to invade the epithelial cells. However, they cannot migrate through the cytoplasm of the cells and become trapped intracellularly.

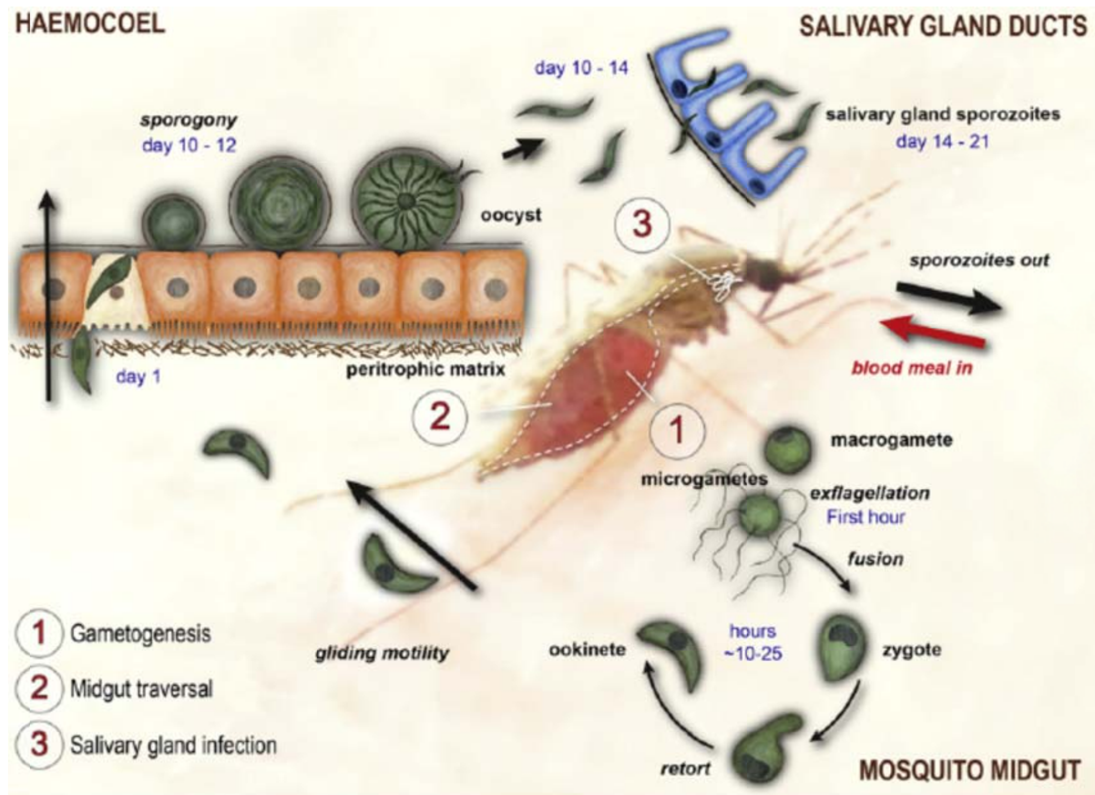


Figure 1.3. The Plasmodium mosquito life cycle

1) Gametogenesis occurs upon activation in the midgut as soon as the female mosquito feeds on a host. The motile, invasive ookinete is formed within the next 24 hrs. 2) The ookinetes invade the midgut epithelium, attach to the basal lamina and transform into oocysts. Within the next two weeks the oocysts increase in volume and sporozoites are formed, which egress and migrate to the salivary gland. 3) The salivary glands remain infected for the rest of the mosquito's life and with every bite sporozoites are injected into the skin of the victim causing a new infection. (Angrisano et al., 2012, Zieler and Dvorak, 2000)

1.5.2 The extracellular oocyst

In the mosquito vector occurs the longest and only extracellular stage of the plasmodium life cycle, the oocyst. When the ookinete has successfully migrated through the midgut epithelium, it attaches itself to the basal side of the midgut lamina and rounds up, developing into an early oocyst (Figure 1.4).

The developing oocyst increases in the size of its capsule and multiple rounds of mitotic divisions result in thousands of nuclei for sporozoite formation. The capsule is made up of mainly mosquito laminin that forms a thick outer layer, where a parasite protein named Cap380 (oocyst capsule protein) is found, as well as an inner oocyst membrane (Nacer et al., 2008b, Srinivasan et al., 2008). A size of 50 - 70 μm diameter is reached by the time the oocyst has come the end of capsule enlargement by day 12 post attachment (p.a.) to the *A. gambiae* midgut epithelial cells (pers. observations). Internal development still continues for another day or two to finish sporozoite formation. Halfway through oocyst development sporogony begins with the budding off of sporoblasts. Sporoblasts are syncytical lobes made out of oocyst plasma membrane invaginations, each with a nucleus that has been produced by multiple mitotic divisions.

A number of mutants have been described where the development of the oocyst is abnormal. A mutant lacking glutathione reductase, which is a component of the glutathione redox system, has been shown to develop into smaller oocysts, which reach a maximum size comparable to the size of the wt oocysts at day 6 - 8 p.a.. No sporozoites are formed in this mutant (Pastrana-Mena et al., 2010).

PbCHT1, a chitinase, leads to a 30 - 90% reduction in oocyst formation, although the traversal of the ookinete is not affected (Dessens et al., 2001). Knockouts of *psop2*, *psop7* and *psop9* (putative secreted ookinete protein) showed normal ookinete formation, but the number of oocysts formed were significantly reduced (Ecker et al., 2008). These three proteins are not related to each other and their function is unknown, although all three are suggested to be secreted from the ookinete.

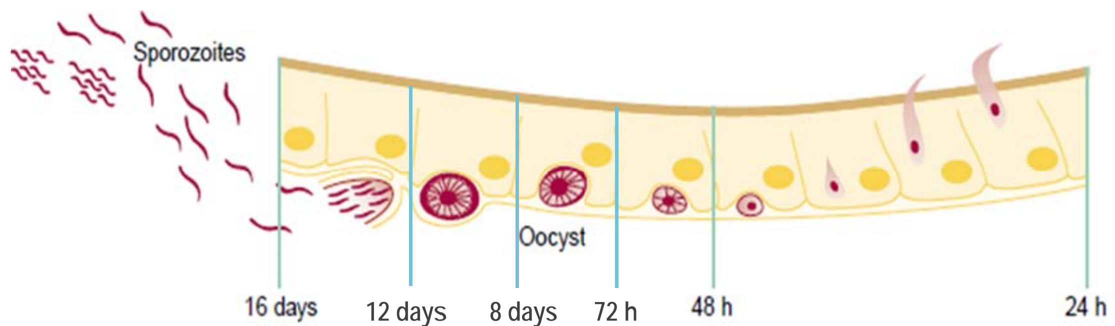


Figure 1.4. Oocyst development in the basal lamina of the mosquito midgut

The ookinete traverses the midgut within 24 hrs. It attaches itself and starts to round up within the next 24 hrs. From now on the oocyst only increases in volume until release of the sporozoites. At day 3 p.a. the oocyst is termed early oocyst, at day 8 mid-stage oocyst and at day 12 mature. From day 12 onwards sporozoites can be observed under the light microscope. From day 14 - 16 the oocysts burst, releasing the sporozoites into the haemolymph, from where they migrate to the salivary glands. (adapted from (Ghosh et al., 2003))

A recent study has shown that deletion of three kinases had a dramatic effect on sporozoite formation in the oocyst (Tewari et al., 2010). Mutants of the genes *pk7* (protein kinase 7), *gak* (G-associated kinase) and *cdlk* (CDPK-like kinase) do not form sporozoites for different reasons. Δ*pk7* oocysts stopped developing around day 10 p.a. and did not form sporoblasts, while Δ*gak* continued to grow to an unusually big size, but did not undergo sporogony. Lastly in the *Δcdlk* mutant initiation of sporoblast formation took place, but granulation occurred which eventually led to oocyst death after day 21 p.a..

After day 14 p.a. the sporozoites are released continuously into the haemocoel. It was previously thought that the pressure of the growing sporozoites leads to the subsequent rupture of the oocyst, however, it was shown that a protease termed egress cysteine protease 1 (ECP1) is involved in egress through its proteolytic activity (Aly and Matuschewski, 2005). The sporozoites migrate, most likely

passively, via the haemolymph into the salivary glands. However, only a fraction of the sporozoites actually arrive at their destination.

Members of the family of the LCCL/lectin adhesive-like proteins (LAP) have been shown to play an important role in the later mosquito stages. They have been extensively studied and in *P. berghei* a deletion of *Pblap1* resulted in normal oocyst numbers, however no sporozoites were produced (Claudianos et al., 2002). A more detailed study of *Pblap1*, *Pblap2*, *Pblap4* and *Pblap6* has shown that deletion of these genes results in abnormal oocyst formation, being either immature in their development compared to the wt or are vacuolated and degenerated (Raine et al., 2007). These genes have been shown to be inherited solely from the female, as the phenotype could only be rescued when the mutants were crossed with wt females. In *P. falciparum* Pflap1 and Pflap4 are required for sporozoite maturation, which is essential for their migration to the salivary glands and subsequent transmission to the mammalian host (Pradel et al., 2004).

A protein being expressed in the male gametocyte but having a defect in the ookinete to oocyst stage has been identified as a regulator in cell cycle progression (Bushell et al., 2009). *MISFIT* has been identified as the first male inherited gene important for the later progression from ookinetes to oocyst stages.

1.6. Transmission from vector to host

Sporozoites remain in the salivary glands until they are injected into the next host together with the mosquito anticoagulant saliva when the blood meal is taken up. The parasites enter the dermis and from there they migrate to blood vessels or the lymphatic system (Amino et al., 2006). Through the blood stream they reach the liver and invade hepatocytes, where each sporozoite gives rise to thousands RBC invading merozoites (see review (Kappe et al., 2003)).

1.7. Parasite-mosquito interactions

Only *Anopheles* mosquitoes can transmit mammalian *Plasmodium* species. Out of the approximately 400 species of *Anopheles* only about 40 are capable of this transmission. However, each parasite species is only able to infect certain mosquito species. Therefore parasite-vector interactions are crucial for determining the susceptibility of the mosquito to the parasite. There are various levels of susceptibility of the mosquito ranging from refractoriness to high levels of infection. Refractoriness of the mosquito is defined as the inability of the parasite infecting the mosquito to be transmitted back to a new mammalian host. However, in some cases parasites can develop in the mosquito, although infective sporozoites are not produced. Some mosquitoes are susceptible to one species of parasite and refractory to another. As an example, infections with *P. berghei* in an *A. gambiae* established laboratory mosquito strain resulted in an average of 67 oocysts in this particular study, while infections in parallel of *A. stephensi* mosquitoes resulted in an average of 362 oocysts per midgut (Kadota et al., 2004). Therefore *A. stephensi* is more susceptible to *P. berghei* infection than *A. gambiae*.

An example of the opposite scenario was the creation of a line of *A. gambiae* that was highly refractory to several parasite strains such as *P. cynomolgi, P. gallinaceum, P. berghei* and four other primate species (Collins et al., 1986). Infection with different strains of *P. falciparum*, however, showed extreme variation in that they were not only able to survive to different degrees in this strain, but also evading the mosquito immune response altogether.

1.8. The mosquito immune system

The mosquito immune system employs various responses to infectious agents, but does not possess antibodies or an adaptive immune system. These responses are either phagocytosis by haemocytes, production of effector molecules such as proteases and molecules having inflammatory or antimicrobial effects and melanisation. The latter defence mechanism is unique to arthropods. Melanisation

occurs mostly in pathogens that are thought to be too large to be phagocytosed. They are encapsulated with melanin which leads to the death of the pathogen. Death is due to the inability of the pathogen to take up nutrients. Reactive intermediate oxygen species produced during melanogenesis also aid in parasite death (Chen and Chen, 1995, Nappi and Christensen, 2005).

As the gametes develop within the blood bolus of the mosquito they are exposed both to the mosquito's digestive and innate immune system as well as to the ingested immune cells from the vertebrate blood, which they were previously protected from whilst residing in the RBC (Margos et al., 2001). Nitric oxide metabolism increases due to the uptake of haemoglobin in the midgut and further decreases ookinete development as it leads to formation of toxic metabolites (Luckhart et al., 1998, Peterson et al., 2007). This ability to increase the cellular and humoral response upon ingestion of the parasite is not always favourable though. Employing reactive oxygen species (ROS) is positive in that they can fight bacteria; a decrease will lead to a higher mosquito mortality rate (Molina-Cruz et al., 2008). But the further increase in ROS upon parasite ingestion can also lead to a higher mortality rate due to host cell oxidative damage. A correlation between high parasite load and increased mosquito death has also been shown (Dawes et al., 2009).

But it is not just the parasite or the blood intake which stimulates the mosquito immune system. Environmental factors can also play a big role such as temperature. It has been shown that a mosquito antimicrobial agent called *defensin* has increased expression at 18°C, while nitric oxide synthase is highest at 30°C (Murdock et al., 2012). In this study melanisation has been found to peak at 18°C.

1.9. Population bottlenecks of *Plasmodium spp*

Within the mosquito stages there are two significant population bottlenecks. The first is the difficult survival and formation into a healthy, mature ookinete. An increased inability to form healthy ookinetes has been observed with increasing parasite loads. In *P. falciparum* this conversion from gametes to oocysts *in vivo* ranges from 0.025% to 42%, while in *P. berghei* this *in vivo* conversion is lower with a maximum observed number of 1.3% (Sinden et al., 2007). *In vitro* this conversion rate is up to 80% (pers. observations). Natural apoptosis accounts for about 50% of the cell death of the developing ookinetes (Al-Olayan et al., 2002). The mosquito plays a great role in the death of the remaining ookinetes, as was described earlier. It has been shown that about 95% of the surviving ookinetes manage to escape the midgut, penetrating the peritrophic membrane and traversing the midgut epithelium (Han et al., 2000). Cell traversal is described as the second population bottleneck, as the development into oocysts is highly unsuccessful. Observations have shown that about 120 ookinetes are needed to produce one oocyst, while adjusting the parasite loads to the best possible scenario only improved this by production of one oocyst from 60 ookinetes (Sinden et al., 2007).

1.10. Actins

Actins are globular proteins found ubiquitously in all eukaryotic cells. They are responsible for important processes within the cell such as cell motility, cell division and muscle contraction. Globular or G-actins are single subunits, which dynamically assemble into filaments leading to polymers called filamentous actin or F-actin. The dynamics of F-actin formation have been studied extensively in other eukaryotic organisms. More than a hundred proteins have been identified in playing a role in regulating actin polymerisation. Many of these regulators of actin polymerisation are, however, missing within the apicomplexan phylum and only a few have been found through similarity searches *in silico*. Only linear filaments are suggested to be found within the apicomplexans as the Arp2/3 complex is missing completely, which is responsible for branched filament formation in other

organisms (Goley et al., 2010). Important actin-binding proteins which have been identified in *Apicomplexa* are the F-actin depolymerising factors ADF1 and ADF2. ADF1 is essential in the asexual blood stages, while ADF2 plays a role in ookinete to oocyst transformation and sporozoite to liver-invading forms (Doi et al., 2010) (Schuler et al., 2005). Profilin promotes actin polymerisation by increasing the concentration of G-actin. This protein is essential in the parasite (Kursula et al., 2008). The beta-subunit of the capping protein (CP) stabilises F-actin and has been shown to be important for locomotion of the mosquito stages (Ganter et al., 2009). Another protein involved in regulating microfilament turnover is the C-CAP protein (Cyclase associated proteins), whose knockout resulted in the loss of the sporozoite stages in the mosquito (Hliscs et al., 2010).

Apicomplexan parasites share a common unique way of locomotion, egress and invasion into host cells called gliding motility, as they do not possess flagella or cilia. It is a substrate dependent motion with no apparent change in cell morphology. The maximum speed is similar to an *in vitro* established actin/myosin motor with several microns per second (Schuler and Matuschewski, 2006). Experiments with the *Toxoplasma* parasite and host cell mutants have shown that actin filaments are required for gliding motility and invasion, as treatment with cytochalasin D, which disrupts filament formation, inhibited these (Dobrowolski and Sibley, 1996). It has also been shown that more than 97% of actin in *Plasmodium* is actually encountered in its monomeric state (Pinder et al., 1998). As an inability to move within the host, invade and egress from the host cell would render the parasite incapable of causing any clinical manifestation in either the mammal or infection in the *Anopheles* vector, it is of great interest to study actins in *Plasmodium.*

1.10.1 Actin II

Compared to other apicomplexan parasites, which encode one actin, *Plasmodium* species possess two genes encoding actin. *Actin I* (PBANKA_145930) is transcribed throughout the life cycle of the parasite and has no intron (Wesseling et al., 1988). In contrast, *actin II* (PBANKA_103010) has an intron, is transcribed at much lower levels, and only in the sexual stages. The two genes share 78% DNA sequence similarity, which is surprisingly low for actins (Morrissette and Sibley, 2002). The sequence diversity may imply significant differences of the functions of the two proteins, such as affinities of actin-binding proteins and the characteristics of F-actin polymerisation (Schmitz et al., 2005). Studies have shown that *Plasmodium* actin only forms very short filaments of about 100 nm in length *in vitro* and has a limited intrinsic ability to polymerise, whereas *Toxoplasma* actin required a 3 – 4-fold lower critical concentration of actin monomers to form filaments, albeit having similar length to *Plasmodium* (Schmitz et al., 2005) (Sahoo et al., 2006). Recently it was shown that actin II filaments can form *in vitro* (Skillman et al., 2011). Compared to actin I they appear curved when stabilised with higher levels of phalloidin, a toxin that binds to F-actin and prevents depolymerisation. The first description of actin II suggested that it is specifically expressed in gametocytes (Wesseling et al., 1988, Wesseling et al., 1989). Proteomic data identified the protein in *P. berghei* in male gametocytes, ookinetes and oocysts, but it was not detected in females or sporozoites (Hall et al., 2005). In *P. falciparum* and *P. yoelii* actin II has been found to be expressed in the sporozoites, but less abundant than actin I, 10 and 7- fold respectively (Lindner et al., 2013).

A detailed analysis of a disruptant of *actin II* in *P. berghei* revealed a defect in male gametogenesis (Deligianni et al., 2011). Male gametocytes were formed and after activation replicated their DNA and assembled axonemes with no detectable difference to the wt. However, the axonemes were immotile and the gametocytes did not egress from the host cell, as both the PVM and the EM were still found to be intact (Figure 1.5). Therefore these mutants were not capable of the typical

radial exflagellation process. On rare occasions exflagellations were observed and subsequent ookinete formation was decreased roughly 50-fold.

The females were not affected as they egressed normally and could be fertilised by wt males. A GFP-tagged (green fluorescent protein)copy of actin II showed the protein to be exclusively expressed in the males and only detected in the cytoplasm. This study only examined the role of actin II in the gametocyte and gamete stages. However, the possible function of this protein in the later mosquito stages has not been studied. This investigation aimed to elucidate this.

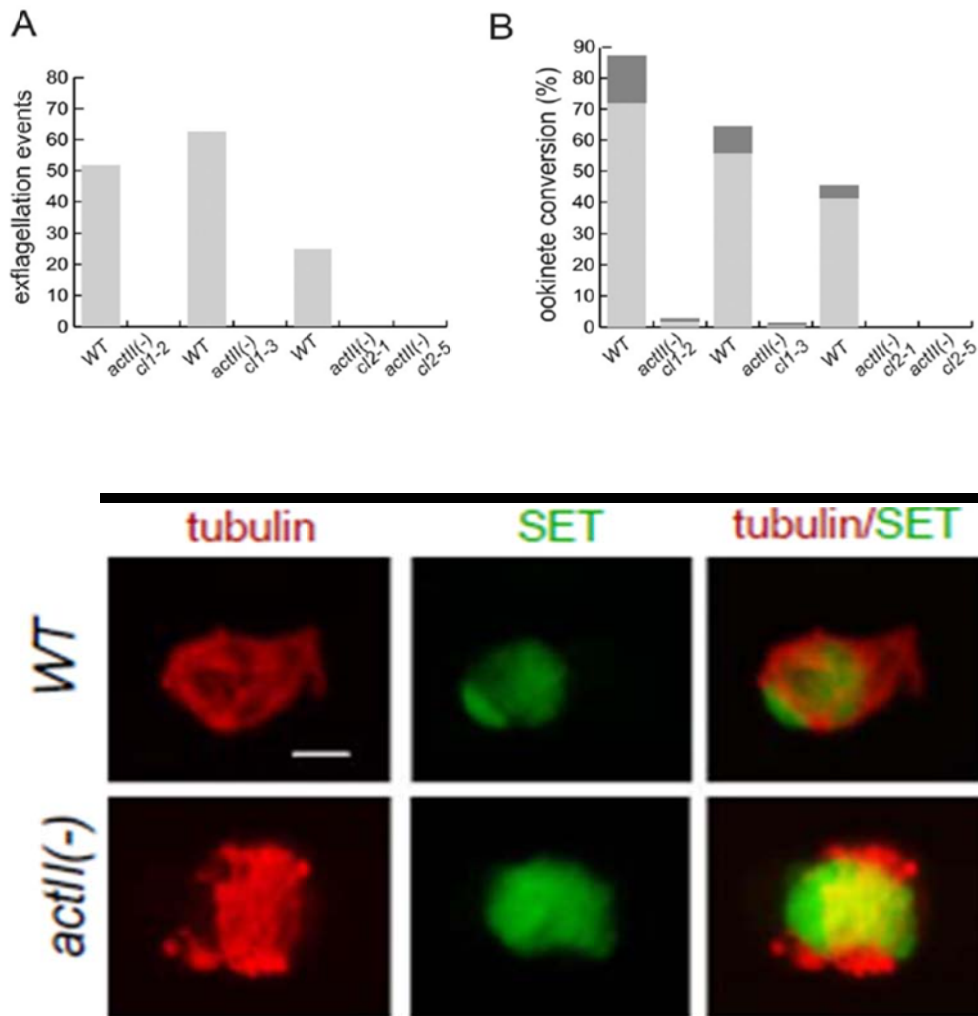


Figure 1.5. Observations from the actII(-) mutant

A) Exflagellation events for wt and *actII(-)*. Only two exflagellation events were observed in cl1-3. Results are normalised per 10 microscope fields B) Ookinete conversions of wt and *actII(-)*. Standard deviation is highlighted in darker colours C) Male gametocytes of the wt and the *actII(-)* were activated and after 15 mins fixed and labelled. The nucleus is labelled with the anti-SET (green) antibody, whereas the axonemes are labelled in red with the anti-tubulin antibody. The axonemes of the wt are beginning to disassociate from the nucleus, while axonemes are also present in the *actII(-)* mutant but they remain close to the nucleus (adapted from (Deligianni et al., 2011))

Objectives of this study

Actin II has been shown to be a very important player in male exflagellation and subsequent parasite transmission to the mosquito stages. In some cases *Plasmodium* proteins do not just serve one single purpose in one specific stage of the parasite life cycle. Determining whether actin II has a second function within the mosquito life cycle proved a challenge, as we needed to circumvent the problem of a lack of males for fertilisation.

