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WELLCOME TRUST CENTRE
FOR MOLECULAR PARASITOLOGY
INSTITUTE OF INFECTION, IMMUNITY & INFLAMMATION

Χρήση μεταβολομικής για την ανακάλυψη ειδικών για τα γαμετοκύτταρα παραγόντων στον κύκλο ζωής του *P. berghei*

Using metabolomics to discover gametocyte specific factors in *P. berghei* life cycle

Κωνσταντίνα Γεωργίου

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ΠΕΡΙΛΗΨΗ

Η ελονοσία αναγνωρίζεται ως μια από τις πιο ολέθριες απειλές υγείας παγκοσμίως, αφαιρώντας τη ζωή σχεδόν ενός εκατομμυρίου ανθρώπων ετησίως, κυρίως παιδιών ηλικίας κάτω των πέντε ετών. Η μετάδοση πραγματοποιείται μέσω του κουνουπιού-φορέα Anopheline. Τα γαμετοκύτταρα είναι οι μοναδικές μορφές του πλασμωδίου που μπορούν να αναπτυχθούν περαιτέρω μέσα στο στομάχι του κουνουπιού. Εφ' όσον τα βιώσιμα γαμετοκύτταρα είναι απαραίτητα για τη μετάδοση της ασθένειας, η παραγωγή τους αποτελεί στόχο για την παρεμπόδιση της μετάδοσης. Σε αυτή την εργασία μελετήσαμε παράσιτα *Plasmodium berghei*, που προκαλούν ελονοσία σε τρωκτικά και χρησιμοποιούνται ως μοντέλα, τα οποία δεν περιέχουν το γονίδιο ανθεκτικότητας σε φάρμακα *mrp1*. Τα αποτελέσματά μας δείχνουν ότι η έλλειψη της MRP1 πρωτεΐνης οδηγεί σε μείωση της παραγωγής γαμετοκυττάρων στο *P. berghei*. Η μεταβολομική ανάλυση έδειξε μεγάλη ομοιότητα μεταξύ των μεταβολικών προφίλ παρασίτων αγρίου τύπου και των MRP1 μεταλλαγμένων, καθ' όλο τον κύκλο ζωής στα ασεξουαλικά στάδια. Διαφοροποίηση παρατηρήθηκε σε ελάχιστους μεταβολίτες, ένας εκ των οποίων ήταν το γ-αμινοβουτυρικό οξύ (GABA), το οποίο βρέθηκε σε χαμηλότερη συγκέντρωση στα MRP1 μεταλλαγμένα παράσιτα συγκριτικά με τα αγρίου τύπου. Στη συνέχεια, προχωρήσαμε στη διευκρίνιση του ρόλου του GABA στα σεξουαλικά στάδια διαγράφοντας ορισμένα γονίδια που πιθανόν εμπλέκονται στο μεταβολισμό του GABA στο *P. berghei*. Καταφέραμε να απομακρύνουμε πέντε από τα έξι γονίδια που στοχεύσαμε. Η φαινοτυπική ανάλυση που έχει πραγματοποιηθεί στις μεταλλαγμένες σειρές παρασίτων ως τώρα, υποδεικνύει ότι η διατάραξη του μεταβολισμού του GABA επηρεάζει την παραγωγή μαστιγίων και την ανάπτυξη των ωοκινών, τα οποία αποτελούν τα κύρια στάδια ανάπτυξης του παρασίτου στο κουνούπι-φορέα.

ABSTRACT

Malaria is recognized as one of the most devastating worldwide health threats that claims the lives of almost a million people yearly, mainly of children under five years of age. Transmission occurs though Anopheline mosquito vectors. Gametocytes are the only forms of the malaria parasite that can undergo further development inside the mosquito midgut. Since viable gametocytes are crucial for transmission of the disease, production of gametocytes is a target for transmission blocking. In this project we studied *Plasmodium berghei* parasites, a rodent malaria model, lacking the multi-drug resistant protein (MRP1) gene. Our results show that lack of the MRP1 protein resulted in reduction of gametocyte production in *P. berghei*. Metabolomics analyses showed high similarity between the metabolic profiles of the wild type and the MRP1 mutant parasites in general across the life cycle in asexual stages except for a few metabolites, one of which was γ-Amino Butyric Acid (GABA) which was found to be in lower abundance in MRP1 mutant parasites compared to wild type. We proceeded to elucidate the role of GABA in the sexual stages with deletion of certain genes that are possibly involved in GABA metabolism in *P. berghei*. We were able to knock out five genes out of six that we attempted and the phenotypic analysis performed on these mutants so far, suggests that disruption of GABA metabolism seems to affect exflagellation and ookinete development which are key events in the mosquito stage development of the malaria parasite.