As we do not know why only the *Plasmodium* parasite possesses two different kinds of actins, determining a function within the mosquito stages could help elucidate the role for this protein, as exflagellation also occurs within other apicomplexans, such as *Eimeria spp,* which do not possess actin II. There are fundamental differences between the apicomplexan species; however, whether e.g. special types of actin filaments are required to carry out *Plasmodium* specific processes is not known. So characterising the function of actin II further within the mosquito midgut stages could allow us to find specific pathways that only *Plasmodium* possesses to find targets for vaccines or other pharmaceuticals to help fight the parasite and therefore malaria, as resistance to current pharmaceuticals is on the rise.

Chapter 2

Expression studies in the mosquito stages

2.1 Actin II

Our laboratory carried out the first detailed analysis into this protein and its possible function in *P. berghei* (Deligianni et al., 2011). As previous reports only suggested it to be found in the sexual stages of this parasite strain, we first had to confirm this. Stage specific whole proteome studies found the protein to be expressed in the asexual blood stages, male gametocytes, ookinetes and oocyst stages in *P. berghei* (Hall et al., 2005).

The *actin II* gene is found on chromosome 10 in *P. berghei* (PBANKA_103010). It contains an intron 448 nucleotides downstream from the translation initiation start site. Actins across different species show high sequence similarity, generally over 90%. But *actin II* is divergent to *actin I* in *P. berghei*, with only 78% identity (Figure 2.1).

As our previous study was focused on the analysis of the protein in the blood stages, we wanted to investigate whether there is a second function in the mosquito midgut stages.

2.2 *actin II* transcriptional expression in blood stages

For RT-PCR (reverse transcription polymerase chain reaction) Primers were used which were specific for *actin II* and for transcripts present only in gametocytes, namely *SET* and *mdv-1/peg3,* for comparison (Pace et al., 2006; Ponzi et al., 2009). cDNA (complementary deoxyribonucleic acid) was synthesised from RNA (ribonucleic acid) extracted from infected blood of several parasite lines containing both asexual and sexual stages. The wt ANKA 2.34 strain, *actII(-)*, a non gametocyte producing strain called HPE, and a strain called ANKA 2.33, suggested to be also a non-gametocyte producer, were used in these experiments (Dearsly et al., 1990, Janse et al., 1989) (See appendix).

A transcript for *actin II* is detected in the wt and 2.33, but not in HPE (Figure 2.1A). The presence of transcripts of the *mdv-1/ peg3* and *SET* genes indicated that

gametocytes were present in all samples except the HPE. These data are contradictory to published data stating that 2.33 is devoid of gametocytes (Dearsly et al., 1990, Deligianni et al., 2011). A stained blood smear of the 2.33 strain showed parasites which appeared to be early gametocytes. Fully mature gametocytes, however, could not be detected. Gametocytes in *P. berghei* can first be detected morphologically as gametocytes around 16 hrs post infection (p.i.). The gametocytes in the 2.33 strain must be younger than 26 hrs p.i., as normally gametocytes are fully mature after 26 hrs. This explains why this strain was previously thought to be a non-gametocyte producer. Therefore these data show that *actin II* is in fact only transcribed in the sexual stages.

P. berghei parasites, different to *P. falciparum*, do not follow a uniform temporal cycle. However, injecting cultured schizonts results in a synchronous infection of the asexual stages for about two cycles. As gametocytes of different ages are also purified together with these schizonts, it has so far been very difficult to obtain a detailed temporal transcription pattern of genes encoding gametocyte specific proteins using PCR. As this contamination with mature gametocytes can be avoided with the 2.33 strain, it was used to follow gametocyte transcription.

In Figure 2.1B *mdv-1/peg3* appears to be transcribed during early gametocytes. *SET* and *actin II* are transcribed at higher levels at an early developmental state and downregulated thereafter.

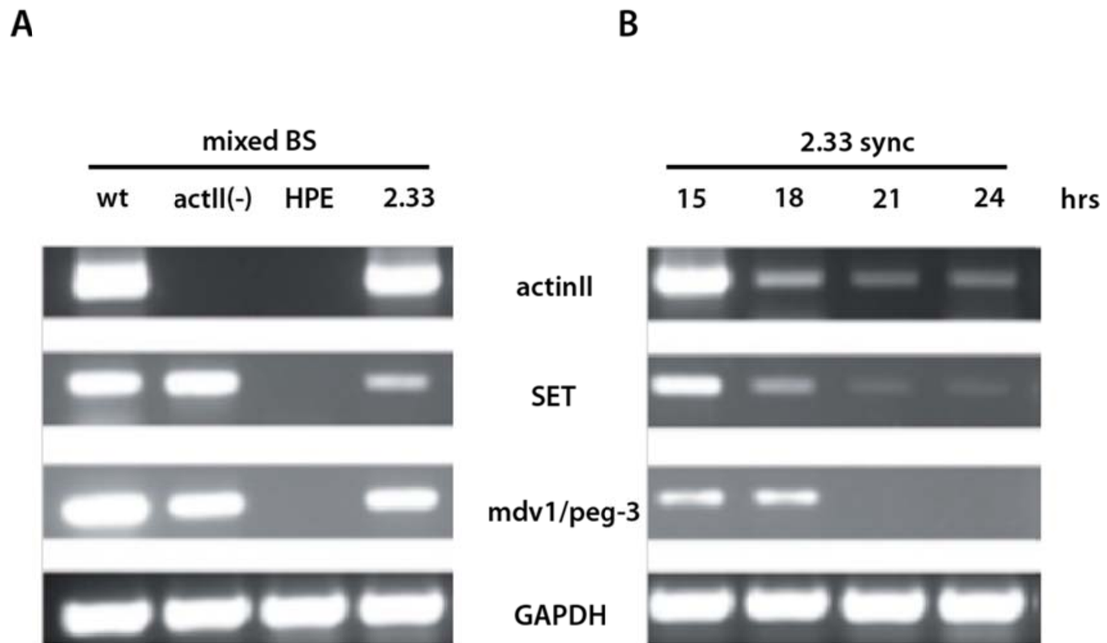


Figure 2.1. RT-PCR of gametocyte specific transcripts and actin II

A: mixed blood stage cDNA of wt, *actII(-)*, HPE and 2.33 reveal that *actin II* is only transcribed in the gametocytes. 2.33 was shown to produce early gametocytes. B: Synchronous infection of the 2.33 strain demonstrates a time dependent expression pattern of gametocyte specific transcripts. GAPDH (Glyceraldehyde 3-phosphate dehydrogenase) was used as a loading control as it is a housekeeping gene transcribed at relatively constant levels throughout the parasite life cycle.

2.3. *actin II* expression in mosquito stages

2.3.1. Transcription of *actin II* in the mosquito stages using reverse transcription PCR

RT-PCR was carried out with cDNA isolated from ookinete stages and oocysts of the wt. Figure 2.2 shows transcription in the mosquito stages to be limited only to ookinetes.

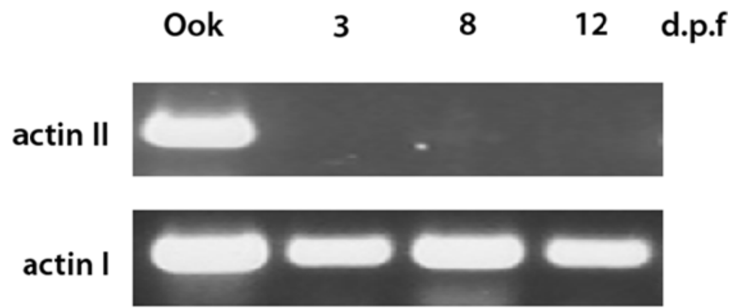


Figure 2.2. Reverse transcription PCR of mosquito midgut stages

actin II transcription is present in the ookinete stages, while completely absent in any of the oocyst stage samples tested. actin I was used as a loading control as it is a housekeeping gene, transcibed at relatively constant levels throughout the parasite life cycle. d.p.f: days post feeding.

2.3.2 actin II antibodies

No commercial antibodies are available against actin II, so peptide antibodies were ordered and tested for their specificity. Two antibodies were raised. One did not give a signal and was not used further (Figure 2.3).

DEAQ
DEAQ
****

RERM
REKM
** :*

MRLD
NRTD
* *

DIEK
EVEE
: : * :

GNIV
NNIV
***

MWIT
MWIT
****

Figure 2.3. Alignment of actin I and actin II

actin I and actin II are 78% identical, with 89% positives. The two peptides in boxes show the position of two antibodies raised against actin II. The second antibodies were not used further

Western blots with crude extracts of wt gametocytes, *actII(-)* gametocytes and wt ookinetes were incubated with the antibody and as shown below the antibody is not specific for actin II (Figure 2.4A). Figure 2.4B corroborates this, as actin II expressed from yeast is recognised, but a protein band in the HPE strain indicated a lack of specificity. Therefore we discontinued the use of this antibody. By this time our laboratory had produced another actin II antibody, which has been proven to be specific and all further western analysis as well as immunofluorescent assays were carried out with this antibody.

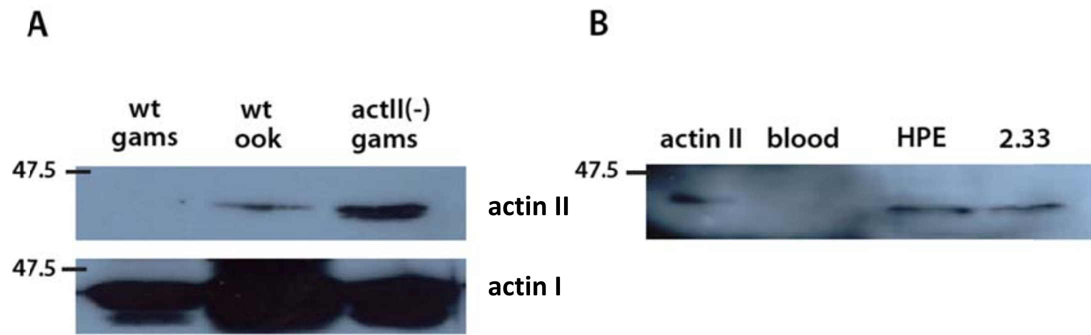


Figure 2.4. Western testing the specificity of the antibody against actin II

A: The antibody recognises a protein/s not present in wt gametocytes, weakly expressed in ookinetes and highly expressed in *actII(-)* gametocytes. B: Further tests confirming the lack of specificity as the blot shows actin II expression in the HPE sample. A band for actin II expressed in yeast suggests that the antibody recognises actin II as well, albeit not specifically. actin I was used as a loading control.

In order to establish what other proteins the antibody might recognise an alignment was analysed (Figure 2.5). The alignment shows that the peptide sequence used to produce the antibody is divergent among actins and actin related proteins. The ubiquitously expressed actin I is not recognised by this antibody, as no signal was detected in the sample of wt gametocytes, although this protein was the most similar to actin II. It could thus not be determined what this antibody recognises.

Figure 2.5. Alignment of actin or actin-related proteins in P. berghei

The boxed area shows the sequence of the peptide chosen as the antigen. There is a minimum of four amino-acid difference between actin II and any other protein.

2.3.3 actin II expression in ookinetes

As previously reported in the literature, expression levels of actin II are not comparable to actin I, which is expressed at higher levels in the parasite (Wesseling et al., 1988). A protocol for western analysis of the ookinete stages was sought for the protein level of actin II from different numbers of wt ookinetes. High numbers of ookinetes are required in order to clearly detect the protein (Figure 2.6). Based on these results $5*10^5$ ookinetes were chosen in the subsequent western analysis of ookinetes.

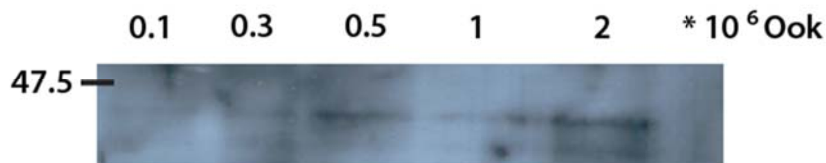


Figure 2.6. Western blot of actin II expression in wt ookinetes

An increasing amount of ookinetes was loaded and actin II expression was determined. This protein is not highly expressed as even at the highest concentration the band is barely visible.

2.4 actin II plays a role in the mosquito stages

The *actII(-)* mutant produces 50-fold less ookinetes than the wt, due to its impairment in exflagellation (Deligianni et al,. 2011). We then carried out dissections of mosquitoes fed on these two strains to count the oocyst load. Three experiments were pooled and no oocysts were ever detected in the *actII(-)*(Figure 2.7). This suggested that the *actII(-)* mutant does not survive to the oocyst stages.

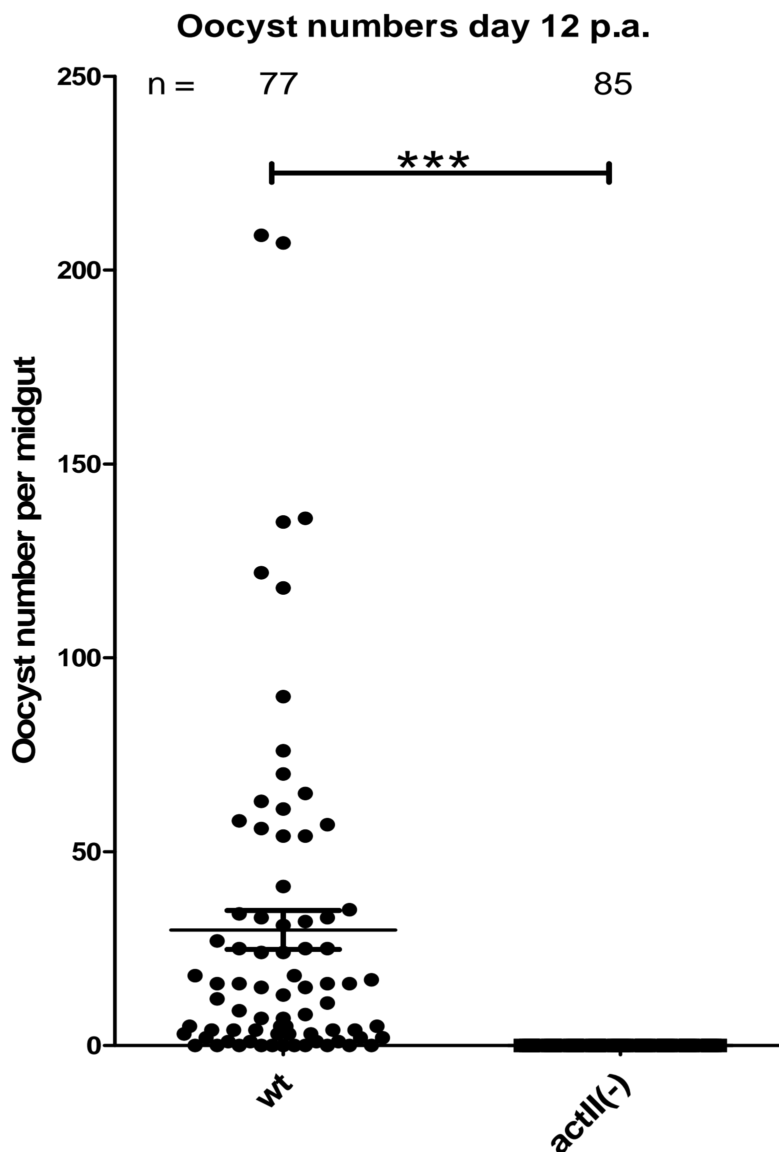


Figure 2.7. Oocyst counts of wt and actII(-)

The wt has an oocyst count across a wide range. The *actII(-)* is completely devoid of oocysts. Oocysts were counted 12 days post attachment (p.a.). (***=p<0.001 using unpaired t test))

To investigate this further we decided to perform genetic crosses of the *actII(-)* mutant with the wt to rescue the mutant so that ookinetes were formed. We could then follow this parasite population through the mosquito cycle. The resulting population consisted of heterozygous ookinetes resulting from the male wt and female *actII(-)* and homozygous wt/wt ookinetes. The two parasite strains were injected in the same animal. After three days blood samples were taken and the mouse fed to mosquitoes. An ookinete sample was taken from an *in vitro* culture and several time points were investigated of the oocyst and the sporozoite stage. The presence of the two parasite strains was determined by genomic PCR with primers specific for each genotype. As can be seen in Figure 2.8 both strains are present at the time of feeding and heterozygous ookinetes are formed. At the oocyst time point of 3 days post feeding (d.p.f.) the *actII(-)* genotype could still be detected. In all subsequent time points during mosquito development the *actII(-)* parasites were not present.

Figure 2.8 Genetic cross of wt and actII(-)

A: Crossing the wt and *actII(-)* leads to a mixed ookinete population. Homozygous wt ookinetes and heterozygous wt (from the male) and *actII(-)* (from the female) ookinetes B: Genotyping of genomic DNA isolated at various time points. BS: blood stages, O: ookinetes; 3 and 8 day oocysts from dissected midguts; Sp: sporozoites. The wt is present in all stages, whereas the *actII(-)* parasite is absent after day 3 C: The mosquitoes were allowed to feed on naïve mice and genomic DNA extracted from the infection. The PCR shows that only the wt genotype was present.

2.5. Discussion

We wanted to investigate transcriptional expression of this second actin gene actin II and whether it has a further function in the mosquito stages. Many proteins have more than one function in addition to their expression in the sexual stages. The already mentioned SET-protein functions both in the asexual stages as well as in the male gametocyte. α-tubulin II is essential in the asexual stages and highly expressed in the male gametocyte. This suggests that these proteins are required during the rapid transformations in exflagellation (Kooij et al., 2005, Pace et al., 2006). These studies have shown increased promoter activity in the male for *α-tubulin II*, while *SET* has a separate promoter specific for the male gametocyte. Transcriptional data from mixed blood stages and synchronised infections shows that in the blood stages *actin II* is exclusively transcribed in the sexual stages (Figure 2.1). In *P. berghei* early gametocytes can be visually distinguished from around 16 hrs p.i., with their commitment to becoming gametocytes only occurring around 4 hrs prior (Mons, 1986). As *actin II* is only transcribed in the sexual stages at an early time point, it could be used as an early gametocyte marker. When the *actII(-)* is fed to mosquitoes it is unable to form oocysts, despite the ability to form 50-fold less ookinetes compared to the wt. *Δpsop2* mutants also have decreased number of ookinetes formed, but are still capable of forming a reduced number of oocysts (Ecker et al., 2008). This suggests a second role for actin II after the ookinetes are formed. Currently most published data on loss of ookinete proteins shows normal wt ookinete numbers, but a subsequent decrease in the formation of oocysts due to motility, invasion or midgut traversal defects (Dessens et al., 2001, Dessens et al., 2003, Ecker et al., 2008, Ecker et al., 2007, Siden-Kiamos et al., 2006). In some cases oocyst formation is completely abolished due to ookinete defects (Dessens et al., 1999, Kadota et al., 2004, Kariu et al., 2006, Hino et al., 2012, Guttery et al., 2012b, Siden-Kiamos et al., 2011).

To verify a second function for *actin II* cross-fertilisation experiments were carried out which would allow the mutant parasite to pass the blockage at the gamete level. Figure 2.8B tested the presence of the *actII(-)* mutant population from

heterozygous ookinetes. This PCR confirms a loss of the mutant parasite pre- to mid-oocyst development. This opened up the question at which stage the defect occurred as there were several possibilities. Dissection of three day old fed mosquitoes sometimes contained midguts which hadn't completely digested the blood bolus, and the mutant parasite DNA could have been from residual undigested parasites. It could also be that the heterozygous ookinetes were not motile, thus not traversing the midgut or that they cannot attach themselves to the basal lamina of the midgut to round up to an oocyst. However, as the mosquito midgut is a very hostile environment for the parasite to reside and initial digestion occurs fairly quickly, it was more likely that the parasite DNA detected was due to early oocysts, which did not develop further (Whitten et al., 2006). This posed the next problem of how to distinguish when the defect occurred. As the wt/*actII(-)* ookinetes possess only one copy of the endogenous gene allele, it was clear that expression from this gene was not sufficient to rescue our mutant to the mammalian blood stages. Unfortunately, this genetic cross did not allow any further analysis as the initially formed heterozygous oocysts cannot be distinguished from the wt homozygous oocysts.

Chapter 3

Promoter exchanges attempt to alter actin II expression

3.1. Promoter-exchange constructs

A common strategy to change expression levels of proteins is to exchange their promoter. Driving *actin II* under a promoter that is active in the male and then active or inactive at various other stages would allow us to analyse the second function further. Several promoters were chosen according to these requirements. α-*tubulin II* is highly transcribed in the microgamete and important for axoneme formation (Kooij et al., 2005). It is not only active in the male, but also in the female (6-folds higher in the male), ookinete, oocysts and at low levels in the asexual blood stages. *mdv-1/peg3* is only transcribed in the gametocyte stage, although in higher levels in females than in males (Ponzi et al., 2009). *cdpk4*, a calcium dependent kinase is expressed in male gametocytes. (Billker *et al*., 2004). These promoters have not been defined and a region ~1.3 kb (kilobase) upstream of the transcription initiation site was chosen for the exchange. *SET* is a conserved nuclear protein implicated in chromatin dynamics (Pace et al., 2006). It has two well defined distinct promoters of which one is exclusively active within the male gametocyte. The protein is found also in asexual stages; this expression is regulated by the other promoter.

3.2. Transfection of four promoter swap constructs

The constructs were each transfected into wt *P. berghei* and cloned by limiting dilution (Figure 3.1). The clones were then analysed by genomic PCR to test for wt contamination. All constructs were successfully transfected into the recipient strain (Table 3.1). For two of the constructs no clone was obtained; the α-*tubulin II* construct was attempted five times and the *mdv-1/peg3* construct was attempted six times. Two mutant constructs were successfully cloned and named *setp-actII* and *cdpk4p-actII*.

Figure 3.1. Construct design

The construct was stably integrated by double crossover. The 5′ FR region is where each of the different promoter regions were inserted. The tgDHFR (*Toxoplasma gondii* dihydrofolate reductase)cassette confers resistance to pyrimethamine and is used to select for successful transfectants.

Promoter	Transfection	Cloning	Line	Name	Gene ID
α-tubulin II	✓	**x5**	**No**	**-**	PBANKA_052270
cdpk4	✓	**x1**	**Yes**	***cdpk4p-actII***	PBANKA_061520
mdv-1/peg3	✓	**x6**	**No**	**-**	PBANKA_143220
SET	✓	**x1**	**Yes**	***setp-actII***	PBANKA_081990

Table 3.1. Construct progress

Of the four constructs transfected only two were successful in their cloning to establish a mutant parasite line.

3.3. Analysis of *setp-actII*

Two clones were obtained from the cloning experiment of the *setp-actII* mutant. In the first clone analysed a very severe growth phenotype was detected within its asexual stages. It took three to four times the usual cycle time to obtain a population with a gametocytaemia level usable for further analysis, meaning 14 - 20 days of *in vivo* culture, comparing to the wt having an average culture time of four to six days. The mutant was able to exflagellate within the normal time frame of 10 - 15 minutes. Ookinetes were formed at a conversion rate comparable to the wt (Figure 3.2). Mosquitoes were then allowed to feed on an infected mouse in order to establish infectivity of these ookinetes. When this parasite line was fed to mosquitoes no oocyst developed (Figure 3.3).

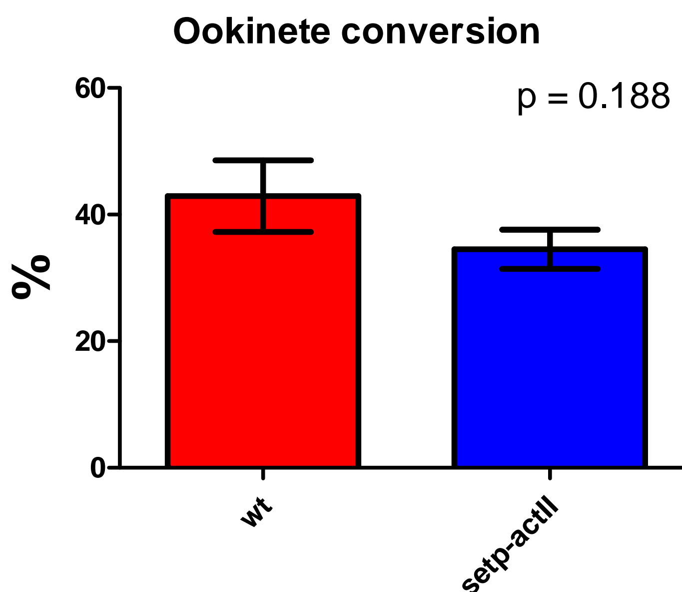


Figure 3.2. Ookinete conversions of the wt and the setp-actII mutant parasite

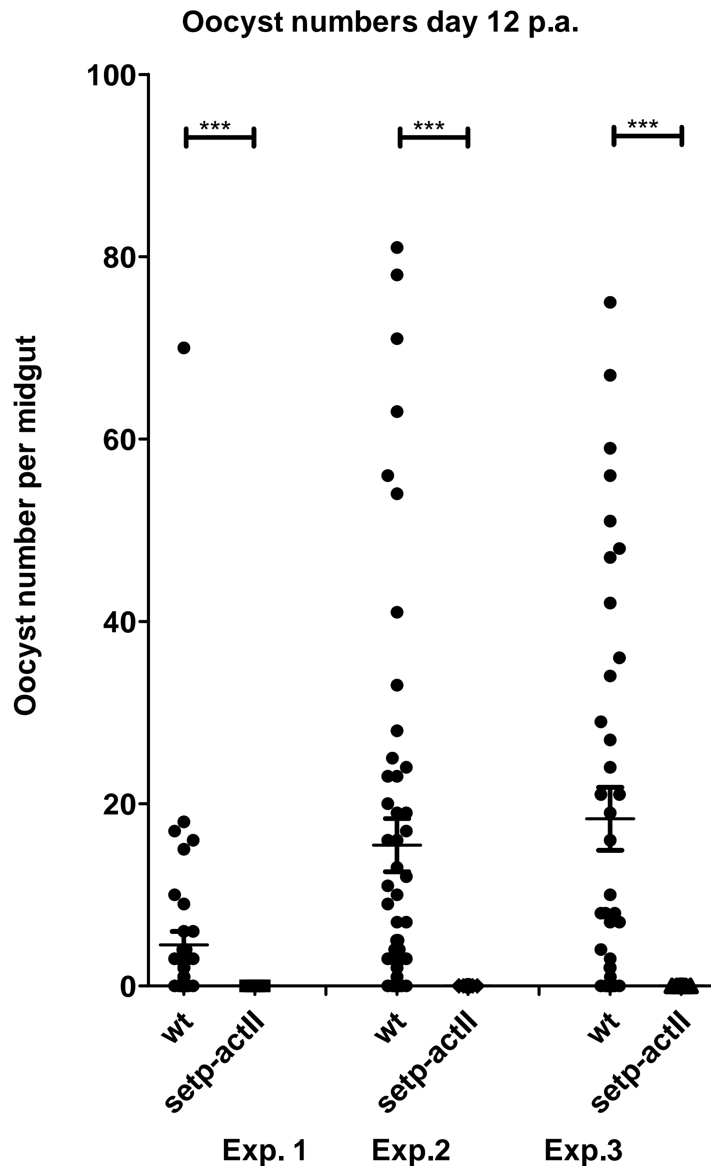


Figure 3.3. Oocyst counts of the setp-actII mutant

No oocysts were observed. *setp-actII* (n= 146; wt n= 143). (***: $p<0.001$ using unpaired t test)

We then tried to determine the defect in the mosquito stages of this mutant. To investigate if these ookinetes were motile, Mos20 cells were added to a purified ookinete culture. The Mos20 cells will stimulate ookinete motility. This experiment revealed that the *setp-actII* ookinetes were indeed impaired in their movement. They were adhering to each other, erythrocytes, the Mos20 cells and/or the glass

slide (Figure 3.4). However, it did appear that these ookinetes were motile, as forward movements, dragging another cell behind, or twirling could be observed. The mutant ookinetes seemed to be connected by elastic structures mainly from the basal side of the ookinete. The ookinetes that displayed actual motion kept being retracted to their connection entity.

Due to the unusual side effect in the blood stage growth it was not clear whether these defects in the mosquito stages were because *actin II* was differently expressed due to the promoter swap or due to an unknown defect. To understand if the growth defects were unique to this clone the second clone was analysed. But it also had the same growth defect in the asexual stages. Therefore no further work was carried out with this parasite line.

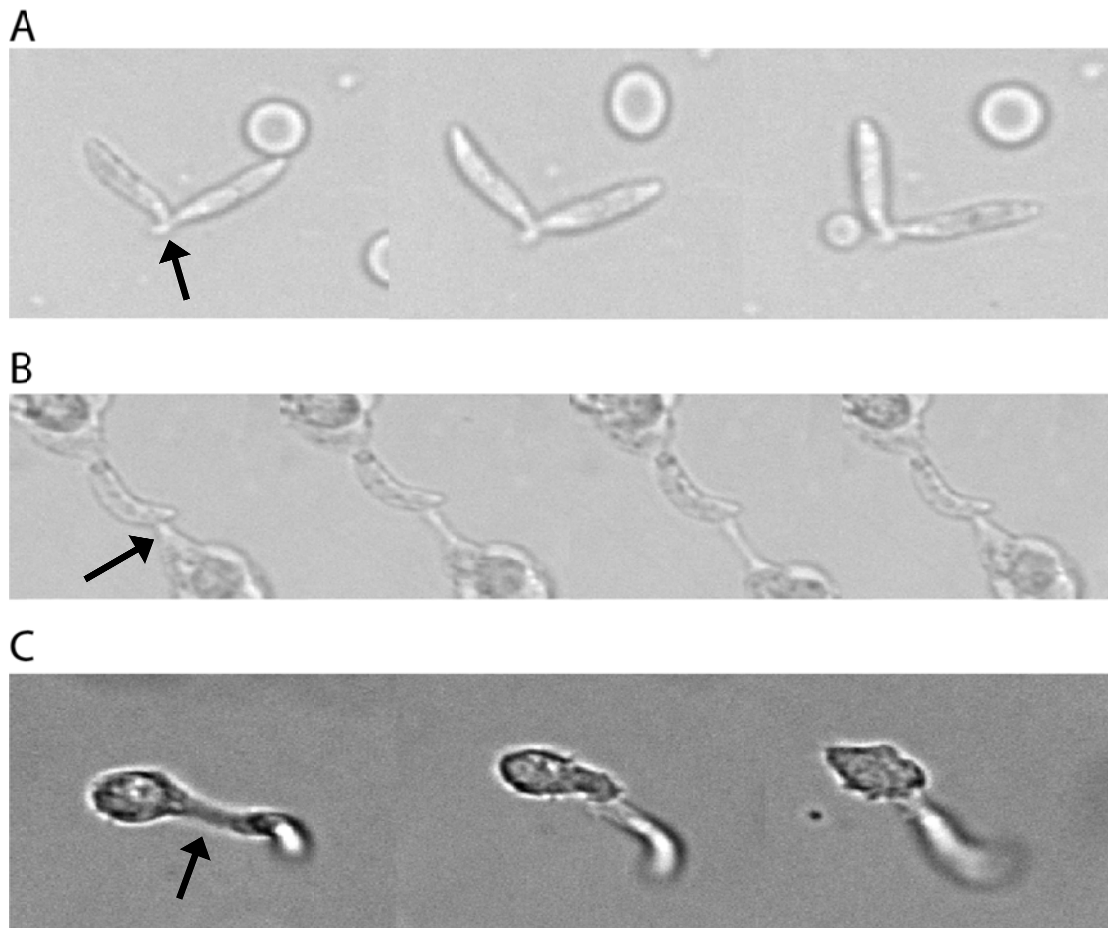


Figure 3.4. setp-actII ookinetes are attached to other culture components

The ookinetes display an abnormal phenotype in that they are attached to other entities. A: To other ookinetes B and C: To Mos20 cells. Net movement only occurred in C. An elastic structure that connected every ookinete is visible (arrow).

3.4. Analysis of *cdpk4p-actII*

The second successfully cloned parasite line was transfected with a construct resulting in the control of *actin II* expression by the promoter of *cdpk4*. The growth of the asexual stages was similar to the wt. Exflagellation appeared normal. Ookinetes were readily formed and the conversion rate was comparable to the wt (Figure 3.5). Ookinete labellings also displayed no different localisation or expression (Figure 3.6). After infection of mosquitoes normal oocysts were detected. No difference was seen in the growth rate, size of the oocysts or the DNA contents (Figure 3.7). Dot plots were created by analysing the images in

detail. The entire oocyst was captured in the confocal microscope and projected to a single image. This was also done with the nucleus. By measuring the area of each oocyst, as well as the area of the nucleus, the percentage of the nucleus compared to the whole oocyst was calculated. From here on this nuclear area and nuclear contents will be referred to as DNA area and DNA contents. The DNA content is not measured in volume but in its area. There was no statistical difference between any of the parameters (Figure 3.8A-F). The transmission through the mosquitoes was not affected, as an infection of a naïve mouse was successful with a similar prepatency period, , i.e. the time for an infection to be established in the mouse, as the wt.

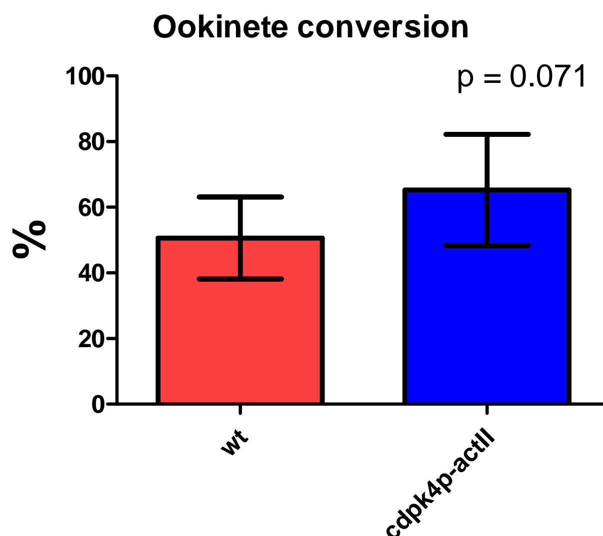


Figure 3.5. Ookinete conversion rates of the cdpk4p-actII

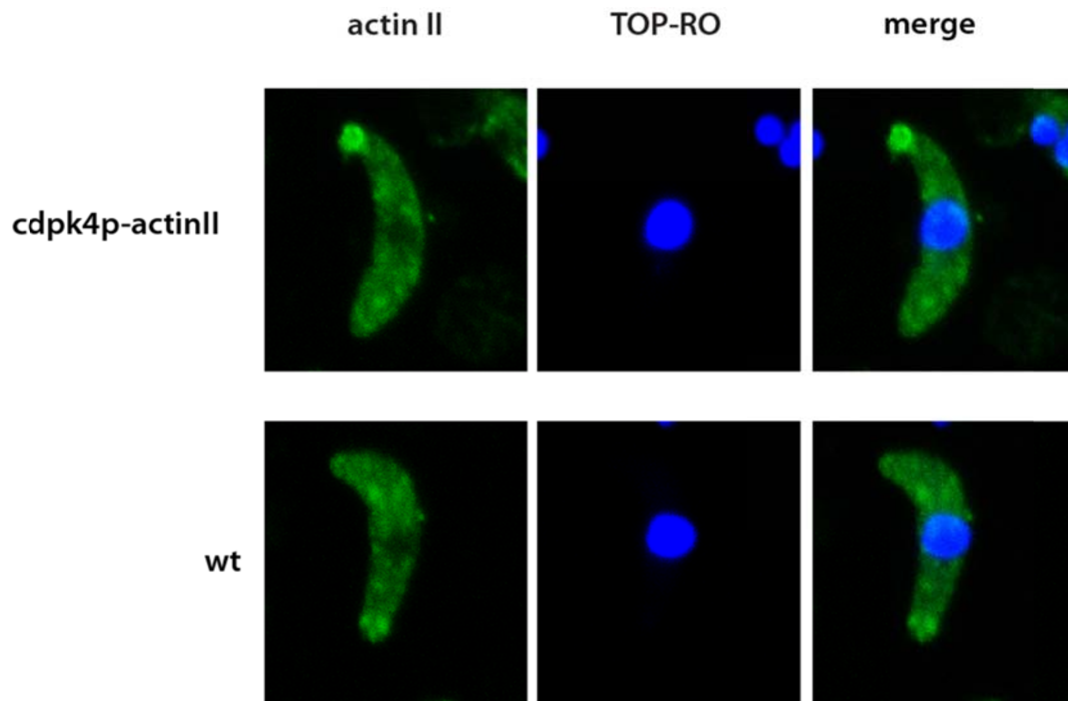


Figure 3.6. Ookinete immunofluorescent assays of cdpk4p-actII

Ookinetes were labelled with an antibody against actin II.

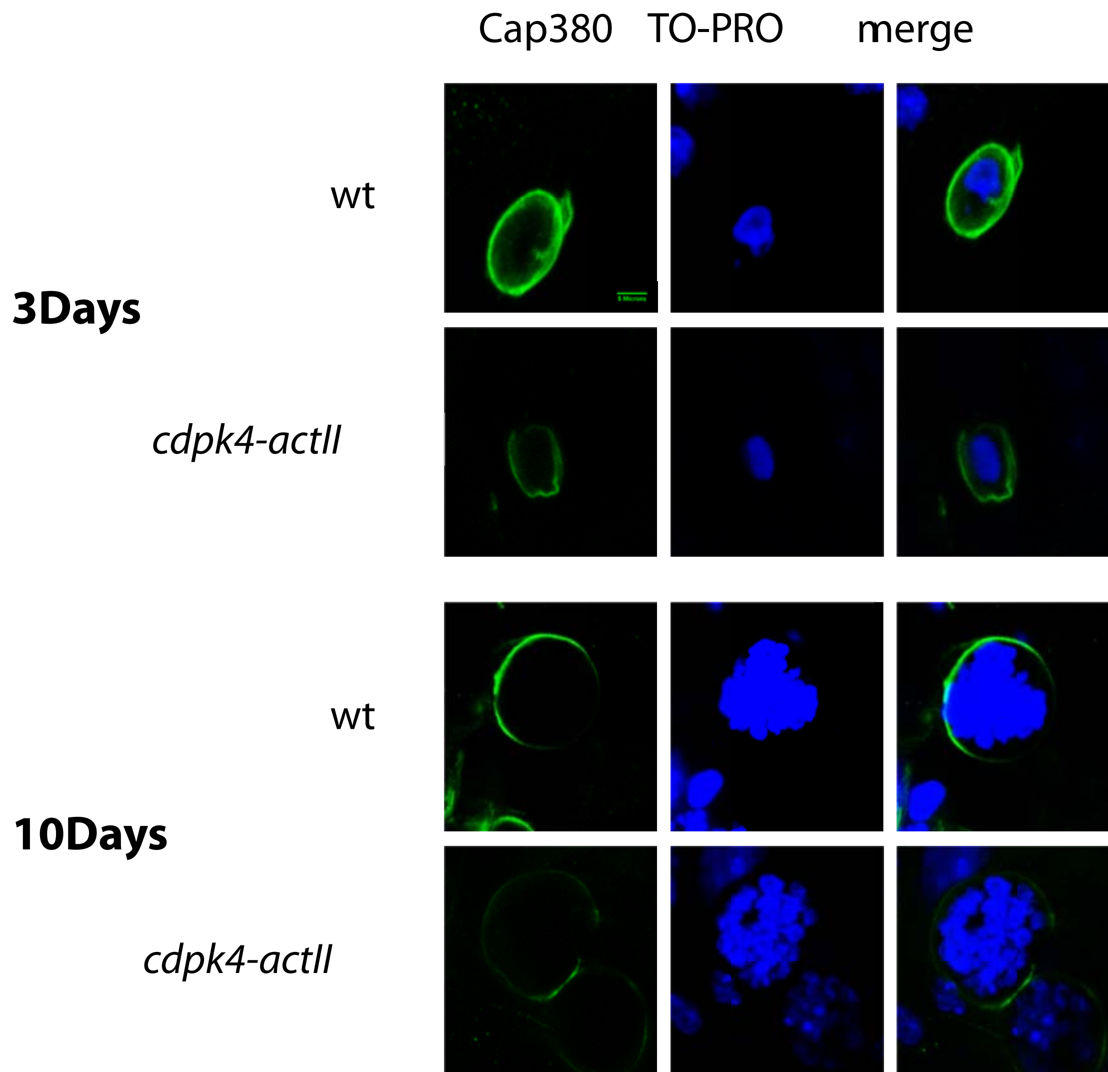


Figure 3.7. Immunostainings of cdpk4p-actII

Oocysts were labelled with an antibody against an oocyst capsule protein called Cap380 (Srinivasan *et al*., 2008) and TO-PRO staining for the nucleus. No difference was observed in the capsule size or DNA contents. Images were all taken at same magnification, scale bar 5 µm.