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1 INTRODUCTION

1.1 General information about malaria

According to the WHO in 2012 approximately 3.4 billion people were living at the risk of contracting malaria. More than a third of these people (1.2 billion) were mostly residents in the African and South East Asian Region. In spite of the worldwide efforts to control and efficiently treat malaria, 207 million cases of malaria were reported in 2012, of which approximately 627,000 resulted in death. Almost 90% of the reported deaths each year occur in sub-Saharan Africa and about 77% in children under the age of 5. Although half the world's population is living at the risk of the disease, there has been a worldwide decrease both in malaria cases and mortality rates. However, the 2013 World Health Organisation report states that malaria is still endemic in 104 countries and territories [1] (Figure 1) and there is a rising threat to current treatment strategies due to increasing drug resistance.

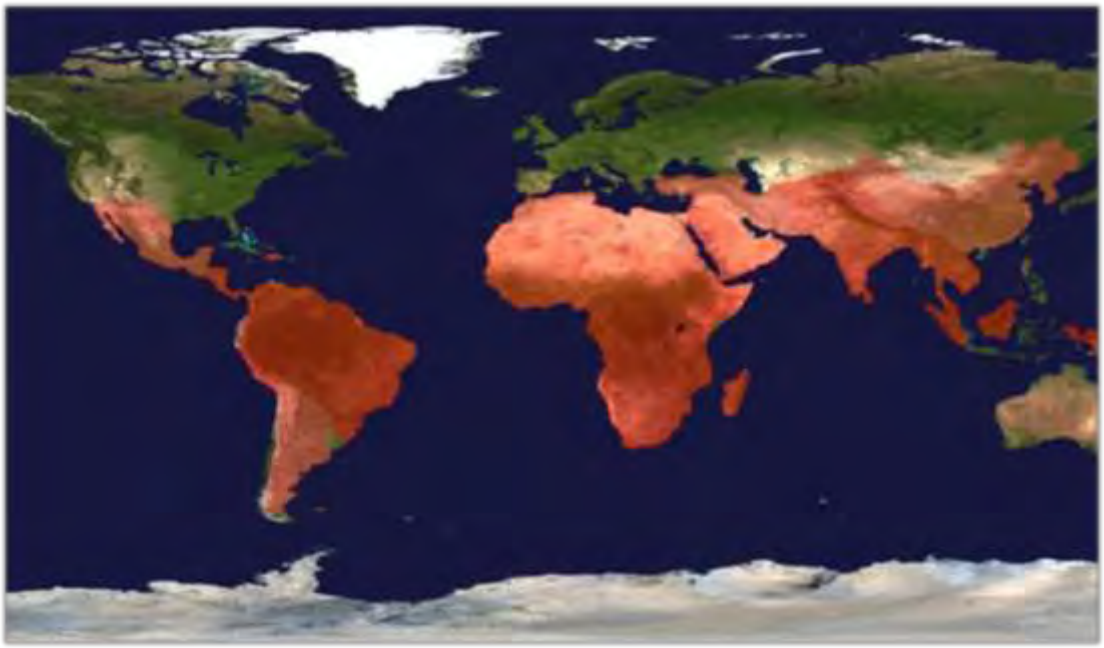


Figure 1: Map showing distribution of malaria around the world.
Regions affected by malaria are marked red.
Wellcome Trust Sanger Institute, 2008

Plasmodium species

Malaria is caused by intraerythrocytic protozoa of the genus *Plasmodium* and is transmitted by the bite of an infected female mosquito of the genus *Anopheles*. Five species are responsible for malaria in humans; *P. falciparum*, *P. vivax*, *P. ovale*, *P. malariae* and *P. knowlesi*. Of the five species, most dangerous and widely spread are *P. falciparum* and *P. vivax*[1]. 85% of all malaria cases and nearly all deaths are caused by *P. falciparum*,

making it the most deadly of the five species. It is possible that *P. falciparum* originated from *P. reichenowi*, a chimpanzee malaria parasite[2].

Plasmodium vivax was considered to be less lethal than *P. falciparum* because it rarely resulted in death. Nevertheless, *vivax* malaria is also a life threatening disease, as *P. vivax* possesses some features that, unlike *P. falciparum*, enable the parasites to survive during winter months and spread to more regions. *P. vivax* can develop in the Anopheles vector in lower temperatures than *P. falciparum* and the parasites can remain in a dormant, hypnozoite stage for long periods of time[3].

Treatment of malaria

Vaccines

An efficient vaccine against malaria would prevent the development of the sexual stages of the parasite and therefore block transmission and simultaneously stop spread and replication of asexual blood parasites which are responsible for the pathology of the disease[4]. The most studied types of vaccine candidates are:

1) Transmission blocking vaccines with intention to block the development of the sexual stages of the parasite in the mosquito midgut or salivary glands.
2) Pre-erythrocytic vaccines, aiming to kill the parasites at the liver stage and prevent their release into the bloodstream of the human host.
3) Blood stage vaccines which are targeted against membrane antigens of the parasite in order to block the invasion of erythrocytes by parasites[5].

Drugs - resistance

Development of new efficient antimalarial drugs has been a struggle because the parasites easily acquire resistance to effective drugs rendering them unsuitable for wide use. Drugs such as chloroquine and sulfadoxine-pyrimethamine used to be the first powerful drugs against the disease and helped eliminate malaria from North America, Europe and parts of Asia and South America before the parasites acquired resistance to the drugs. Some of the new drugs used to treat malaria are artemisinin and its derivatives, atovaquone, proguanil and chloroquine; chloroquine is mostly used for *P. vivax* infections as *P. falciparum* resistant lines have been reported[6]. The first cases of chloroquine resistant parasites were reported in the late 1940s and within a short period of time the resistance spread to most areas where malaria is considered endemic. *P. falciparum* has acquired resistance to almost all the antimalarial drugs that have been used to date. Although artemisinin and its derivatives have proven to be powerful drugs, the recent indications of acquisition of artemisinin resistance makes the need for new effective drugs imperative[7].

Malaria control efforts

Since 2010 malaria control programmes have been able to expand their control policies due to increased financing. As a result, there has been a worldwide reduction of malaria incidents and deaths with a 45% drop in mortality rates noted in all ages in the period of 2000-2012 (Figure 2). In 2013 the funding that was internationally disposed for malaria control efforts was 1.97 billion dollars[1]. Although this amount is not sufficient to reach the global targets set by the WHO it is a remarkable improvement over the 100 million dollar funding of 2010.

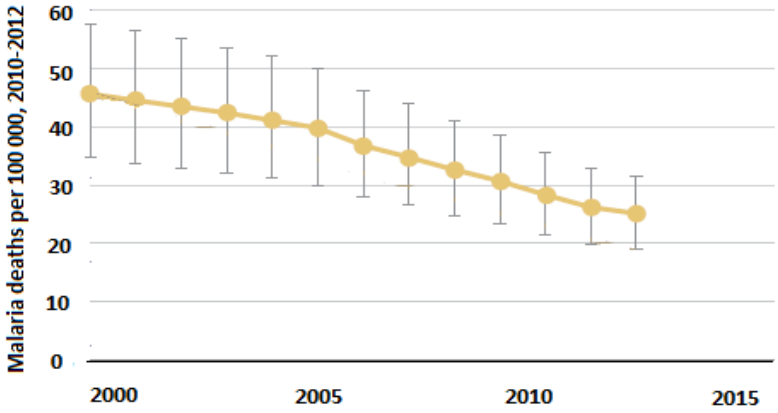


Figure 2: Mortality rates 2000-2012.
WHO report 2013

The strategies implemented in order to control and eliminate malaria include vector control, diagnostic testing and appropriate drug treatment.