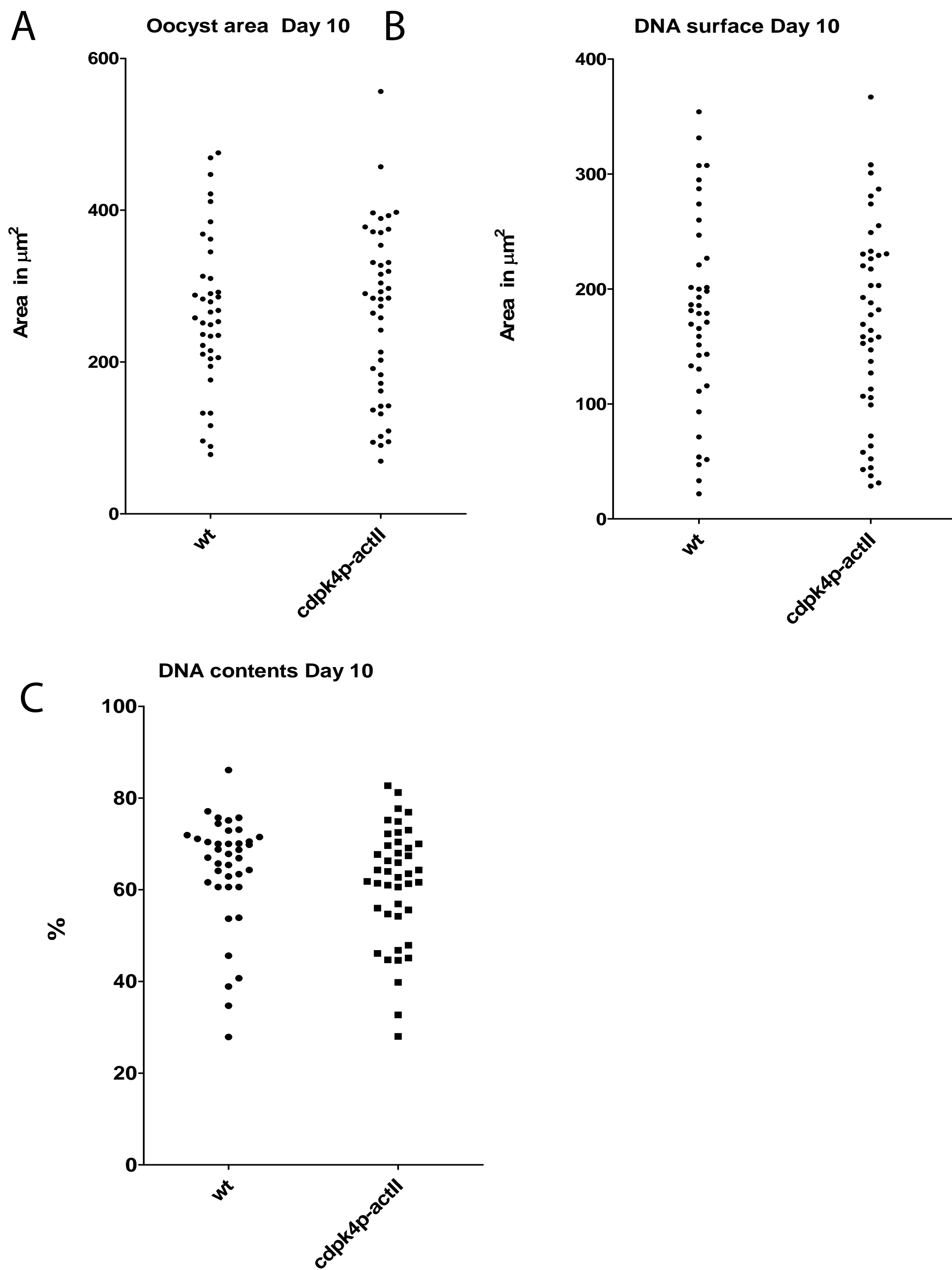


Figure 3.8. Dot blots of cdpk4p-actII versus wt data

Data analysed at day 10. A: Oocyst area B: DNA surface and C: DNA contents.

3.5. Discussion

The only method for conditional mutagenesis in *Plasmodium* parasites until very recently was based on promoter exchange. This method of altering a protein's expression levels, is used to overcome the limitation when the protein is known to be essential at a specific stage of the life cycle (Siden-Kiamos et al., 2011, Raabe et al., 2011) (Laurentino et al., 2011). We employed this method using promoters of genes known to be expressed in male gametocytes. Even though transfection was successful for each promoter exchange, two of the constructs were unsuccessful as several cloning attempts proved futile. Expressing a protein in asexual stages, where previously there was none, could lead to unviable parasites. This could be the reason why the *α-tubulin II* promoter construct was not clonable. Unfortunately the only promoter having no known expression beyond the gametocyte stages, *mdv-1/peg3*, was also unsuccessful. The two promoter exchanges that were successfully cloned did not shed any new light on the function of actin II in the mosquito stages.

The *SET* locus has previously been described in great detail (Pace et al., 1998, Pace et al., 2006). A knockout could not be established due to it being essential in the asexual stages and expression beyond the gametocyte stage has not been proposed (Pace et al., 1998). Even though the coding region is the same for both asexually and sexually expressed *SET*, the mRNA transcript differs from each other due to separate transcription initiation sites. Here, the endogenous *actin II* promoter region was exchanged with the promoter responsible for sexual expression. However, as a severe growth phenotype in both the asexual and sexual stages was observed; therefore it cannot be ruled out that the *SET* promoter has additional functional properties that so far have not described. Another possibility to this unexpected growth phenotype was that the exchange of the promoter changed the sequence on the reverse DNA strand altering the sequence of a gene present there. Judging from Figure 3.9 the gene PBANKA_103020 could have been affected in its 5' flanking region (FR). This protein is of unknown function as yet, but conserved throughout all of the *Plasmodium* species.

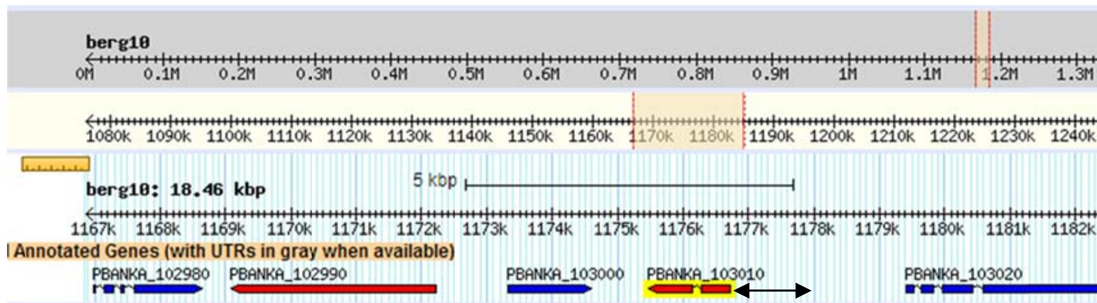


Figure 3.9. Plasmodium berghei *Chromosome 10 region*

The *actin II* gene is highlighted in yellow. The double arrow indicates where the *SET* promoter was inserted. (taken from www.plasmodb.org)

Another possibility could be that the schizonts that were transfected already had a defect due to an unexpected mutation. But this is highly unlikely due to the fact that a second clone also displayed this growth phenotype.

When the *in vitro* ookinete culture of this mutant was studied another defect became apparent. The ookinetes that were formed were trapped in that they were either bound to each other or other components from the culture (Figure 3.4). They were still mobile as twirling was observed as well as location displacement with an attached cell dragging behind. The attachment appeared to be an elastic structure initiating from the basal end. This phenomenon has not been mentioned before. However, structures termed nanotubes have been identified in *P. falciparum* which have been found to contain actin II (Rupp et al., 2011). They appear upon gametocyte activation and are thought of as cell to cell connections only between sexual cells. There has been no mention of such filaments in *P. berghei*. It is possible that these cell to cell connection in this mutant appeared due to the differential expression of actin II. But, as the blood stages already had a defect, it is not possible to make any clear deductions. One thing that is obvious from these data is that these structures hinder the ookinetes, which could be the reason for no oocysts being formed (Figure 3.3). As this effect in the ookinetes cannot be contributed for sure to the promoter exchange no deductions about the function of actin II can be made from this mutant parasite.

The *cdpk4* promoter region exchange parasite did not display a growth defect. Taking all the data obtained and summarising them, it can be said that this mutant behaves exactly like the wt. Very recently it was reported that *cdpk4* is transcribed at the same stages as *actin II* (Rita Tewari, pers. comm.). This may suggest that the two promoter regions are similar. But when comparing the relevant sequences it was found that the alignment did not show a high similarity (44%) (data not shown) and because of the high AT content no clear motifs could be identified. Transcriptional regulation in *P. berghei* and actually all *Plasmodium* species is poorly understood. Genes are mostly regulated through transcriptional, post transcriptional and translational repression. Histone modifications have also been shown to be of importance (Lopez-Rubio et al., 2009, Salcedo-Amaya et al., 2009). Transcription factors are largely unknown, apart from the recently discovered Apetala 2 (AP2) protein family. There are 26 AP2 transcription factors that are conserved throughout all Plasmodium species, with only one additional found in *P. falciparum* (Painter et al., 2011). We then looked at the predicted AP2 binding sites (Figure 3.10). However, the binding sites of the two promoter regions are differently localised. No data is available for the expression of the cdpk4 protein or its function in the ookinete, but the fact that the two promoter regions appear interchangeable is worth noting. As far as the author is aware no two unrelated protein have so far been identified having such a similar regulation. Unfortunately again, this data does not infer any information on the function of actin II in the mosquito stages.

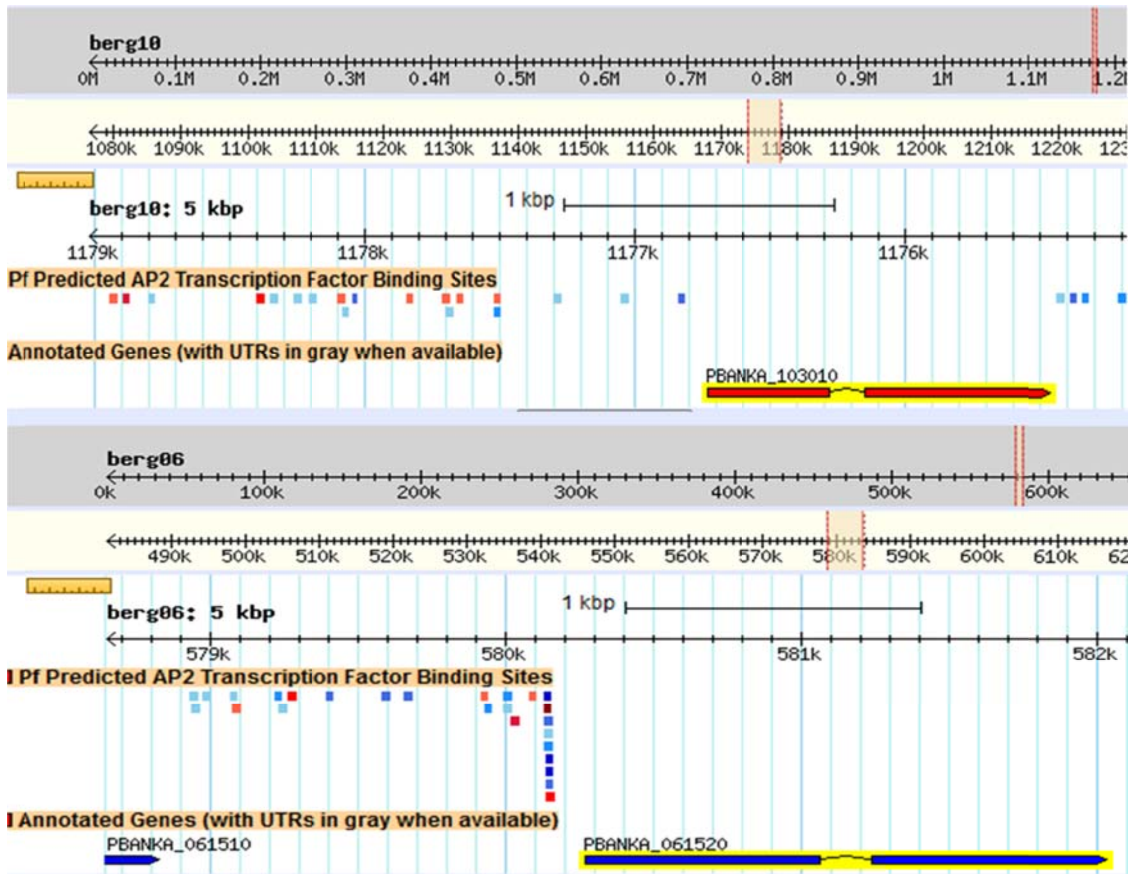


Figure 3.10. Predicted AP2 transcription factor binding sites

Above the *actin II* gene (PBANKA 103010) and below *cdpk4* (PBANKA 061520) gene. The predicted AP2 sites are situated differently for the two genes.

Chapter 4

Genetic crosses producing only heterozygous ookinetes

4.1. First series of experiments

4.1.1. Heterozygous ookinetes developed into abnormal oocysts

Crosses with the wt and the *actII(-)* revealed a defect in the mutant strain. Repeating this genetic cross with a strain that only produces fertile males called *Δ47* led to formation of only heterozygous ookinetes, as the *actII(-)* mutant only produces fertile females. Thus all subsequent oocysts will have the same genotype. *In vitro* ookinete cultures were set up containing equal amounts of blood from both parasite strains. The purified and counted ookinetes were fed through membranes to mosquitoes. Three successful independent experiments were carried out in this first series. In the first experiment samples were taken at 24 hrs, 72 hrs, 6 days and 10 days after blood feeding. In the subsequent experiments only samples taken at day 3 and 10 were analysed in detail. These experiments were difficult because the mosquitoes often died before the end of the experiments.

The detailed analysis of these oocysts was to measure the amount of DNA in the wt and in the mutant cross oocysts as well as their actual size (Figure 4.1). In Table 4.1 data are presented of oocyst counts. At three days the numbers of detected oocysts were comparable in the two samples. However, at the day 10 samples the difference between these two populations is clearly pronounced. The number of detectable oocysts has decreased dramatically to an average of two oocysts per midgut, while the wt had an average of 24 oocysts per midgut. The prevalence had decreased substantially in the mutant cross from 77.7% to 31.9%, which is roughly a 3-fold decrease from the wt with 92.4%.

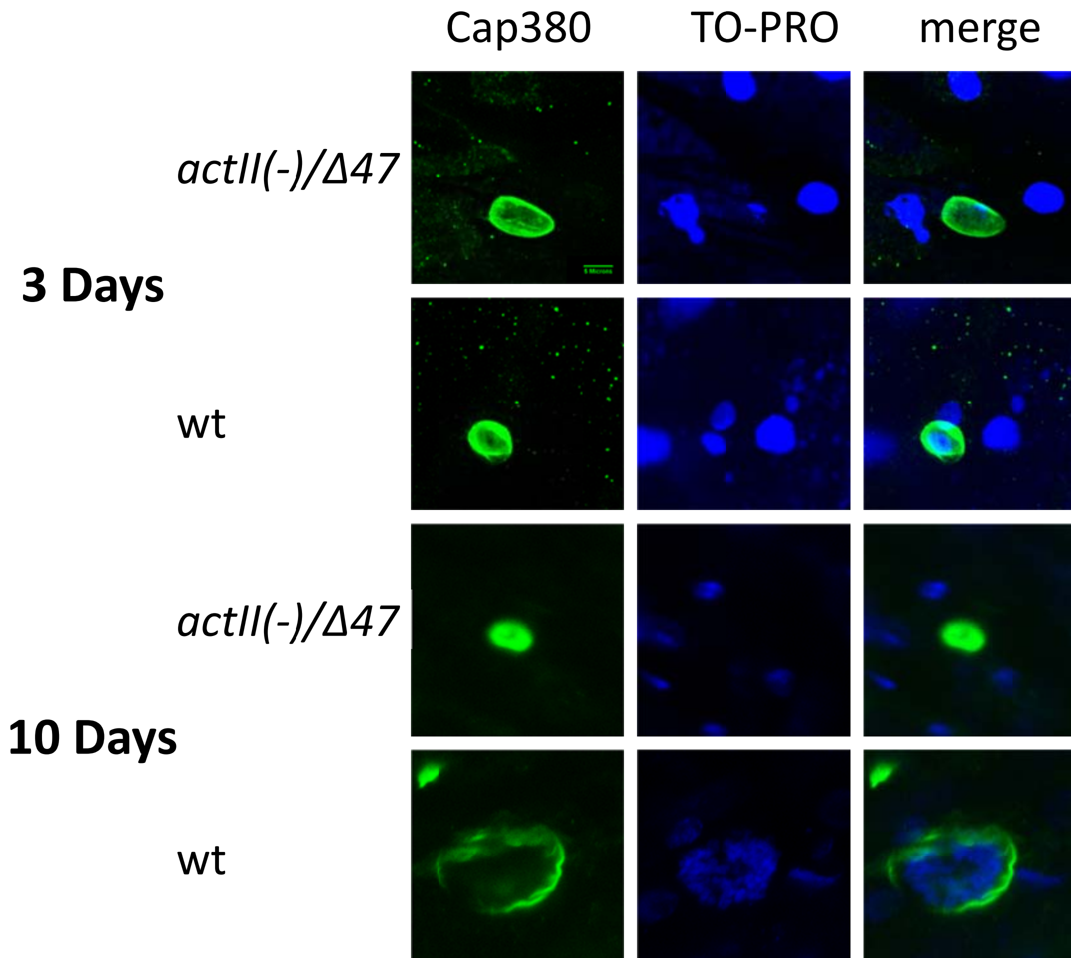


Figure 4.1. Immunofluorescent labelling of wt and mutant cross oocysts

At day 3 the heterozygous oocysts contained DNA. At day 10 growth and DNA replication had increased the size of the wt oocysts, while small empty oocyst capsules were detected in the heterozygous oocysts. Images were taken at same magnification, scale bar 5 µm.

D. p. f.	Parasite	Number of midguts	Average of oocysts	Prevalence
3	wt	19	17	89.5 %
	actII(-)xΔ47	18	12	77.7%
10	wt	31	24	92.4 %
	actII(-)xΔ47	24	2	31.9 %

Table 4.1 Midguts and labelled oocysts counts in the wt and actII(-)xΔ47 cross infection
Striking differences can be seen between the wt and the mutant cross at day 10 as well as a decrease in oocyst prevalence in the mutant cross from day 3 to day 10. Data pooled form three independent experiments.

D.p.f.	Parasite	Oocyst Area in μm^2	DNA Area in μm^2	DNA contents (%)	No. of Oocysts
3	wt	54.1	17	29.8	25
	actII(-)xΔ47	42.2	3.3	7.5	34
10	wt	266.9	160.1	58	54
	actII(-)xΔ47	30.5	0	0	42

Table 4.2. Oocyst analysis of the wt and actII(-)xΔ47 cross
At day 3 there was already less DNA contents in the mutant cross. By day 10 the mutant oocysts contained no DNA. D.p.f.: days post feeding

Table 4.2 shows that the DNA area and subsequently the DNA contents is significantly reduced from 17 μm^2 to 3.3 μm^2, comparing the wt to the mutant cross, and from just under 30% to 7.5%. Within the next seven days the oocyst increases its volume and undergoes multiple rounds of replication to allow sporogony to take place. The wt oocysts have increased their volume substantially and replicated their DNA contents to an average of 58%, the highest contents to be found to be 79% (data not shown). In the mutant cross not a single oocyst was found to contain any DNA.

When plotting the DNA contents at day 3, two distinct groups could be made out for both the wt and the mutant cross. These data were separated and labelled according to their nuclear content. Oocysts having a DNA content below 15% were labelled as abnormal and the ones above as normal. Comparing the normal oocysts of the wt and the mutant cross with each other, one can find no statistical difference between them (Figure 4.2B). This is also the case with the abnormal oocysts (Figure 4.2C). However, when comparing the normal oocysts to the abnormal ones, there is significant statistical difference. Figure 4.3B shows that the day 10 mutant cross oocysts had arrested in their size development since day 3.

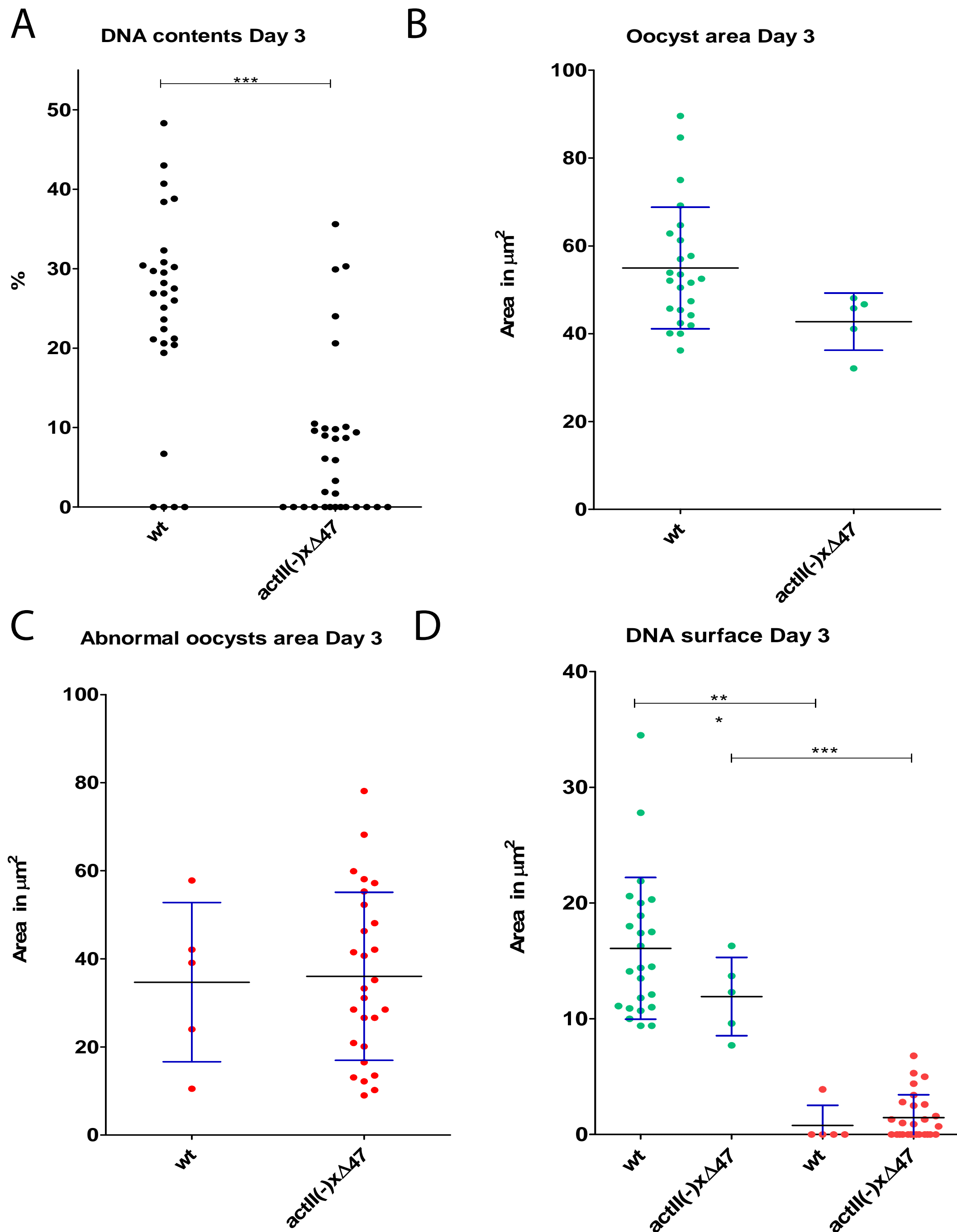


Figure 4.2. Dot plots of analysed oocyst data day 3

Oocysts were measured and plotted according to A: their DNA contents, B: and C: their surface area and D: DNA area (wt: n=29; *actII(-)xΔ47*: n=32). A: Two distinct oocyst populations could be distinguished based on their nuclear contents; normal > 15% (later in green) and abnormal < 15% (later in red). These populations were separated in B, C and D to show the differences and similarities between them (wt: n=24 and n=5; *actII(-)xΔ47*: n=5 and n=27). (****p*<0.001 using unpaired t test)

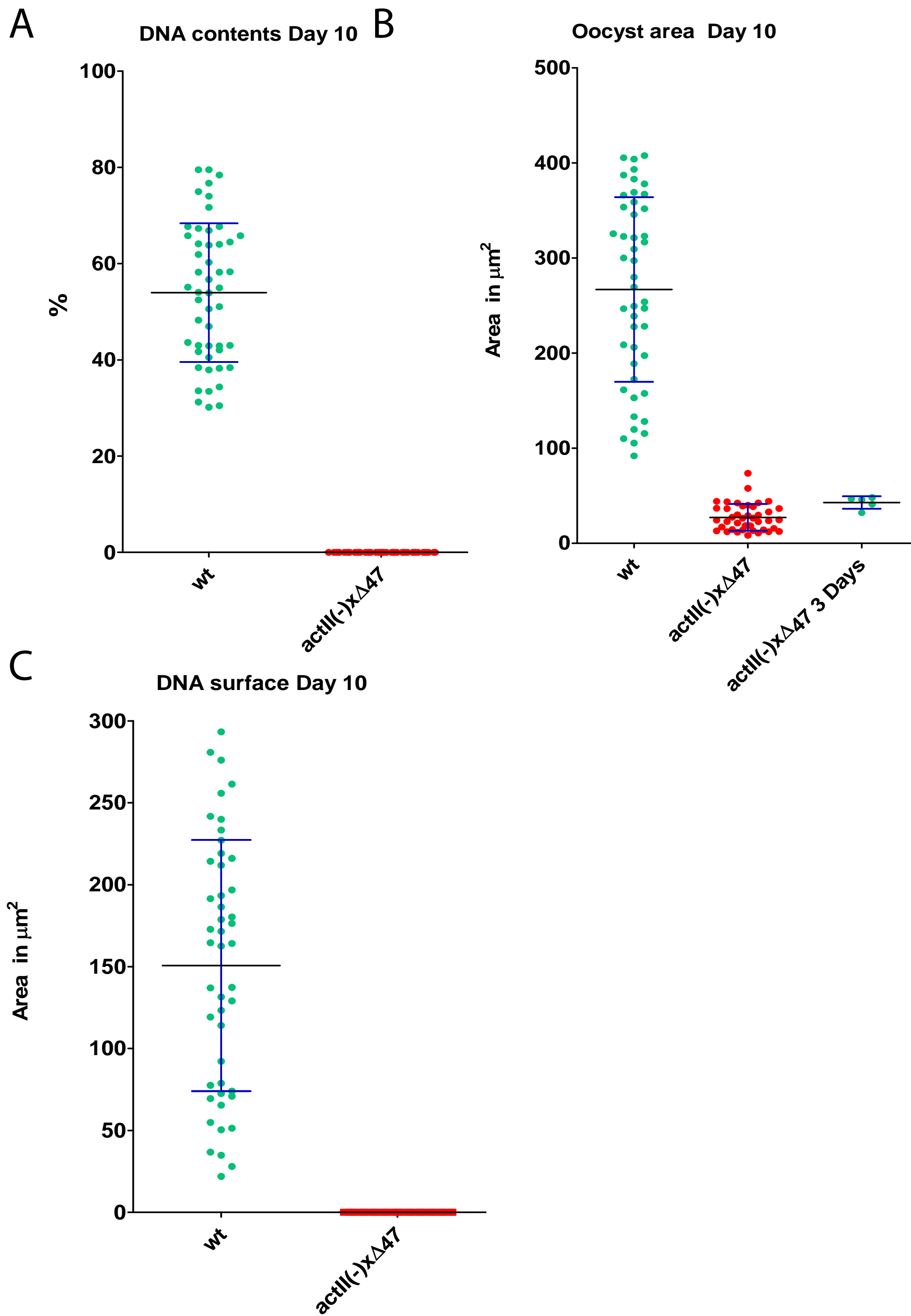


Figure 4.3. Dot plots of analysed oocyst data day 10

At this time point no DNA was found in the mutant cross oocysts. Heterozygous oocysts had not grown in size after day 3. (wt: n= 47; *actII(-)xΔ47*: n=42) Normal oocysts in green, abnormal in red. A- C: $p<0.001$ using unpaired t test

4.1.2. Oocyst capsule size is irregular in the *actII(-)xΔ47* cross

The oocysts of the *actII(-)*x*Δ47* appear have an irregular shape compared to the round oocysts of the wt. At day 10 the few oocysts that can still be observed appear as if the capsule may have collapsed into itself (Figure 4.4).

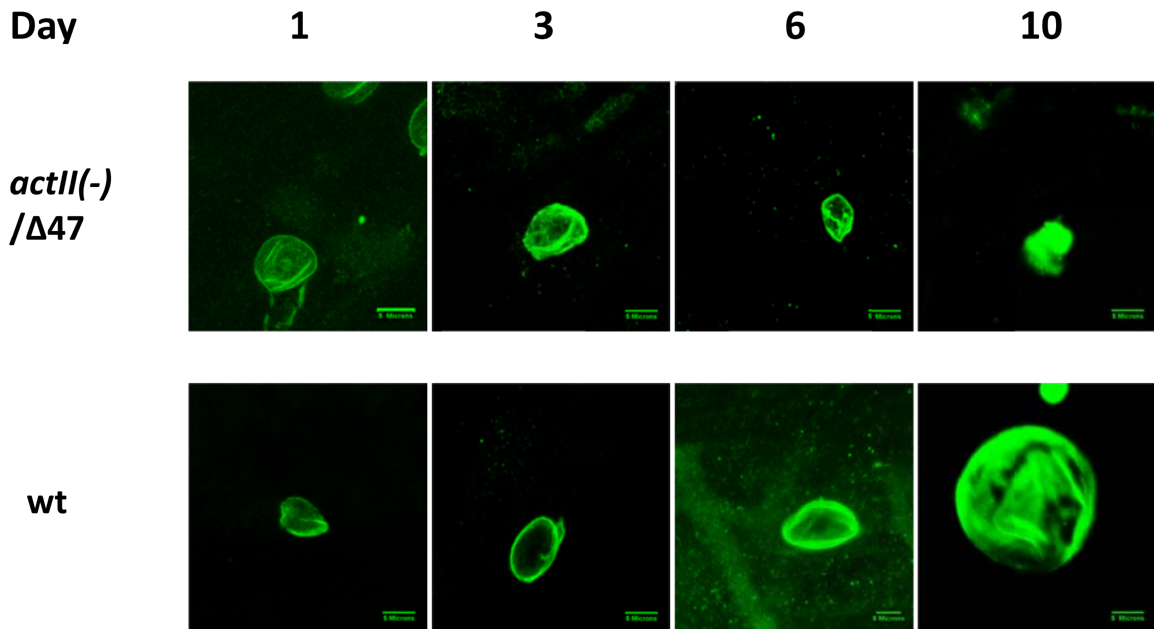


Figure 4.4 Oocyst surface labelling with anti-Cap380

The *actII(-)*xΔ47 cross grow in size after day 3. The wt grows in size after day 6. At day 10 the structure of the capsule of the heterozygous oocysts has changed their morphology.

4.2. Second series of experiments

4.2.1.Heterozygous ookinetes developed into delayed oocysts

In the first set of experiments only between 24 and 31 mosquitoes at day 10 were analysed, because the vast majority of mosquitoes died before this time point. In order to increase the number of samples the experiment was repeated. However, in this second series of experiments heterozygous oocysts containing DNA were

observed, although the abnormal, empty oocysts were also detected. The following dot plots present the data from these experiments (Figure 4.5). The heterozygous oocysts can be divided into two populations (Figure 4.5A). One, which is consistent with the previous data, without DNA detectable in the oocyst at day 10, and the other population in which DNA is detectable. These oocysts, are however significantly smaller and contain less DNA than their wt counterparts. Upon observation in the light microscope oocysts were observed that contained sporozoites. But transmission experiments back to naïve mice with the secons series were never successful. In the four transmission experiments performed the prepatency period of the wt was four to five days, the heterozygous oocysts did not result in infectious sporozoites. This suggests that these oocysts are also defective.

We then plotted all of the data from the first and the second set of experiments into the same graphs (Figure 4.6). This shows that the defect of the heterozygous oocysts is not as pronounced as with the first set alone, but still present.

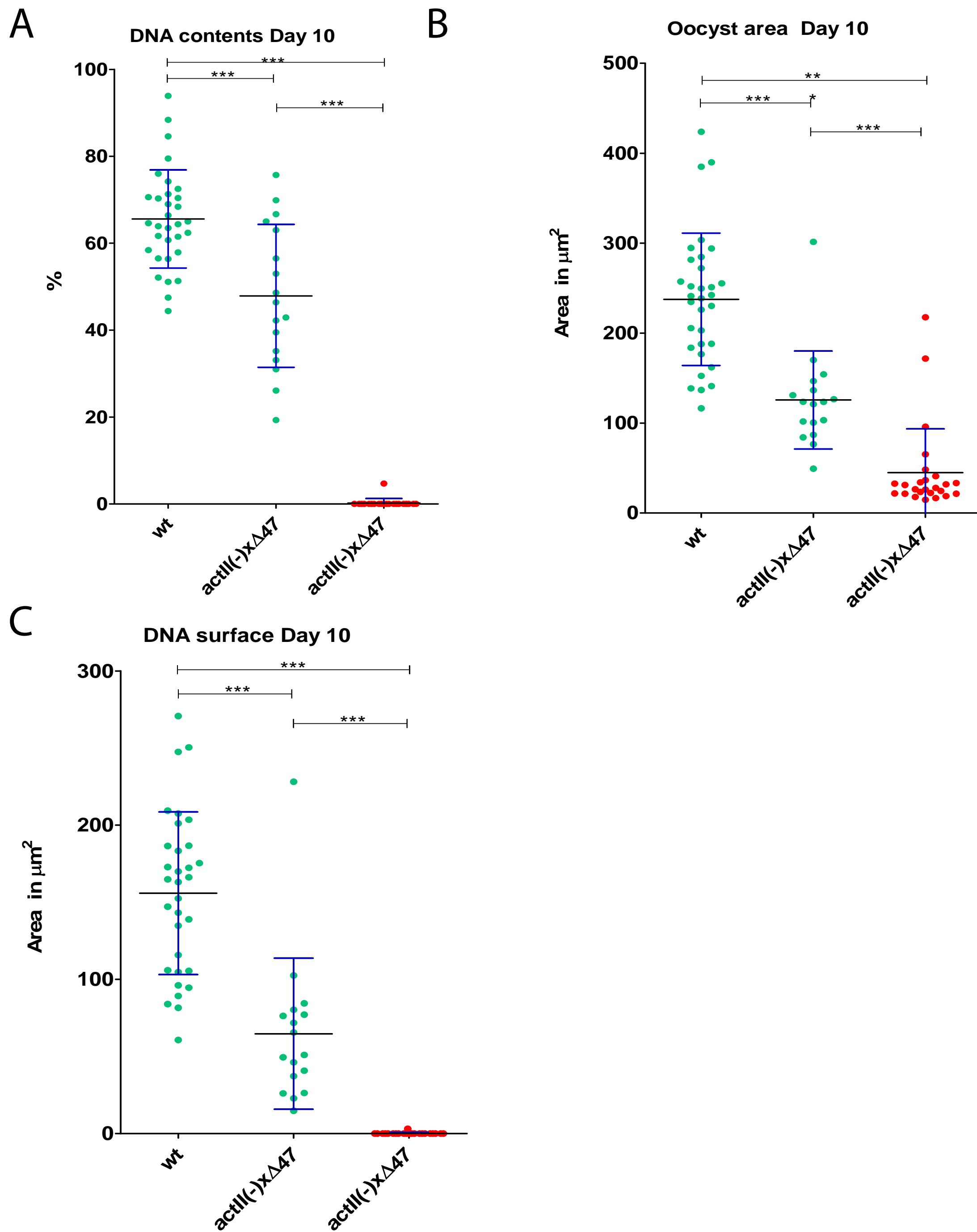


Figure 4.5. Dot plots of actII(-)xΔ47 oocysts containing DNA

A: 17 heterozygous oocysts contained DNA, while 21 oocysts were empty. (wt: n= 32) . B: Oocyst areas were measured. The heterozygous oocysts were significantly smaller. C: DNA surface area was significantly smaller compared to the wt. (***: $p<0.001$ using unpaired t test)

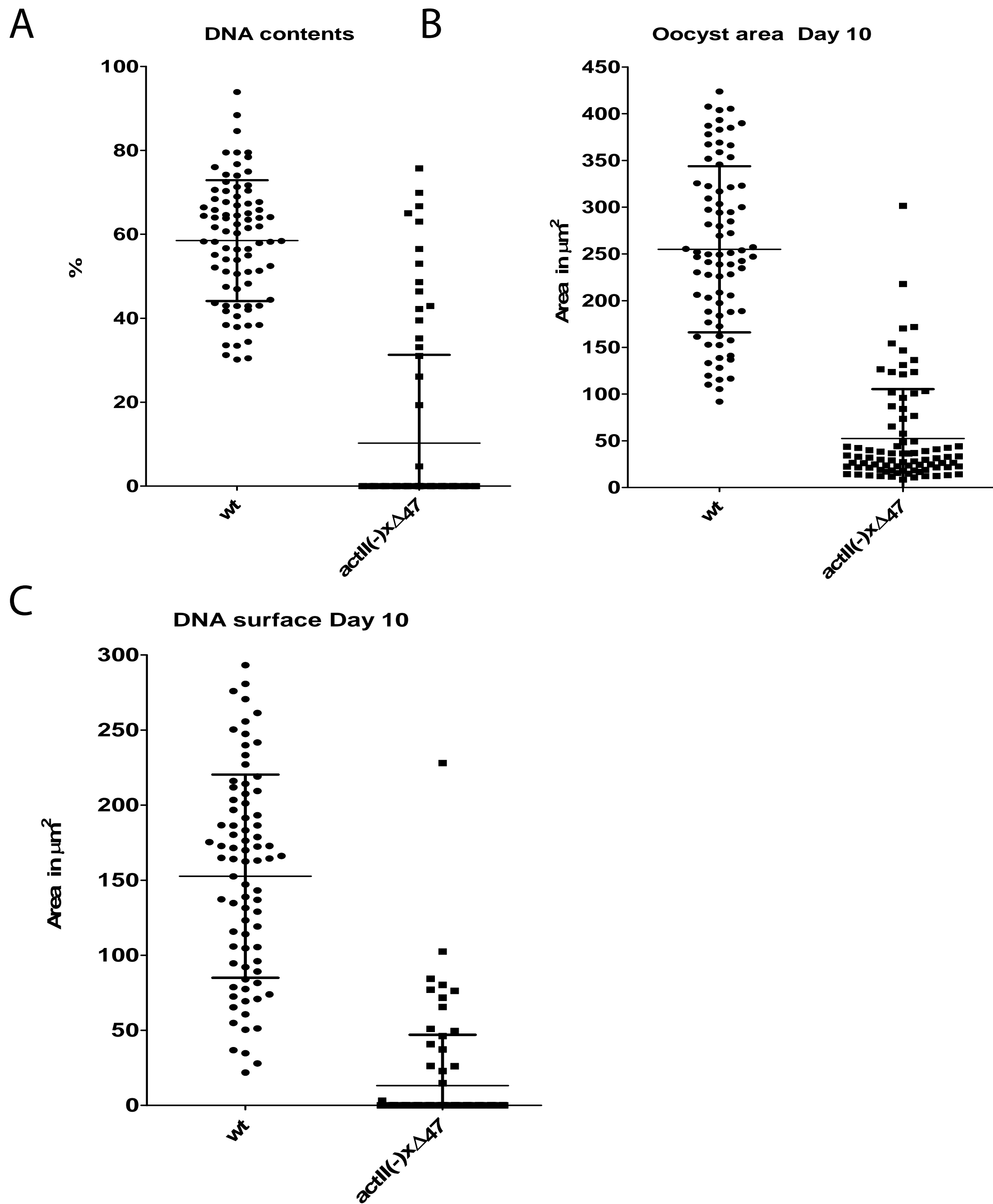


Figure 4.6. Dot plot of combined oocyst data of both sets of experiments

The defect is not as pronounced, however all in all plots the data sets are still significantly different from each other. n: wt= 82, *actII(-)xΔ47* = 80. A – C: $p<0.001$ (using unpaired t tests).

4.3. Control experiments

As the two sets of experiments had a somewhat different outcome (the phenotype was more severe in the first set), trouble-shooting took place with several possible solutions to explore. The first was that a contamination of the sample had occurred. Control cultures with only pure blood were set up in parallel each time the *actII(-)* was mixed with the *Δ47 in vitro*. On no occasion was any ookinete detected in these controls, nor exflagellation in the *actII(-)* strain. Another possibility is a difference in the population of the mosquitoes used. This is not easy to determine. Our mosquitoes are kept in cages in a separate room and can only breed within the cages. No new mosquitoes have been introduced during the time the experiments were conducted. We tested the mosquito DNA with the only means we had available. *A. gambiae* exists in molecular forms called M and S-form, which can be differentiated based on genotyping. A PCR was performed on mosquito DNA samples from the first experiments and the last series of experiment. Figure 4.7 shows that both mosquito populations are pure M-forms. No population shift or contamination was detected with this method.

The next possibility was a difference in the mosquito immune response that was due to different starvation methods used in the first set of experiments and the last set. Starvation was extended from 5 hrs to 16 hrs to be more time efficient. To test if this difference was responsible for the difference between the two series, a genetic cross with the wt and the *actII(-)* was carried out again. In parallel two cages that had been starved for either 5 or 16 hrs, were fed on mice containing the genetic cross. Genotyping was performed on samples isolated at day 3 and day 8 from these mosquitoes. The mutant parasites were present at day 3 in both the long (16 hrs) and short (5 hrs) starvation experiments (Figure 4.8). At day 8 the *actII(-)* parasite population was no longer detected for either sample. This is consistent with the previous *actII(-)* and wt cross experiments. Importantly none of the two primer pairs amplified fragments from uninfected mosquito DNA. As no difference could be seen in survival of the *actII(-)* parasite population using the

different starvation methods, it can be concluded that the difference in the two series of experiments is unrelated to this parameter.

Figure 4.7 PCR of molecular forms of Mosquito DNA

Due to an insertion for the M-form at locus S200 X6.1 the band obtained would be 479 bp, while it is absent in the S-from leading to a band of the size of 249 bp (Santolamazza et al., 2008). Sample 1 was extracted from mosquito guts from the first *actII(-)xΔ47* cross feed, sample 2 was taken from the second series of experiments, where mutant oocysts containing DNA were detected. The size of the band of both samples is consistent with the band corresponding to the M-form.

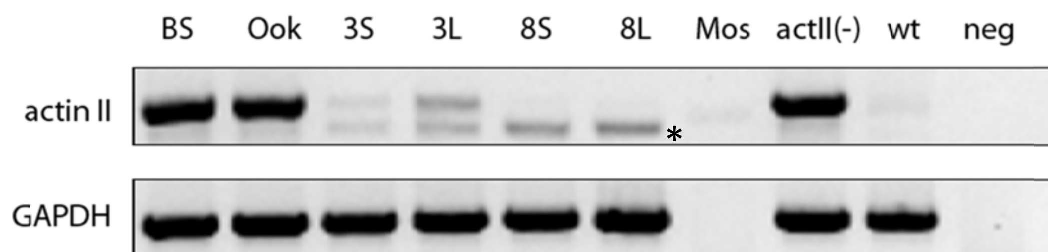


Figure 4.8. PCR of different starvation methods

Two different starvation methods were tested, L: 16 hrs with H_2O at 28°C, S: 4 hrs without H_2O at 19°C. The *actII(-)* strain is present in both samples at 3 days, but is lost at day 8. BS: blood stages; Ook: ookinetes; Mos: uninfected mosquito DNA. GAPDH was used as a loading control. (*: nonspecific transcript from oocysts)

4.4. Discussion

As the promoter exchange construct did not yield any conclusive results about a possible second function of actin II in the mosquito stages, it was decided to try a second approach by crossing the *actII(-)* strain, which only produces fertile females, with a strain generating only functional male gametocytes. As was already shown during the *actII(-)* cross with the wt, the defect caused by the lack of actin II cannot be rescued by providing one copy of the wt *actin II* gene, inherited from the males. The analysis of the mutant cross showed the defect to be primarily during oocyst development as some oocysts containing DNA were observed until three days. It is worth noting that the number of oocysts in the both series of experiments with the *actII(-)xΔ47* crosses was generally lower than the wt, possibly indicating that the mutant ookinetes could have difficulties crossing the midgut epithelium or attaching themselves. Many mutants have been described where this is the case. Noteworthy here are the *Δpplp5* and *Δpsop7* mutants, which both form ookinetes at a normal rate, but have strongly reduced oocysts numbers (Ecker et al., 2007, Ecker et al., 2008). *ΔPSOP7* lacks sporozoite formation altogether just as the *actII(-)*. However, an inability to cross the midgut and reduced oocysts is no indication of loss of infectivity. *ΔCTRP* and *ΔSOAP* mutants have been shown to be able to re-establish infection if directly injected into the haemocoel (Dessens et al., 1999, Nacer et al., 2008a). Therefore the phenotype we have observed of the degradation of oocyst DNA and growth arrest indicates an actual defect in the oocysts. The plotted data in Figure 4.2A shows two distinct oocyst populations even in the wt sample. In the wt it is not uncommon to find many abnormal oocysts due to the harsh environment the ookinete has been exposed to (Whitten et al., 2006). But this explanation cannot solely be responsible for the results in the mutant cross. In the first set of experiments we never observed DNA beyond the three day time point. If there was no other defect other than exposure to the midgut, we would have observed at least some oocysts following normal development. As the presence of DNA is in direct relation to sporozoite formation, the transmission blockage of the *actII(-)* is explained. The common method for oocyst studies is to visually observe midguts between days 10

and 12, where only fully formed oocysts can be distinguished. Deteriorated or early growth arrested parasites could be missed; therefore it is possible many mutants said to be unable to form oocysts indeed form oocysts, albeit growth arrested ones. One study utilised GFP expressing parasites to investigate oocyst growth of *Δc-cap* (Hliscs et al., 2010). These oocysts exhibited a growth arrest comparable to the wt at day 8 and had DNA present. Nuclear division was reduced and did not result in sporogony. It is suggested that C-CAP is an actin-regulator in the oocyst responsible for mitotic spindle arrangements and vesicular trafficking. Actin II might also function as an actin I regulator. We have shown previously the absence of actin II in the gametocyte leads to an absence of actin I redistribution in the gametocyte (Deligianni et al., 2011). In non-activated wt gametocytes actin I is present in both the cytoplasm and the nucleus. Upon activation the actin I found in the nucleus is entirely redistributed into the cytoplasm. In the male *actII(-)* gametocytes this redistribution was not observed and actin I remained in both the nucleus and cytoplasm.