Vector control: the policies that are being applied in order to reduce the population of mosquitos as well as the human – vector interaction are:

- Insecticide Treated mosquito Nets (ITN): World Health Organisation suggested the free distribution of ITNs to malaria endemic regions. Even though the number of ITNs delivered and used by people at risk is increasing, 100% ITN coverage has not been reached yet.
- Indoor Residual Spraying (IRS): efforts for IRS have been restricted because of the confirmed resistance of mosquitos to at least one insecticide. In order to manage the threat of insecticide resistance, more costly non-pyrethoid insecticides are in use thus limiting the number of people that can be protected.
- Intermittent Preventive Treatment (IPT): according to recent evaluations, IPT benefits 35 million pregnant women and a significant amount of the 26 million infants born, each year.

Diagnostic testing: diagnostic testing must be applied in public and private sectors in order to reduce the mistaken use of antimalarial drugs in suspected cases of malaria and increase the number of properly treated people with confirmed infections[1].

1.2 *Plasmodium berghei*

P. berghei is one of the four *Plasmodium* species that infect murine rodents. Introduction of rodent parasites to the laboratory has contributed enormously to understanding the biology and immunology of malaria parasites. Rodent malaria parasites are commonly used for research purposes. Use of rodent malaria parasites has helped advance knowledge of the disease as well as the development of therapeutic measures. Despite some dissimilarity between rodent and mammalian malaria, rodent parasites have proven to be useful practical models since their structure, physiology and life cycle resemble those of mammalian parasites.

Plasmodium berghei is an excellent research model for a number of reasons. Firstly, it is easily studied *in vivo* since it is not infectious to humans. Secondly, the basic biology, genetics and genome organisation remain similar between human and rodent malaria. The sexual stages of *P. berghei* can be cultivated *in vitro* and the purification of the parasite at various stages of the life cycle is possible. The genomes of both the parasite and the mosquito are known and there are technologies available for their genetic modification. Finally, there are several constructed lines that have been genetically modified in order to be advantageous for laboratory use, such as mutant parasites expressing reporter genes (Green Fluorescent Protein and Luciferase)[8].

Life cycle of *Plasmodium berghei*

An infection begins with the bite of an infected female *Anopheles* mosquito and the subsequent release of the sporozoites in the blood stream of the vertebrate host (rodent) which are carried by the peripheral circulation and home to the liver invading its cells. Within the following 47-52 hours, the sporozoites grow inside the hepatocyte, divide and mature into schizonts containing haploid merozoites. In some species, such as *P. vivax*, the parasites can remain inactive in the liver for extended periods in the form of hypnozoites. In *P. berghei* there is no evidence of the hypnozoite stage in the liver. The merozoites are released from mature liver schizonts, enter the circulation and invade red blood cells. *P. berghei* has a strong preference for reticulocytes (immature erythrocytes) but can also invade mature red blood cells. Within an erythrocyte the merozoite grows from a ring stage into a mature trophozoite. The parasite then enters the schizont stage during which the parasite divides forming 8-24 merozoites. After the egress from a lysed erythrocyte, the newly formed merozoites are released in the circulation and reinvade erythrocytes. The lysis of a significant amount of erythrocytes during the asexual development of malaria parasites is responsible for the clinical manifestations of the disease. Some of the merozoite-infected blood cells do not continue the cycle of asexual multiplication, instead they develop into sexual precursors, called gametocytes (Figure 3). In each blood stage cycle, 5-25% of the parasites are committed to sexual differentiation. It is not known when

commitment to sexual differentiation takes place in *P. berghei* parasites. Even though there is evidence of the influence of innate and environmental factors in the switching mechanisms, the molecular mechanisms that induce and regulate the switch from asexual multiplication to sexual differentiation have only recently started to unravel[9]. Gametocytes through sexual reproduction are responsible for the transmission of the disease. There are some differences in the development of *P. berghei* and *P. falciparum* gametocytes. In *P. berghei*, the merozoites of liver schizonts are able to differentiate directly into gametocytes after invasion of the erythrocyte[10]. The developmental time needed for *P. berghei* gametocytes is 26-30 hours which is quite short compared to the 8-11 days *P. falciparum* gametocytes require to develop. Also the morphology of the gametocytes of these two species is different, *P. falciparum* has banana-shaped gametocytes and *P. berghei* has round to oval shaped gametocytes. When a mosquito feeds on an infected vertebrate host, it ingests *Plasmodium* infected erythrocytes including gametocytes, the only cells capable of further development in the mosquito midgut. In the mosquito midgut, gametocytes activate and differentiate into gametes. Differentiation of the female gametocyte produces a single female gamete, the macrogamete. The male gametocyte differentiates into eight sperm-like motile microgametes. The maturation process of the microgametocyte is known as exflagellation. The differentiation of the gametocytes has been shown to be generated by three environmental factors: a temperature drop of at least 5°C in the infected red blood cells (temperature of the red blood cells inside the mouse is 37°C, whereas in the mosquito it is 21°C), a rise in pH from 7.3 to 7.8-8.0 and the presence of a gametocyte activating factors such as xanthurenic acid. After activation male and female gametes fuse to form diploid zygotes. Within 18-24 hours the spherical zygote develops into a banana-shaped, motile ookinete which traverses the wall of the mosquito midgut and develops into an oocyst on the basal lamina. Growth and division within each oocyst produces thousands of haploid sporozoites; 1500-8000 sporozoites can be contained in each oocyst. Upon sporozoite maturation, after 8-15 days, the oocyst bursts releasing sporozoites which then travel to and invade the mosquito salivary glands (it has been found that only a small percentage of the sporozoites, about 2%, reach the salivary glands). The sporozoites can survive in the salivary glands for weeks before being injected into a new host. The cycle of infection re-starts when the mosquito takes a blood meal, injecting the sporozoites from its salivary glands into the bloodstream of the vertebrate host[8, 11].