There have been several mutants described with growth arrest of the capsule or an absence of sporogony. A protein kinase pk7 has recently been identified to be growth arrested at day 10 while undergoing no sporogony (Tewari et al., 2010). It is not mentioned whether any nuclei were present, but it is suggested that this kinase functions prior to nucleation and sporoblast formation. The same was said for the kinase gak, however in this mutant, despite the lack of sporozoites, the oocyst was not growth arrested, but continued to enlarge by day 21 p.a. (Tewari et al., 2010). Despite oocyst growth *Δpblap1* equally failed to undergo sporulation (Claudianos et al., 2002). This suggests that oocyst capsule growth does not necessarily need to coincide with DNA replication, but DNA replication requires oocyst growth.

As the prevalence of oocysts in the *actII(-)xΔ47* cross midguts was very low, it was decided to carry out further repeats. The results showed that in these experiments oocysts were found containing DNA, in addition to the previously detected empty oocysts. Closer analysis of the heterozygous oocysts containing DNA revealed a

significant difference in size and DNA contents compared to the wt (Figure 4.5A-C). We tried to explore the reasons why this difference in the outcome in the two series of experiments had occurred. There are two molecular forms of *A. gambiae* that are morphologically indistinguishable species having specific single nucleotide polymorphisms. It has been shown that not only are there differences between the forms in their behaviour and genetics, but also in their ability to respond to *Plasmodium* infection (White et al., 2011, Esnault et al., 2008, Gimonneau et al., 2012). These forms are said to have diverged due to larval predation and inter-form competition (Lehmann and Diabate, 2008). Mosquito populations contain considerable heterogeneity. During repeated growth cycles especially after bottle necks a shift in the population may take place. This could have occurred if some rare S-form mosquito would have been present in our population so that this shift could take place. Exploration of this option however proved that the molecular form was the same with all mosquitoes being M-form.

An increase in melanisation of the heterozygous ookinetes in all of the *actII(-)xΔ47* crosses was observed in the dissected midguts, suggesting that the mosquito immune system could have been regulated differently than before. Wounding of the epithelial cells during traversal initiates the melanisation immune response by encapsuling the parasite in the brown-black pigment of melanin. This essentially traps the parasite and blocks further movement and oocyst development. Temperature has been known to affect the immune response of the mosquito (Murdock et al., 2012). As the protocol for starvation prior to feeding was adapted to be more efficient, the mosquitoes were now starved for a longer period in their usual environment at 28°C and only stored in the 19°C incubator upon feeding, unlike the previous method of short starvation at 19°C. Control experiments showed that this was not the reason for differential outcome of this series of experiments. Even though ookinete culture carried out in parallel to each feeding and these never produced ookinetes, it cannot be ruled out that the DNA containing oocysts were homozygous for the *Δ47* strain, as a very low self-fertilisation level of 0.1% has been reported (van Dijk et al., 2010). Nevertheless, it was not possible to transmit the parasites from the mosquitoes containing these

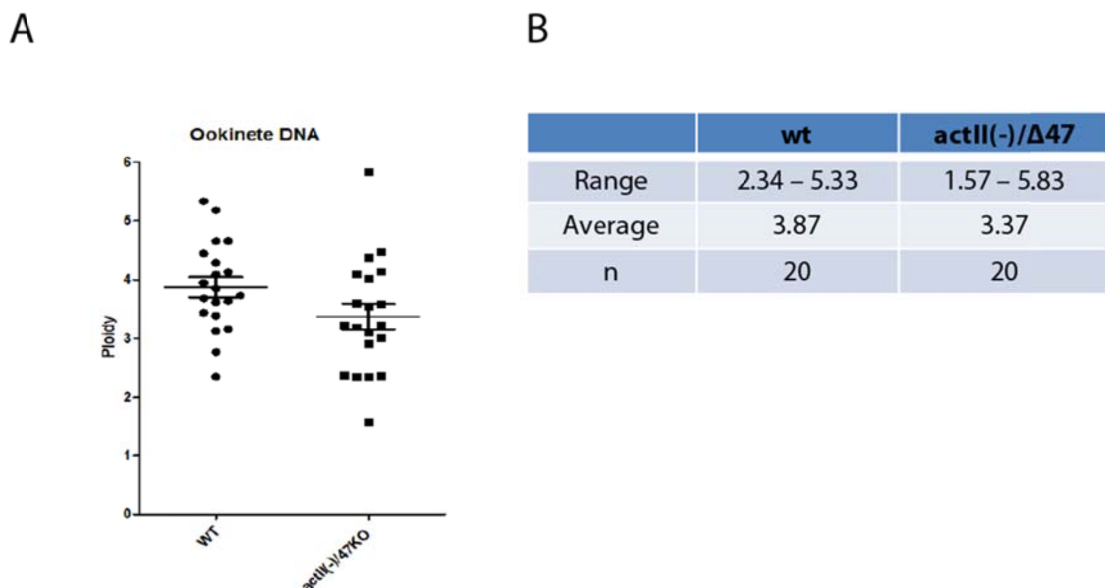
oocysts to naïve mice. Thus suggesting that even though these oocysts contained DNA and on rare occasions formed sporozoites, they still contained a defect.

Chapter 5

Ookinete studies via episomal overexpression of GFP-actin II

5.1 Heterozygous ookinetes have a normal DNA contents but actin II is absent

A defect in oocyst formation can in some cases be due to malformations at the ookinete level. Therefore a more detailed analysis was carried out on *actII(-)*x*Δ47* ookinetes. As there was a DNA replication defect in the oocyst, it was speculated that this might be due to a block in meiosis, which occurs normally after the formation of the zygote and which results in the ookinete being tetraploid. Determining the ploidy is achieved by using a DNA stain, whose intensity is proportional to the amount of DNA present such as Hoechst 33342. Known haploid parasites are measured as controls and the increased fluorescence from ookinete nuclei converted to the ploidy of their genome. The average ploidy of the *actII(-)xΔ47* ookinetes is close to their expectant ploidy with no significant difference compared to the wt (Figure 5.1). This suggests that the meiosis in the *actII(-)*x*Δ47* zygote undergoes its normal processes.



	wt	actII(-)/Δ47
Range	2.34 – 5.33	1.57 – 5.83
Average	3.87	3.37
n	20	20

Figure 5.1. Chromosome number identification of actII(-)xΔ47 ookinetes

Measuring the nuclear fluorescence of haploid parasites makes it possible to deduce the ploidy of ookinetes. A: Dot plot of each ookinete counted. B: Range and average of the DNA content of the ookinetes

5.2 Ookinete immunolabellings revealed a lack actin II in the cytoplasm

It has been previously shown with a GFP-tagged actin II that the protein is found in the cytoplasm of the ookinete. (Elena Deligianni, unpublished results). Carrying out immunofluoresence assays with the wt and *actII(-*)x*Δ47* using an antibody against actin II indicated that there was no actin II in the mutant cross ookinetes, although the presence of actin II in the wt can be easily distinguished (Figure 5.2). This result was somewhat unexpected as the *actin II* allele carried by the *Δ47* males is present in these ookinetes. To verify this result a Western blot was also carried out with the same results (Figure 5.3). No actin II protein was detected.

Then the question arose why there was no actin II present in ookinetes even though the male copy of the gene was present. Our lab had created a transfectant expressing GFP-tagged actin II. This strain expresses the protein episomally under its endogenous promoter together with an antibiotic gene giving resistance to pyrimethamine. From previous experiments it was known that when actin II is overexpressed due to high pyrimethamine pressure no ookinetes are formed.

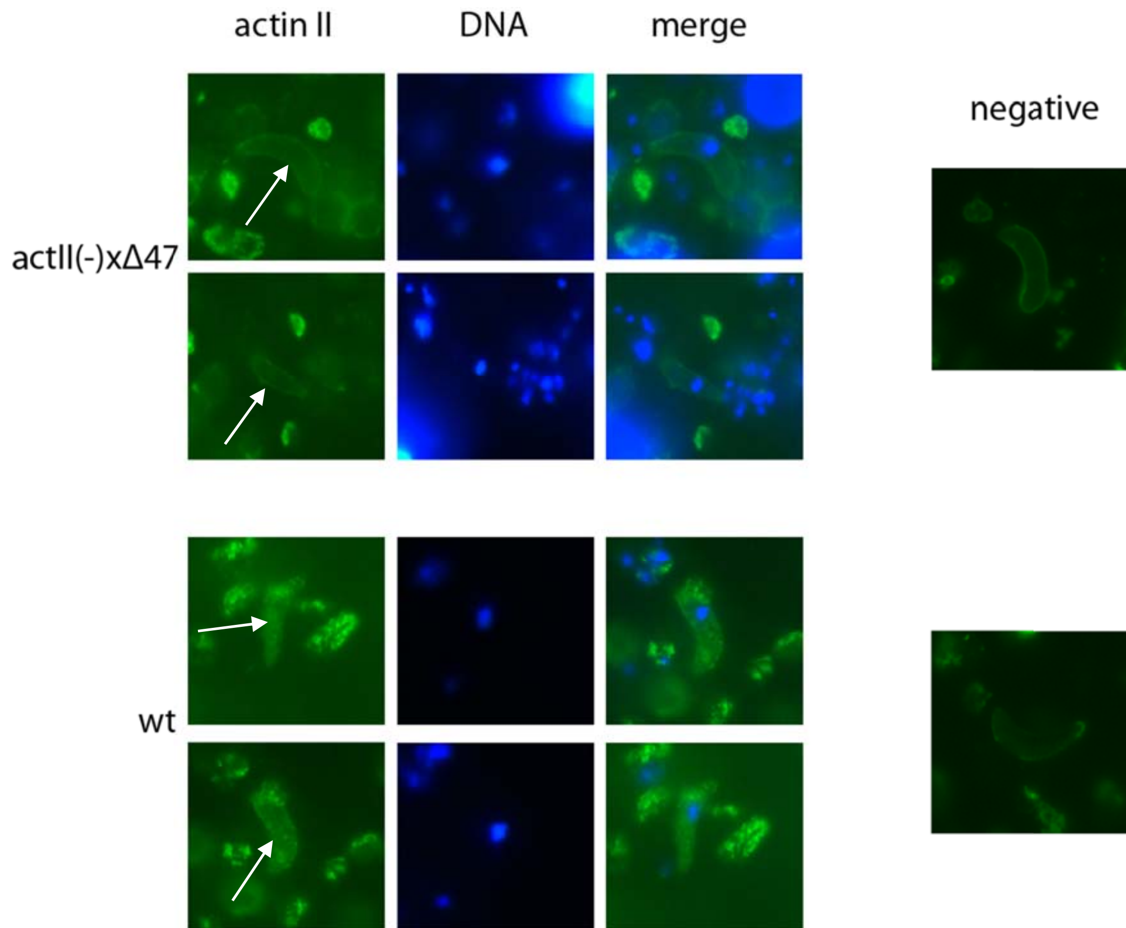


Figure 5.2. actII(-)xΔ47 ookinetes lack actin II

Ookinetes (arrows) were labelled with the actin II antibody and signal was detected in the cytoplasm of the wt. The mutant cross revealed a complete absence of the protein despite presence of the *actin II* gene. The negative was not labelled with actin II. A non-specific peripheral staining can be observed here as well as in the mutant cross.

47.

Figure 5.3. Western blot of wt and actII(-)xΔ47 *ookinetes*

The actin II protein is present in the wt ookinetes. The mutant cross does contain actin II. SET was used as a loading control.

5.3 Actin II relocalisation during ookinete development

Expression of the GFP-actin II fusion protein was followed during ookinete development. GFP fluorescence could be detected during exflagellation in the male gametocyte (Figure 5.5A). Zygotes are formed around 2 hours and around 12 hrs post fertilisation the first so called retorts can be detected with an antibody against the Pbs21 surface antigen of females, zygotes and ookinetes (Figure 5.4). Figure 5.5B shows the presence of the GFP-actin II in the residual cell body of the zygote (white arrow). The elongated retort, when emerging does not show at first any GFP fluorescence. Later on, the emerging ookinete elongates and simultaneously the fluorescence in the residual cell body decreases. This suggests that the protein is redistributed across the cytosol during maturation. Mature ookinetes do not demonstrate increased fluorescence, suggesting that no new protein is produced.

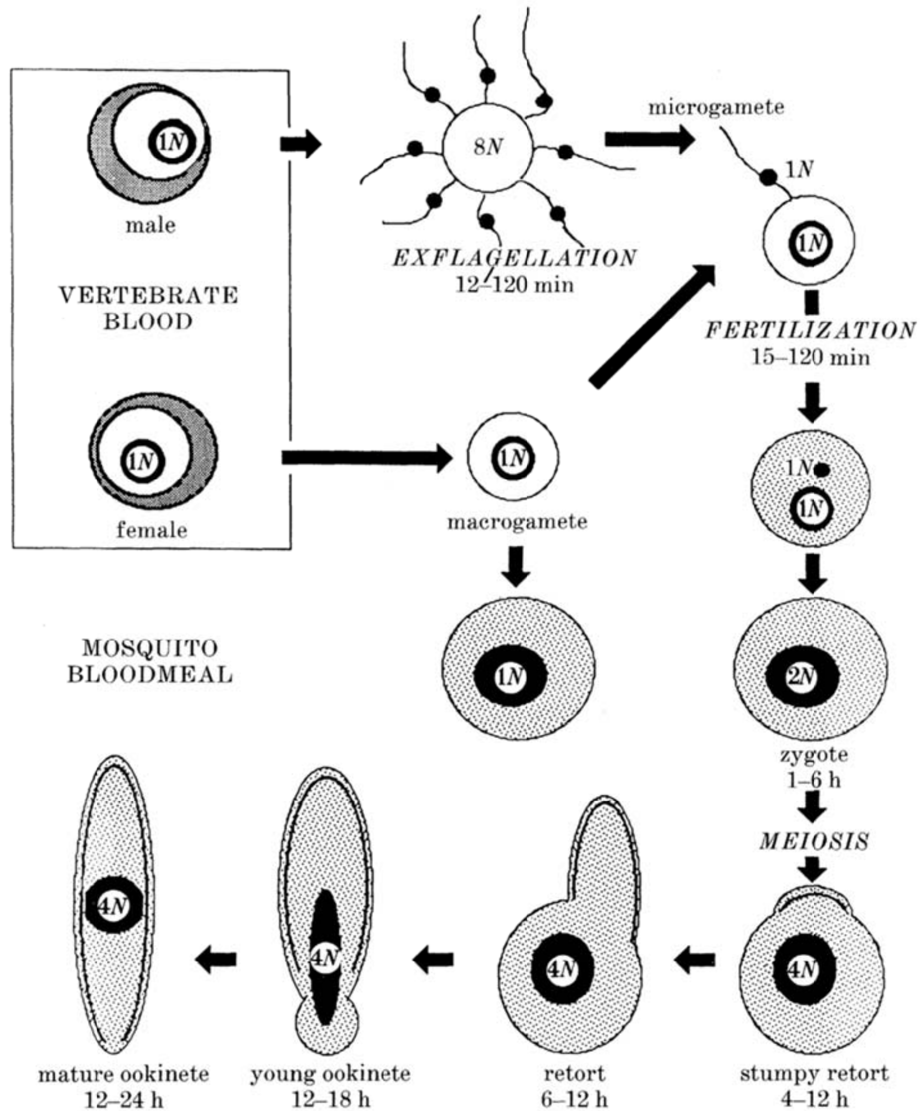


Figure 5.4. Ookinete development

The zygote is formed upon fusion of the male and female gamete. After about 6 hrs a retort emerges from the cell body developing within the next 6 to 12 hrs into a mature ookinete. Adapted from (Sinden et al., 1985).

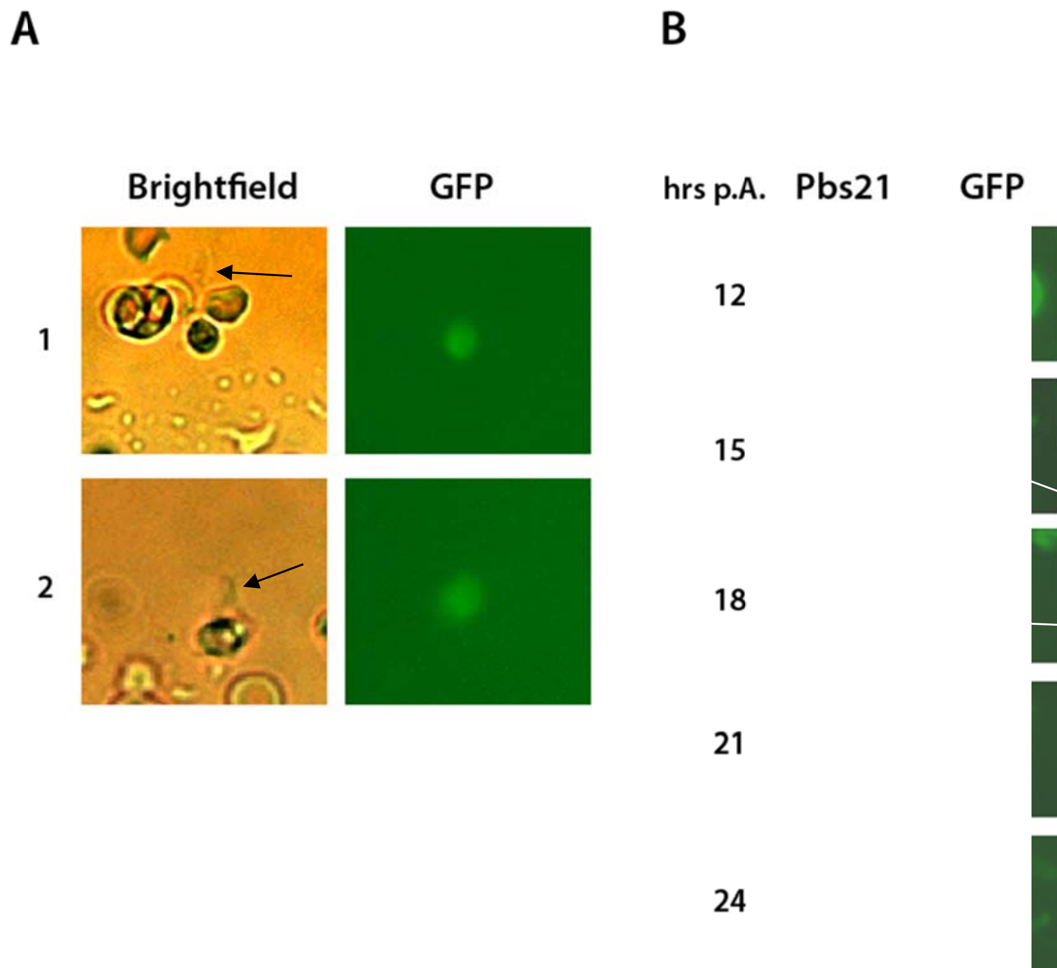


Figure 5.5 Time dependent expression and relocalisation of GFP-actin II

A: GFP-actin II expression in live male gametocytes. Fluorescence is restricted to the residual body of the male gametocyte. Flagella are seen in brightfield (arrow) B: Fixed samples labelled with an anti-GFP antibody show localised actin II presence in the residual zygote cell body (white arrow) which become redistributed upon maturation of the elongated ookinete. (*: autofluorescent RBC, as no Pbs21 labelling is detected)

5.4 Defect in the female results in developmental arrest

We knew from previous experiments that overexpression of the GFP-actin II resulted in a block in ookinete formation. To achieve over expression we increased the number of episomes carrying the *GFP-actin II* fusion gene by raising the concentration of antibiotics during growth of the parasite in the mouse. To determine if the block in ookinete formation was due to a defect in the male or

female gametocytes we crossed the *GFP-actII* parasite with the *Δ47* strain, which produces only males, or the *Δ45/48* strain, which produces only fertile females (see appendix). In this case exflagellation did take place in the *GFP-actII* strain and ookinetes were also formed in the latter cross, confirming that the *GFP-actII* males are fully fertile. No ookinetes were formed in the cross with the *Δ47* mutant. This gave us the possibility to cross the *GFP-actII* strain with the *actII(-)* mutant, which results in ookinetes having actin II and the GFP fusion protein expressed for the male derived genome. We carried out immunofluorescent assays with crosses of the *GFP-actII* males with either the *Δ45/48* or *actII(-)* females. GFP fluorescence was determined, as well as the presence of actin II. Figure 5.6 shows that the ookinetes in the *actII(-)xGFP-actII* did not express either actin II nor GFP. This is in contrast to the *Δ45/48xgfp-actII* ookinetes, which express actin II. This suggested that the actin II present in the ookinete is expressed from the gene present on the female derived genome, as we observe normal expression when crossed with the *Δ45/48*, but no actin II expression when *GFP-actII* is crossed with the *actII(-).*

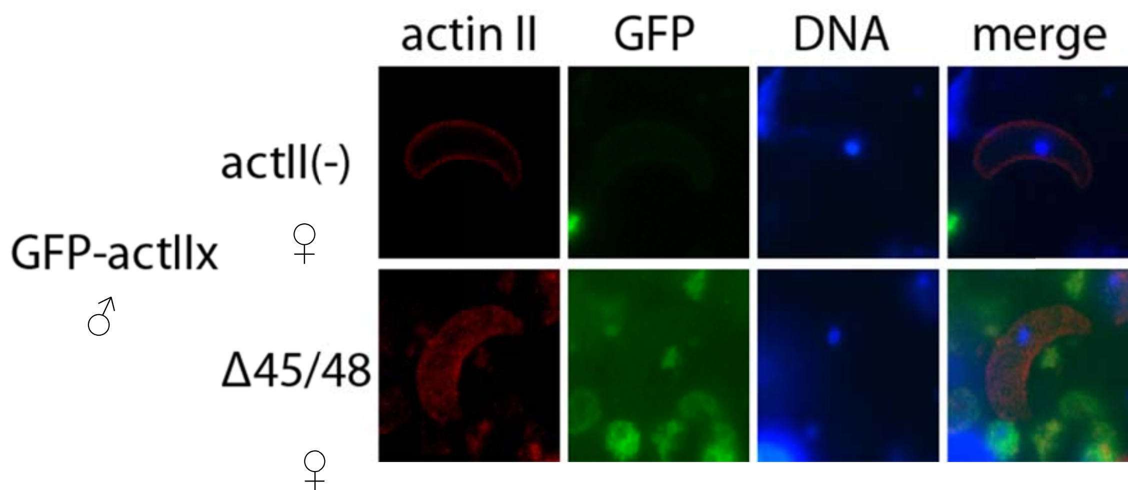


Figure 5.6. actin II in crosses with the GFP-actII strain

Top row: Ookinetes resulting from a cross with GFP-*actIIxactII(-)* showed no actin II expression despite the male carrying an overexpression vector in addition to the wt gene of *actin II*. The *Δ45/48* ookinetes displayed actin II expression, although no GFP was observed

Furthermore no GFP labelling of neither of the ookinetes was observed so even the presence of an episome which leads to overexpression of the protein in the male did not contribute to the protein levels in the ookinete. Overexpression of actin II in the female, on the other hand, resulted in a block in ookinete formation. This provides further evidence that expression of actin II in the zygote dependent in the gene derived from the female. We tried to pinpoint whether the defect in the female is due to lack of egress, an inability to be fertilised or that the zygotes cannot progress through to ookinete development. The markers that are currently available do not allow distinction between females or zygotes. However, we know that the diploid nucleus of the early fertilised zygote remains initially for the first few hours, and that meiosis forming the tetraploid ookinete occurs thereafter. An early zygote is pointed out with the arrow in Figure 5.7, which is distinguished by its smaller nucleus. These observations suggest that when the GFP-actin II fusion protein is expressed at high levels zygotes are formed but these zygotes cannot transform to ookinetes.

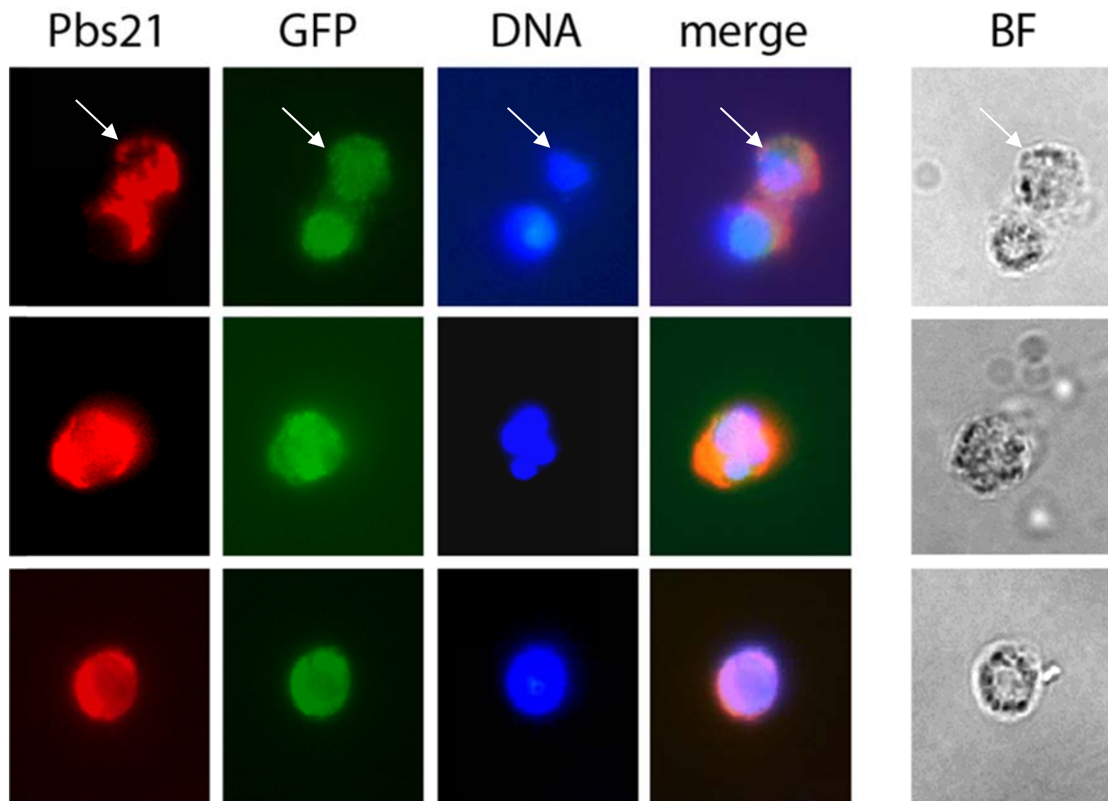


Figure 5.7 Three zygotes formed from self-fertilisation of the GFP-actII *strain*

Zygotes formed after 3 hrs displayed increased GFP expression as well as a larger nucleus due to a step of meiosis. The arrow indicates an early zygote not having undergone meiosis. No ookinetes were formed from this sample.

5.5 Discussion

The use of a strain expressing GFP tagged actin II allowed us to perform several experiments suggesting that the protein in the zygote and ookinete is expressed from the female copy of the gene and primarily during the zygote stage. The time course experiment revealed new insights into the expression of actin II. In *P. berghei* the stages of ookinete development have not been strictly defined, while in *P. falciparum* five stages have been defined (Ghosh and Jacobs-Lorena, 2013). Between one and six hours after fertilisation the zygote persists. From this round cell, a 'bud', where the so called apical complex is, is laid down at four hours and this is the first evidence of the development of the polar cell. Further extension of the microtubule cytoskeleton away from the zygote cell body results in the elongated, so called retort being formed. Immature ookinetes are visible after ~12 hrs and after approximately 18 hrs the ookinete is mature and ready to invade the midgut (Sinden et al., 1987). From Figure 5.5 it can be deduced that actin II expression occurs pre-ookinete formation and that the protein is concentrated in the round residual zygote cell body. Fluorescence increases until 12 hrs and the protein continues to be restricted to the cell body. Then fluorescence decreases, suggesting that no new protein is produced; the protein is no longer restricted to the cell body, but appears to be present in the whole cell. From these data one cannot determine with certainty when and where the protein is needed, although the strong signal in the zygote stage may indicate a function at this early time point.

By using a method to increase the expression of a GFP-actin II fusion protein we obtained functional male gametes, that could be used to cross fertilise our *actII(-)* mutant, which forms fertile females. These males did not rescue actin II expression in the ookinete (Figure 5.6). This led us to hypothesise that gene expression in the zygote of *actin II* is dependent upon the female derived copy of the gene. Crosses with the *Δ45/48* females and the fusion strain resulted in actin II expression as in this case the females contain the wt allele of the gene. However, also in this case GFP expression was not observed. One reason for this could be that the episome is

not passed with the male gamete in sufficient numbers to allow visualisation in the female. Another possibility could be that the episome did not contain the relevant regulatory regions for zygote actin II expression.

Further examination of the reason for the lack of ookinete formation in the mutant strain overexpressing GFP-actin II revealed that the female gametocytes are fertilised by the male. The typical exflagellation centres facilitating zygote formation were present indicating normal commencement in fertilisation (Eksi et al., 2006). However, upon closer examination the zygotes formed were arrested in their development. Retorts, which are formed around six hours, were not observed. These data suggest that there is a function of actin II required in the zygote. The over expression of the GFP-actin II fusion protein possibly interfered with essential mechanisms in the zygote and was therefore blocked in further development. This created an antimorph, which is a term for dominant negative mutations. This dominant negative effect with GFP tagging has been described in other studies (Miao et al., 2010, Rapicavoli et al., 2010). When the actin II protein is completely absent, on the other hand, development of ookinetes is normal and the lack of the protein only becomes apparent in the oocyst stages.

Chapter 6

General discussion

In all eukaryotic organisms actins play a vital role in essential cellular functions. Many different isoforms exist, with some being redundant. In apicomplexan species only one actin has been found, with the exception of *Plasmodium spp*.. Previously, we studied a mutant lacking the second actin, called actin II. It was found that the male gametocyte was severely impaired in exflagellation resulting in a block in transmission through the mosquito. After our detailed study of the function of the gene in the blood stages, we went on to analyse the function in the mosquito stages. Figure 6.1 depicts the protein expression of actin II we discovered during this study. We determined that expression of actin II is sex specific from the female in the mosquito stages. The protein is transcribed in the activated female gamete and translation occurs upon zygote formation. Actin II initially remains in the residual cell body from the emerging retort. In the absence of actin II ookinetes are still formed, but a defect can be detected in the oocysts stage. The oocysts do not develop into mature oocysts, because of a growth defect as well as a lack of DNA replication. The transmission cycle in the mosquito is blocked.

The inability to form male gametes of the mutant lacking actin II posed a challenge for the study of the gene in the insect stages. I here studied expression of the gene using RT-PCR analysis and specific antibodies detecting the protein. It was determined that the cognate transcript as well as the protein was present in gametocytes and in ookinetes but not in later stages.

The idea of using promoter exchange constructs to alter actin II expression was intended to be used to pinpoint the requirement of actin II by absence or addition to certain stages. Previously this has been shown to be highly successful and with the ease of transfection protocols in *P. berghei*, the results were highly anticipated. Creating a knock-down in the gametocyte stages, where the histone chaperone protein FACT is normally expressed utilised this technique (Laurentino et al., 2011). As the gene is essential in the asexual blood stages, the promoter was exchanged with one only active in these stages. This led to a downregulation of the protein in gametocytes, resulting in the delay of DNA replication and gamete formation in the male. No effect was found in the female. The oocysts formed with the mutant

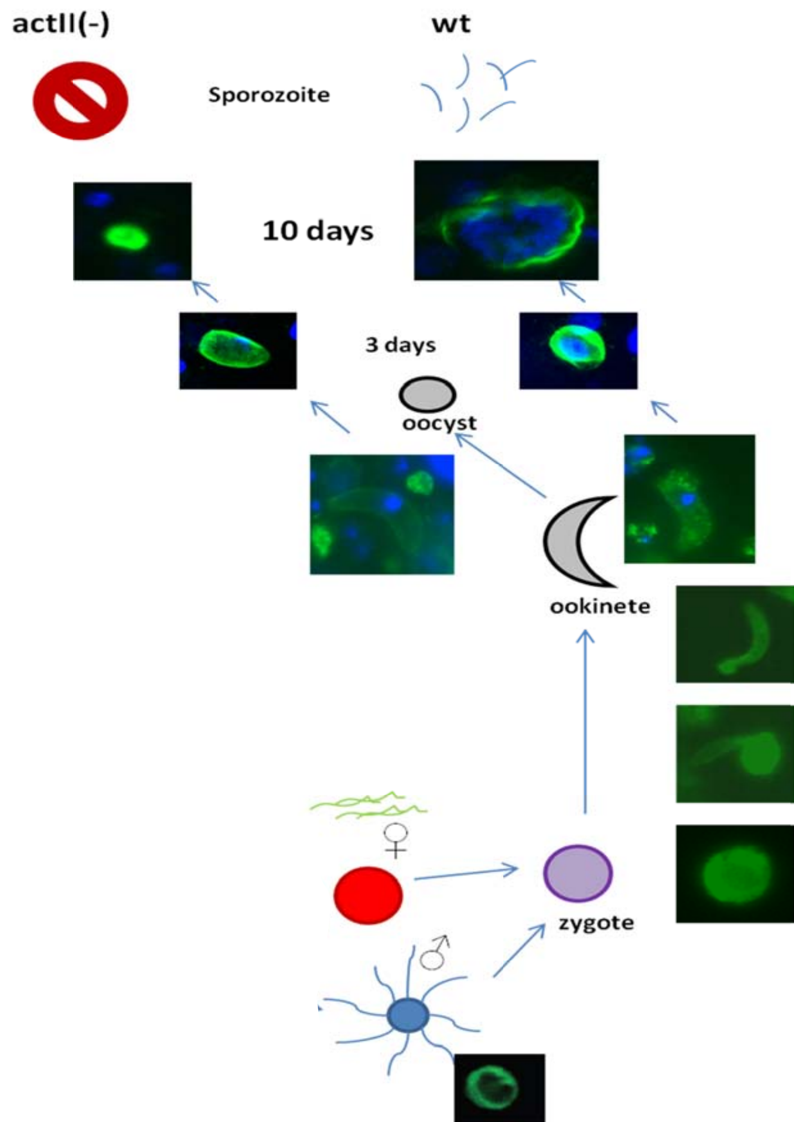


Figure 6.1 Actin II in P. berghei

Actin II transcription and expression is followed through the life cycle. Actin II protein expression in the blood stages is limited to the cytoplasm of the male gametocyte (green in insert), while only the transcript (curved green lines) is found in activated females. The protein is the again expressed in the zygote, from the female derived genome (in green). In the ookinete the protein is redistributed into the cytoplasm from the residual cell body in the last stages of development (green).The ookinete then develops into the oocysts where two scenarios can take place (Blue :DNA; green: Cap380). Left pathway: Absence of actin II leads to ookinete formation but a transmission block due to the oocysts not developing. Right pathway: Oocysts develop normally and sporogeny can be observed.

male gamete were arrested early in their development and did not show sporulation or transmission. But our promoter exchange experiments were unsuccessful. Since then several new methods have been devised to alter protein expression in the parasite. These could now be employed to gather information. Using a tetracycline-repressible transactivator system one can regulate gene repression (Pino et al., 2012). However, due to the sex-specific regulation in the zygote stages, this option would not be applicable for the actin II protein.

Female gametocytes have been shown to have an active *actin II* promoter, although no protein is expressed (Deligianni et al., 2011). This study has shown that protein expression is initiated upon zygote formation for actin II and that transcription had to have occurred in the female, not the male. This adds *actin II* to the continuously emerging proteins required sex-specifically for expression in the mosquito stages. Such genes include the family of lap genes. *PbLAP1, 2, 4* and *6* all have vital functions in the mosquito stages, specifically from the transmission from mosquitoes back to the host (Raine et al., 2007). The wt allele had to be inherited from the female gametocyte. The *actII(-)* can still form ookinetes, as the female inheritance of the gene is only required for the oocyst stage. This requirement of a protein not directly needed in the stage it is expressed in, is not the first finding of its kind. The nuclear formin-like protein MISFIT has been described to be arrested in the ookinete to oocyst transition (Bushell et al., 2009). The mutant phenotype displays degeneration of oocysts after day 3. This had been attributed to the reduced quantity of DNA at the ookinete level in this mutant. It has been proposed that MISFIT is a regulator for cell cycle progression in the mosquito, much like the cdpk4 kinase is found to be a key regulator in cell cycle progression in the male gametocyte (Billker et al., 2004). Sex-specific expression was also required; only for the MISFIT protein the male allele is required for expression. But a common pathway cannot be suggested for cdpk4 and MISFIT. Even though we observe a DNA replication defect in the oocyst in the *actII(-)*, the ookinete is still tetraploid. This begs the question on what the function of actin II could be. MISFIT suggests that the DNA replication mechanisms are somewhat conserved in the ookinete and oocyst. While for actin II the two pathways seem to be have no relation to each

other, as actin II is somehow relevant for oocyst DNA replication, but not in the ookinete. In both cases however, the expression of the protein is taking place much earlier, than the time the phenotype is detected.

It is very difficult to assign a function to actin II in the mosquito stages, due to the delayed defects, which could mean that actin II is acting not as a conventional actin but rather in a different context. Actins form filaments which are required in important cellular process such as motility, cell division, cytokinesis and organelle movement. Actin I in the *Plasmodium* parasite is known to fulfil these functions by forming filaments. Only recently it was shown that actin II can also form filaments (Skillman et al., 2011). *In vitro* they require higher levels of the filament stabilising agent phalloidin than actin I. Also, they display a curved filament, rather than straight like actin I. This could indicate that actin II fulfils a different role in the parasite. Our group has created an exchange of the *actin II* coding sequence with the *actin I*. This resulted in a rescue of exflagellation, suggesting, that at least for the male gametocyte, actin I can carry out the function actin II fulfils (Maria Andreadaki, unpub. data). Whether this is also true for the mosquito stages remains to be investigated. It is possible that at least for the ookinete stages, actin II could also act as a transcriptional regulator. Actins, especially nuclear actins, have recently become strong contenders for regulating transcription (Miyamoto and Gurdon, 2012). In fact, F-actin polymerisation has been shown to activate phenotypic variance of the var-gene family of surface proteins (Zhang et al., 2011).

The only transcription factor protein family so far known in *Plasmodium,* is AP2 (Coleman and Duraisingh, 2008). AP2-O has been shown to control stage-specific gene expression in the ookinete, while AP2-L is responsible for expression of genes required for liver stage development (Iwanaga et al., 2012, Yuda et al., 2009). But this sex specific expression that we detected could also be due to translational repression release. The female gametocyte is known to store silent messenger RNA's (mRNA) which are found in the cytoplasm. These require long term maintenance and stabilisation until they are activated in the zygote. The DEAD-box RNA helicase DOZI is responsible for this preservation (Mair et al., 2006). If DOZI is

not present the zygotes do not develop into ookinetes, as the mRNA required for translation is degraded. It has been proposed that all of these translationally repressed mRNA encode proteins that are essential for the transition of the zygote to the ookinete (Mair et al., 2010). We have shown that an over expression of actin II resulted in arrested zygotes narrowing the timeframe of actin II requirement during ookinete development down to the first few hours. We therefore propose that actin II is also translationally silenced and maintained by DOZI in the female gametocyte. Very recent data provides further evidence for the storage of silent mRNA's and them being essential for transition of zygote to ookinete. Using actinomycin D and amanin to block transcription at fertilisation results in the formation of zygotes, which however, are arrested there after (Elena Deligianni, unpub. Data.). Using cycloheximide to block translation does not result in zygote formation. Therefore translation is essential for zygote formation, while transcription only becomes essential at a later stage. A kinase has been shown to be required for activation of these silenced mRNA's. cdpk1 is expressed in all life stages of the parasite, it has been shown that specifically in the female macrogamete this kinase activates mRNA's required in the zygote (Sebastian et al., 2012). The histone chaperone FACT and the RNA-binding protein Puf2 have also been shown to play a role in gene regulation (Laurentino et al., 2011, Muller et al., 2011).

The requirement for female inheritance suggests a switch from male to female requirement after activation, but we do not know enough yet about the differences of transcriptional regulation between the two and what they contribute to the later mosquito stages to make an informed hypothesis of its function. But as *Plasmodium* is the only genus of Apicomplexa which possess this gene, suggests that actin II is required for a function unique to this parasite. Actin II could possibly redefine the way we see actins in *Plasmodium*.

Chapter 7

Materials and methods

7.1. Parasitological methods

7.1.1 Parasite strains

Parasite strains used in this study were the wild-type ANKA 2.34 and the *actII(-)* strain described in (Deligianni et al., 2011) . In short, the knock-out of *actin II* was created by introducing the antibiotic resistance cassette DHFR/TS into the coding region via double crossover. Non-gametocyte producing strains HPE and ANKA 2.33 were used to distinguish between asexual and sexual expression (Dearsly et al., 1990, Janse et al., 1989). The genetic cross experiments were carried out using knockouts which only produced healthy males or females, *Δ47* and *Δ45/48* respectively (Khan et al., 2005, van Dijk et al., 2001). The *GFP-actin II* construct was also described earlier (Deligianni et al., 2011), resulting in expression of a fusion protein where GFP was fused via a linker of actin II. The construct was introduced as an episome.

7.1.2 Parasite maintenance

The parasite strains were maintained in 6 - 10 week old Theiler's original mice by intraperitonal (i.p.) injection of infected blood. All animals were carried out in full conformity with Greek regulations and the protocols approved by the Ethics committee of FORTH, Crete. Parasitaemia was determined by staining blood smears with Giemsa solution (Sigma) in the light microscope. 100 µl of infected blood was passaged to the next mouse until a maximum of 10 passages had occurred. After this a new stab from frozen was injected to ensure no chromosomal changes can occur. In order to increase gametocyte load for each culture, 200 µl of phenylhydrazine at 6 mg ml^{-1} was injected into each mouse at the time of passage. A parasitaemia between 15 – 20% with at least 8 exflagellations per field were used in experiments. Parasitaemia is calculated as a percentage of infected erythrocytes to non-infected erythrocytes, not distinguishing between sexual or asexual stage infected RBC's.