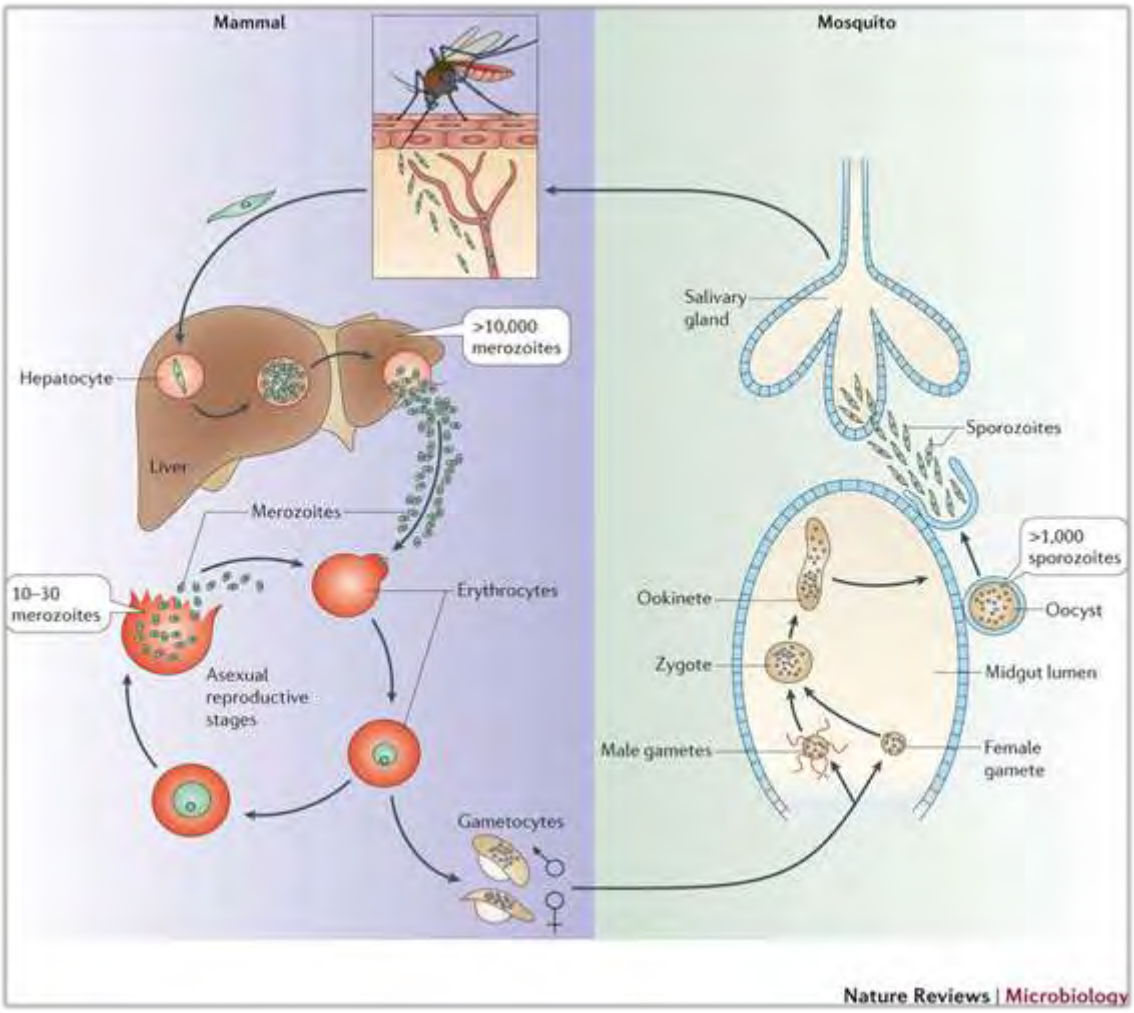


Figure 3: Life cycle of Plasmodium.
Nature reviews/ Microbiology 11,701–712 (2013)

Sexual differentiation in *Plasmodium berghei*

The developmental switch from asexual multiplication to sexual development is induced and regulated by molecular mechanisms. As a result, a cascade of gene expression, involving a number of sexual-stage specific genes and their protein products, is activated. There is evidence that in both *P. falciparum* and *P. berghei*, the switching mechanism is regulated by innate and environmental factors. In *P. falciparum* some of the environmental conditions that can affect gametocytogenesis are the presence of erythrocyte lysate, host immune sera and lymphocytes, the drug chloroquine and high number of asexual parasites[12].

There is also evidence of the association of sexual commitment with heterotrimeric G proteins and the specific signal transduction pathways that can be activated by these proteins. When a G-protein coupled receptor binds to a ligand, the α subunit is activated and can interact with several effector molecules, such as adenylate cyclase or phospholipase C. The effector molecules can subsequently activate different enzymes such as Protein Kinase A (PKA) and Protein Kinase C (PKC) and finally result in activation of

transcription factors and expression of specific genes required for gametocytogenesis (Figure 4). The involvement of G proteins in sexual differentiation was supported by data which showed that gametocytogenesis is stimulated by the addition of cholera toxin to *P. falciparum* cultures. Cholera toxin is an activator of the Gas class of heterotrimeric G-proteins. Furthermore, it was demonstrated in *P. falciparum* that expression of the Gα subunits was stage-specific, with highest levels of expression during asexual development and in mature gametocytes. Since expression of G-proteins is considered a marker of the cell's sensitivity to environmental conditions, increased expression of G-proteins in the free merozoite could account for the parasite's response to the environmental conditions through different metabolic processes; processes which can, amongst other factors, define the number of parasites that will differentiate into gametocytes[13].

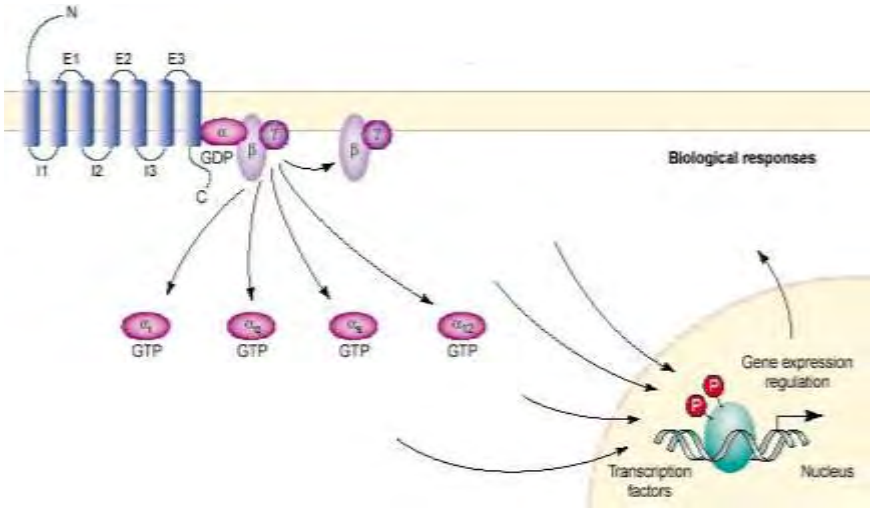


Figure 4: G-protein based signalling.
Dopamine Receptor D4 Database (G-Protein Coupled Receptors)