7.2. Mosquito methods

7.2.1. Mosquito rearing

A susceptible mosquito population of the G3 strain *A. gambiae* was kept in the insectary at a constant temperature of 28°C, 80% humidity and a 12/12 hour light/dark cycle. The female adult mosquitoes were allowed to feed once on naive mice to produce eggs. The larvae were kept in distilled water and fed with powdered cat food. Adult mosquitoes were fed a 10% glucose solution given on cotton placed upon the cages. For maintenance of the mosquito line female mosquitoes were allowed to feed on anaesthetised Theiler's original mice.

7.2.2. Mosquito feedings

The mosquitoes were starved in their cage at either a short period of time without any form of hydration for 3 – 5 hrs in an 19°C incubator or overnight with cotton soaked in distilled water for 16 - 20 hrs in the insectary and only placed into the 19°C incubator just before the feeding. Infected mosquitoes were kept at 19°C and in humid conditions.

In vivo feedings were carried out by letting mosquitoes feed directly on *P. berghei* infected mice. When more than one mouse was used for feeding during an experiment care was taken that the number of exflagellations per field were similar. The mice were anaesthetised and the mosquitoes were allowed to feed for 20 mins.

For *in vitro* feedings an overnight (o/n) *in vitro* ookinete culture was purified with ammonium chloride and counted. 400.000 – 600.000 ookinetes were used in each feeder. A feeder is a small cylindrical shape with two pipes on the top allowing water to pass through the hollow middle which warms up the feeder according to the water temperature. At the bottom a membrane, in this case stretched Parafilm©, is attached forming small pocket for blood to be offered to the mosquitoes. The water pumped through the feeders has a temperature of 37°C to mimic the vertebrates' body temperature. The ookinetes were mixed with fresh

blood from a naive mouse, mixed and 200 - 300 μl injected into each feeder. Starved mosquitoes were allowed to feed for 40 mins (Sinden, 1997).

The fed mosquitoes were left without food for 24 hrs and then separated according to whether they had fed or not. Glucose was offered from there on and replaced every three to four days.

7.2.3 Transmission to naïve mice

To assess if the parasite is able to be transmitted from the mosquito back to the vertebrate host 'bite back' experiments were performed. Mosquitoes which have been infected were kept for 21 days in order for sporozoites to develop and migrate to the salivary glands. On day 21 post infection mosquitoes were starved for three to five hours and allowed to feed for 30 mins on naïve C57BL/6 mice, a strain which is more susceptible to mosquito infection by sporozoites (Scheller et al., 1994). The mice were closely monitored for parasites and prepatency period determined as the number of days until the first day of detection of parasites in Giemsa stained blood smears. This is used as an indication of the infectivity of the parasite strain.

7.3. Parasitological methods

7.3.1. Schizont cultures

The culturing of schizonts allows for the reintroduction of pure schizonts into the mouse, used for synchronous infections or for the use in transfections. Schizont cultures were established by culturing 1 - 3% infected blood overnight at 36.5°C in a 75 ml flask on a slowly moving shaker. The schizonts were purified after 17 - 21 hrs with a 15% Nycodenz gradient. For a synchronous infection the schizonts were injected intravenously into the tail of a naïve mouse and 100 μl blood samples

taken from the tail at 15, 18, 21 and 24 hrs p.i.. See transfection protocol below for further use of schizont cultures.

7.3.2. Transfections

The wt ANKA 2.34 strain was used as a recipient strain for transfection of plasmids constructs. The procedure was carried out as described previously (Janse et al., 2006). In short, a purified schizont culture was incubated with 10 μg of the linearised transfection construct and electroporated using the Nucleofector© device from Amaxa GmbH, programme U-33. The transfected schizonts were immediately injected intravenously into the tail into two naïve mice. The parasites were allowed to multiply for 24 hrs and then during the following three days injected with 100 μl of pyrimethamine, 2.5 mg ml^{-1} i.p. to select for transfectants. Mice were bled at a parasitaemia of 5%, stabs frozen and DNA extracted for genotyping.

7.3.3 *In vitro* ookinete cultures

Ookinetes were produced *in vitro* as described before (Sinden, 1997). Briefly, blood from an infected mouse was collected by cardiac puncture into a heparinised syringe and diluted 1:10 into 19°C ookinete medium and incubated in the same temperature for 18 - 24 hrs. The ookinete medium contains RPMI-1640 with Hepes (25 mM), L-glutamine (2 mM), 2 g L^{-1} $NaHCO_3$, 10 ml penicillin (5000 U ml^{-1}), Streptomycin (5 mg ml^{-1}), 10% foetal calf serum, pH 8.0.

The culture was spun down at 250 g at room temperature (RT) for 10 mins. The ookinetes were then purified using one of two methods. The first method utilised magnetic beads (Dynabead©) coated with the 13.1 antibody detecting the surface antigen Pbs21 (Siden-Kiamos et al., 2000).

The second method was used when the quantity of ookinetes was crucial. The parasite pellet was resuspended in 0.17% M ammonium chloride (Sigma) 50ml per

0.7 - 1 ml of infected blood. The suspension was incubated on ice for 20 mins, leading to a lysis of the RBC's. The parasites were collected by centrifugation at 250 g at RT for 10 mins. The solution was washed twice in phosphate buffered saline (PBS), pH 7.2. The remaining pellet contained free blood stage parasites, ookinetes as well as RBC ghosts. The number of ookinetes was counted in a haemocytometer under the light microscope.

7.3.4 Ookinete conversions

In order to assess the parasite's ability to form ookinetes 5 μl of an ookinete culture was added to 10 μl of an antibody mix (100 μl of PBS, pH 7.2; 1 μl of 13.1 mAb and 1 μl of anti-mouse Alexa Fluor-555). The mix was incubated at RT in the dark for one hour and then viewed with a Zeiss Axioskop 2 plus microscope. Macrogametes, zygotes and ookinetes were counted; at least 100 cells were counted in each experiment. The conversion rate is the percentage of ookinetes as a fraction of the total number of counted cells.

7.3.5 Immunofluorescent assays of *in vitro* cultured ookinetes

7.3.5.1 Using the actin II antibody

All steps were carried out at RT unless otherwise stated, washes were in PBS. 1 - 5 x10^4 ookinetes were diluted in 400 μl of PBS. Each sample was collected on a poly-L-lysine coated coverslip (ø 13 mm) by centrifugation at 500 g for 10 mins. The supernatant was removed and the samples incubated in 500 µl of fixative (4% formaldehyde, 0.1% Triton in microtubule stabilizing buffer (MTSB, 10 mM MES, 150 mM NaCl, 5 mM EGTA, 5 mM glucose, 5 mM $MgCl_2$ pH 6.9)) for 10 mins. This fixative is used to stabilise the actin filaments. After removal of the supernatant 200 – 300 μl of ice cold Methanol is added to each well and incubated at -20°C for

2 mins. This was followed by two washes before incubation with AffiniPure Fab Fragment Donkey Anti-mouse IgG (Jackson Immuno Research) diluted 1:50 in PBS with 5% NGS (Normal Goat Serum) for 1 hr at 37°C. The antiserum directed against actin II was pre-adsorbed for 15 mins with *actII(-)* gametocytes. Two washes were performed before incubation with the primary actin II antibody at a concentration of 1:200 in PBS and 5 % NGS o/n at 4°C. As a negative control parallel samples were incubated only in PBS. The two samples were washed twice in PBS, before the secondary antibody anti-mouse Alexa-488, diluted 1:1000 in PBS, 5% NGS was added and incubated for 1 hr. DNA was stained using two methods. For staining with TO-PRO the sample was first treated with RNAse for 20 mins on ice and then stained with TO-PRO diluted 1:500 in PBS for 30 mins. Alternatively, Hoechst 33342 (1:1000) was added for 10 mins. The samples were washed twice before mounting in Vectashield. When stained with Hoechst the samples were viewed with a Zeiss Axioskop 2 plus microscope fitted with an Axiovert CCD camera (Zeiss). With TOP-RO the samples were analyzed using a Zeiss LSM 510 confocal laser scanning microscope with Biorad lasers. Images were analyzed with ImageJ software (http://rsbweb.nih.gov/ij/).

7.3.5.2 Timecourse GFP stainings

The mice harbouring parasites transfected with the episomal *GFP-actin II* construct were treated with pyrimethamine (14 μg ml^{-1} diluted in drinking water, pH 3.5 - 5). To enrich for gametocytes mice were treated with sulphadiazine, 2.5 μg ml^{-1} in drinking water 40 hrs prior to the experiment (Beetsma et al., 1998). In these experiments 100 μl pyrimethamine (2.5 μg ml^{-1}) was injected i.p. once per day. 0.5 - 1 ml of blood was collected by cardiac puncture was added to 10 ml of ookinete culture medium. Samples were fixed and stained with an anti-GFP (Invitrogen) and the 13.1 antibodies to distinguish the developing ookinetes from the other parasite stages

For the 0 hr sample 200 μl of the blood culture was added immediately to 500 μl of 4% paraformaldehyde in PBS and incubated for 15 mins. The samples were then

collected on poly-L-lysine coated coverslips (ø 13 mm) by centrifugation at 500 g for 10 mins. The 3, 6, 12, 15, 18, 21 and 24 hr samples were collected on the cover slips and then incubated with the fixative. The samples were permeabilised with 0.1% Triton X-100 for 3 mins. This was followed by two washes, before blocking with the AffiniPure Fab Fragment Donkey Anti-mouse IgG (Jackson Immuno Research) diluted 1:50 in PBS, 5% NGS for 1 hr at 37°C and after two washes in PBS the antibodies were added as described The DNA was visualised with Hoechst 33342 (Dilution1:1000). The samples were viewed with a Zeiss Axioskop 2 plus microscope fitted with an Axiovert CCD camera (Zeiss). Images were analyzed with ImageJ software (http://rsbweb.nih.gov/ij/).

7.3.6. Determination of DNA content in ookinetes

Fixed ookinete samples that were stained with Hoechst 33342 according to the above protocols were examined with a Zeiss Axioskop 2 plus microscope fitted with an Axiovert CCD camera (Zeiss). Images were analyzed with ImageJ software (http://rsbweb.nih.gov/ij/). Fluorescence intensities were normalised to haploid parasites in the same fields. Increased fluorescence was indicative of diploid/tetraploid ookinetes according to their calculated ratio.

7.3.7. Oocyst detections

7.3.7.1 Via light microscopy

Mosquitoes were dissected in PBS at day 12 p.i. and their midguts placed on a glass slide under a cover slip and examined at a magnification of 400x. At this magnification and time point oocysts are detectable as round shapes on the surface of the midgut, with sometimes observable sporozoites. Oocysts are counted to assess the ability of the parasite to develop from ookinetes to oocysts.

7.3.7.2 Immunofluorescent assays

In order to monitor oocyst growth midguts were stained with fluorescent antibodies, as it is not possible to discern healthy oocysts with the naked eye until day 10. The protocol is as follows. The mosquitoes were dissected in PBS and their midguts immediately placed in a 2 ml Eppendorf tube containing 1 ml of fixative (4% formaldehyde (Polysciences), 0.2% saponin (Sigma) in PBS). The midguts were incubated on ice for 45 - 60 mins. All following steps were carried out at room temperature unless specifically mentioned. The midguts were then washed twice for 15 mins in PBS with 0.2% saponin on a shaker at low speed. Blocking was carried out for 30 mins in PBS with 0.2% saponin and 5% normal goat serum (NGS). The primary antibody was diluted 1:250 in PBS with 0.2 % saponin and 5% NGS and kept stationary at 4°C o/n. The guts were washed three times 15 mins in PBS with 0.2% saponin and 5% NGS. For all the following steps the tube is wrapped in foil to minimise light exposure. Incubation with the secondary antibodies was at a concentration of 1:1000 in PBS with 0.2% saponin and 5% NGS for 1 hr. Two 15 min washes in PBS with 0.2% saponin and 5% NGS. The midguts are incubated with 1 μl of RNAse (Qiagen, 100 mg ml^{-1}) in PBS with 0.2% saponin for 25 mins on ice. Then the DNA is stained with TO-PRO (Invitrogen) at 1:500 for 10 mins in PBS with 0.2% saponin. Lastly, two 10 min washes in PBS before mounting of the guts in Vectashield (Vectro Laboratories). The samples were analyzed using a Zeiss LSM 510 confocal laser scanning microscope with Biorad lasers. Images were analyzed with ImageJ software (http://rsbweb.nih.gov/ij/).

7.3.8. Sporozoite dissection

Sporozoites were isolated from dissected salivary glands and from midguts from day 14 post attachment to day 21 p.a.. Salivary glands or midguts were collected in RPMI 1640 and crushed with a pestle. They were centrifuged at 250 g for 1.5 mins at 4°C. The supernatant was collected in a fresh tube and put on ice, while 100 μl of fresh medium was added to the pellet, crushed again, spun and the supernatant added to the other supernatant. The sporozoites were spun at 700 g for 1.5 mins

at 4°C. The sporozoites were now contained in the pellet, which was resuspended in 30 μl of medium. The sporozoites were examined in the light microscope.

7.4. Plasmid generation for transfection constructs

The constructs were inserted into the transfection vector pL0001. The plasmid and DNA sequence of pL0001 are available from MR4 (http://www.malaria.mr4.org). The pL0001 plasmid contains the *T. gondii* DHFR/ts cassette which gives resistance to pyrimethamine.

The 5'FR sequences of *cdpk4* (PBANKA_061520), *α-tubulin II* (PBANKA_052270) and *mdv1-peg3* (PBANKA_143220) and *SET* (PBANKA_081990) were amplified from *P. berghei* ANKA 2.34 with primers shown in Table 7.1. Two fragments within the 5' FR of the *actin II* gene were the sites of the double crossover to stably integrate the construct. The right integration site was 456 bp from the coding region start site.

Genotyping was carried out using the primers in Table 7.2..

Gene sequence	Primers	Size in bp
***cdpk4* 5′FR**	cdpk4promF-EcoR1 GGAATTCAAATATAGCTATAAAAATTTACCT cdpk4promR-BamH1 CGCGGATCCTTTTATTATATATATAGGTATAT	1447
***α - tubulin II* 5′FR**	atubIIpromF-EcoR1 GGAATTCGGTAAGAGAGACTCCTGATGTGC atubIIpromR-BamH1 CGCGGATCCTTTCGAATAAATTTATCTAAAATAG	1256
***mdv-1/peg3* 5′FR**	mdv1promF-EcoR1 GGAATTCGACTAATTTTATTTGAAAAAAATA mdv1promR-BamH1 CGCGGATCCTGTGAATAGTATATATTTTTT	1324
***SET* 5′FR**	setpromF-EcoR1 GGAATTCGATCCACACATATTTGTATGCA setpromR-BamH1 CGCGGATCCTGGGGATATTACATATAGTCTT	1308
***actin II* 5′ FR**	actII5UTRF-KPN1 CGGGGTACCGCGAATTTACAAGTTCTTT actII5UTR-R-HindIII CCCAAGCTTCTAAAAATAAACAATTGTGAACA	377
***actin II* 5′ FR**	actIIcodF-BamH1 CGCGGATCCATGCCAGAAGAATCAATAGCTTTAGTG actIIcodR-Not1 ATAGTTTAGCGGCCGCTGTTATACCTGTGGTTCTTC	456

Table 7.1. Construct primers of promoter regions

Only the SET promoter has been defined. The actin II 5′ FR sequences were the sites of the double crossover. Restriction sites are underlined.

Primers	Purpose

L665: GTTGAAAAATTAAAAAAAAAC L635: TTTCCCAGTCACGACGTTG	Presence of episome
L695: AATATTCATAACACACTTTTAAGC actII5UTRF-KPN1	Presence of construct
cdpk4: cdpk4promF-EcoR1 *mdv-1/peg3*: mdv1promF-EcoR1 *SET*: setpromF-EcoR1 *α-tubulin II:* atubIIpromF-EcoR1 a2yoe1R: ACCCTCATAAATTGGAACAGTG	Correct right integration
A2KO5F: gg<u>ggtacc</u>ACCCAATTTTATATCATTCAATAC a2yoe1R	wt contamination

Table 7.2. Primers for genotyping of the constructs

Restriction sites are underlined.

7.5. Molecular methods

7.5.1. DNA extraction

The same protocol was used for DNA from parasites or mosquito midguts. The sample pellet was resuspended in 700 µl of TNE buffer (10 mM Tris pH 8.0, 5 mM EDTA pH 8.0, 100 mM NaCl), 200 μg ml^{-1} of RNAse, 1% (v/v) SDS and incubated for 10 mins at 37°C. 200 μg ml^{-1} of Proteinase K was then added followed by one hour incubation at 37°C. The sample was extracted once with phenol, once with phenol:chloroform:isoamylalcohol (25:24:1) and once with chloroform. DNA was precipitated with ethanol, washed with70 % EtOH, and the DNA pellet resuspended in ddH_2O and stored at -20°C.

7.5.2. RNA extraction

RNA was extracted from ookinetes and mosquito midguts using the TRI® reagent and from blood samples the TRI® reagent BD. 50µl of blood, 10 - 15 midguts or a small pellet of ookinetes was resuspended in 500 µl of TRI®. 100 µl of chloroform was added and shaken vigorously for 15 seconds, followed by incubation at RT for 10 mins. The sample was spun at 19000 g for 15 mins at 4°C. The aqueous phase was precipitated with isopropanol, the pellet washed with 75% EtOH, briefly air dried, resuspended in 10 - 20 µl of DEPC treated ddH_2O and kept at – 80°C.

7.5.3. cDNA synthesis

cDNA was synthesised according to the protocol of the Invitrogen Thermoscript™ system with the following alterations at the beginning. The RNA sample was DNAse treated. 1 µl of RQI DNAse (Promega), 1.1µl of 10xRQI buffer and 1 µl of Superase™ inhibitor (Invitrogen) was added to the first reaction mix. After the first incubation step the DNAse was removed by adding 1 µl of RQI DNAse STOP solution and incubated for 10 mins at 65°C. Then the company protocol was followed.

7.5.4. PCR

Reverse-transcription PCR was carried out with the primers in Table 7.3.

The primers used to determine the genotype of the parasites in the genetic cross experiments are shown in Table 7.4.

For detecting of the M- and S-form of mosquitoes MSF: TCGCCTTAGACCTTGCGTTA and MSR CGCTTCAAGAATTCGAGATAC (Santolamazza et al., 2008).

Gene transcript	**Forward primer**	**Reverse Primer**
actin II	a2yoe1F GGGATGGAACAAAAAGAATGC	a2yoe1R
SET	setMGS-F1 CCCAGTTAATATAGTATTGATGTGTT	setMGS-R1 GTTTTAAGCGCCTCTAAATG
mdv-1/peg3	mdvMGS-F1 AAAAATGGCTACGTGAAAAA	mdvMGS-R1 TTTCTTCTTCAGGGTCTTCA
gapdh	gapdhIntron-F1 GCCGTATCGGTCGTTTAGTATTC	gapdhIntron-R1 GTTATATTTCTCGTGGTTAATACcatg
actin I	act1F2 AGATGCCCAGAAGCATTATTTC	act1R2 AGATCCTCCGATCCAAACAG

Table 7.3. Primers used in reverse transcription PCR's

Genotype	Forward primer	Reverse Primer
wt	AIIYXF GCGGATCCATGCATCATCATCATCATCATGCTTCACCAGAAGAATCAATAGC	a2yoe1R
actII(-)	L665	AIIYXR gacAGATCTTTAGAAGCACTTTCTGTGAACG
actII(-) in control experiments	DHFF-CLA1 ccatcgatGTTTTTCTTACTTATATATTTATAC	a2yoe2r GTAATATGTAATCCAGGACATTCCC.

Table 7.4. Primers used for genotyping of genetic cross experiments
Restriction sites are underlined

7.6 Western blot

Samples were sonicated on ice, then 2 μl of β-mercaptoEtOH, 1 μl of PMSF and 0.2 volumes of 5xSDS loading buffer was added and the samples boiled before loading on a 12% SDS-PAGE gel. After gel electrophoresis the proteins were transferred to nitrocellulose membrane filters. The membrane filters were blocked in 5% skimmed milk for one hour. Incubation with the primary antibodies directed against actin II, diluted at 1:1000 in TTBS (50 mM Tris pH 8.0, 150 mM NaCl, 0.1% Tween 20) was carried out for one hour followed by three 10 min washes in TTBS. Secondary antibodies conjugated with horse radish peroxide were added for another hour at a dilution of 1:70000 in TTBS, followed by three washes of 10 mins. The signal was detected using the SuperSignal West Pico solution (Pierce Biotechnology).

7.7. Statistical analysis

Paired and unpaired data comparisons were carried out to determine statistical significant differences between samples using the unpaired t test in Microsoft Excel and GraphPad Prism 5.

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Appendix

Parasite strain	Fertile Gametocytes	Ookinetes	Oocysts	Sporozoites/ Transmission	Reference
actII(-)	Only ♀	Yes	Yes, but do not develop	No	(Deligianni et al., 2011)
ANKA 2.34 (wt)	Yes	Yes	Yes	Yes	
ANKA 2.33	No, only immature gametocytes	No	No	No	(Dearsly et al., 1990)
cdpk4p-actII	Yes	Yes	Yes	Yes	This study
GFP-actII*	High: Yes	No	No	No	(Deligianni et al., 2011)
	Low: Yes	Yes	Yes	Yes	
HPE	No	No	No	No	(Janse et al., 1989)
setp-actII	Yes	yes	No	No	This study
Δ45/48**	Only ♀	No	No	No	(van Dijk et al., 2001)
Δ47**	Only ♂	No	No	No	(Khan et al., 2005)

Parasite strains used in this study and their parasite stages

*High refers to pyrimethamine concentration of 70 μg ml^{-1} and low 14 μg ml^{-1}

** These strains produce ~10% of wt ookinete number when allowed to be fed in vivo. Therefore all experiments were carried in vitro to obtain only one of the sexes