Gametocyte non-producer lines of *P. berghei*

The capability to produce gametocytes can be lost during prolonged periods of multiplication in the vertebrate host or in *in vitro* cultures when passage of the parasite through the mosquito does not occur. This is due to the loss of selection pressure as the parasite can continue its growth without transmission by the invertebrate host. It has been shown that lack of only one gene, AP2G gene (PBANKA_143750), is sufficient for loss of gametocytogenesis[9]. Line G401cl1 is an AP2G knock out and does not produce any gametocytes.

Gametocyte low-producer lines of *P. berghei*

In our laboratory we have observed that the level of production of gametocytes can be affected by the deletion of certain genes from *P. berghei*'s genome. Parasite lines

1025cl1 and 1512cl1 are gene deletion mutants of the MRP1 gene (PBANKA_144380) and produce low number of gametocytes.

- Line 1512cl1 is made in 820 background meaning that the female gametocytes produce RFP and the male gametocytes GFP.
- Line 1025cl1 is made in 507 background resulting in the production of GFP from the parasites all through the cell cycle.

1.3 ABC transporters

ATP binding cassette transporters are amongst the largest evolutionary conserved protein families. ABC transporters bind and hydrolyse ATP molecules to provide energy for the transportation of their substrates. These transporters are responsible for the translocation of various substrates, ranging from ions to macromolecules[14]. ABC transporters are located on the membrane and are categorised as importers or exporters depending on their function. Importers are only found in prokaryotes and are responsible for nutrient uptake[15]. ABC exporters are present in both eukaryotes and prokaryotes and play a key role in cellular exportation of endobiotics and xenobiotics[15, 16].

Overexpression of certain ABC exporter proteins contributes to drug resistance in organisms ranging from human neoplastic cells to parasitic protozoa. Although these proteins have mostly been studied for their role in cancer multidrug resistance, some members of the ABC family use the energy acquired from ATP hydrolysis for cellular processes, such as DNA repair and mRNA translation[17].

Multi-drug Resistant Protein 1 transporter

The ABC transporter family is divided into 7 subfamilies, named from ABCA to ABCG. MRP1 belongs to the ABCC subfamily.MRP1-related proteins have been identified in mammals and in a variety of non-mammalian organisms including yeast[18], protozoa, nematodes[19] and plants[20].MRP1 was the first gene of the ABCC subfamily to be identified in humans. The gene was identified because its overexpression resulted in resistance against many chemotherapeutic agents in a human lung cancer cell line[21].

Structure: Usually MRP1 proteins consist oftwo Nucleotide Binding Domains (NBDs) and two Membrane Spanning Domains (MSDs). Mammalian MRP1 transporters have a 5-domain structure constituted by two NBDs and threeMSDs (Figure 5)[22].

Substrates and Function: MRP1 is responsible for the transportation of a wide range of substrates. It is suggested that overexpression of MRP1 leads to development of multidrug resistant cells. In humans, the transporter is considered critical mostly due to its ability to efflux variousanticancer drugs, such as anthracyclines and methotrexate, as well as plenty of other types of drugs e.g. anti- HIV drugs[23].

Conjugates of glutathione, glucuronides and sulphate can be transferred across the cellular membrane by MRP1. These conjugates are products of Phase II metabolism in cells and their efflux by MRP1 is part of the cell detoxification from endo and xenobiotics. The transporter also has a high affinity for the conjugated cysteinyl leukotriene C4 (LTC4) which is a mediator of inflammatory response[24]. Several drugs can be effluxed by MRP1 both as unconjugated and conjugated (usually with glutathione) organic anions. It has been shown that even in circumstances where the drugs do not form a conjugate with glutathione, their transport requires the presence of GSH. It was therefore suggested that glutathione is co-transported with some unconjugated substrates of MRP1[25], [26]. MRP1 also participates in the protection of the cell from oxidative stress since it can transfer glutathione and its oxidized form, GSSG. Maintenance of a low GSSG:GSH ratio is crucial for prevention of oxidative stress and normal cell activity[27]. Therefore, in every organism where it is present, MRP1 contributes to the removal of toxins and the protection from endo and xenobiotic-induced damage.

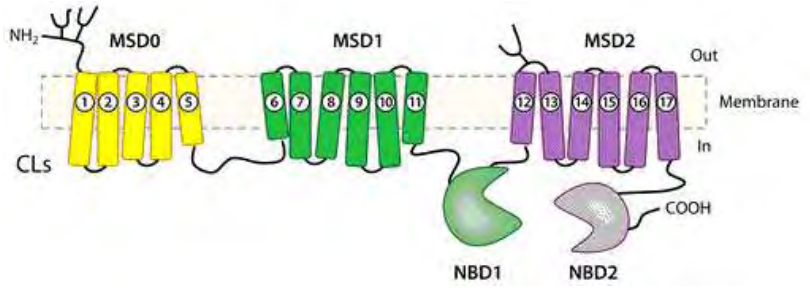


Figure 5: Structure of MRP1 protein. Mammalian MRP1 proteins have a 5 domain structure; non-mammalian MRP1 proteins lack the MSD0.

1.4 Metabolomics

Metabolomics is the scientific study of chemical processes involving metabolites which are low molecular weight molecules, mostly intermediates or products of biological pathways in any organism. In global metabolomics studies, the metabolome, which consists of all the metabolites of an organism, is studied in detail. The genome, the transcriptome and the proteome are comparatively more stationary nodes of flow of information within an organism, whereas the metabolome is dynamic and changes much more rapidly as a result of environmental factors, as well as gene expression and protein function and regulation. The metabolome is closest to the phenotype than the other three 'omes' mentioned above. Changes in the metabolome are the ultimate answer of an organism to genetic alterations[28].

Metabolomics offers qualitative and quantitative information about metabolites thus helping to define metabolic changes associated with conditions of interest, such as drug exposure, parasite-host interactions and mutations. Metabolomics studies usually aim to generate a snapshot of the physiological state of a system and determine how an external or internal stimulus can alter the biology of the system studied.

A metabolic analysis can be either targeted, when the aim is to detect variations of a particular set(s) of metabolites, or untargeted, when all potential metabolites that can be detected are examined. In targeted approaches the techniques used are nuclear magnetic resonance (NMR) spectroscopy and chromatography can be used to achieve good resolution before detection by mass spectrometry (MS). In untargeted approaches, liquid chromatography coupled with mass spectrometry (LC/MS) has proven to be the most effective combination of techniques for the measurement of the highest possible number of metabolites in a sample[29].

1.5 Tricarboxylic acid cycle in *Plasmodium spp*

Plasmodium species primarily use glucose as the main energy source and depend on glycolysis for ATP synthesis. The product of glycolysis in *Plasmodium spp.* is lactate, via lactate dehydrogenase, rather than pyruvate. Pyruvate dehydrogenase is the enzyme that catalyses the decarboxylation of pyruvate into acetyl-CoA inside the mitochondrion. Upon its formation, acetyl-CoA feeds into the TCA cycle. The localisation of pyruvate dehydrogenase in the apicoplast of *Plasmodium spp.* suggests the lack of a TCA cycle[30]. Despite the fact that *Plasmodium* parasites do not use oxidative phosphorylation for ATP generation, the presence of a single mitochondrion seems to be essential for their growth[31]. In addition, although the parasites rely on glycolysis for energy production, all the necessary genes for the tricarboxylic acid cycle are present in the genome of *Plasmodium spp.* It was suggested that the branched chain α-keto acid dehydrogenase (BCKDH) complex, which is located in the mitochondrion, is involved in the conversion of pyruvate to acetyl-CoA[32].

The results of flavoprotein deletion in *Plasmodium* support the hypothesis of a functioning TCA cycle. Flavoprotein (Fp) is one of the subunits of the mitochondrial complex II, which catalyses the oxidation of succinate to fumarate. Deletion of gene *Pbsdha*, coding for Fp, had no effect in the development of blood stage parasites or gametocytes in *P. berghei*; it did however affect the mosquito stages and resulted in a block in transmission[33]. Finally, recent metabolomics studies in *P. falciparum* carbon metabolism revealed that both asexual and sexual stage parasites utilize the TCA cycle. This targeted analysis also demonstrated that although in asexual stages, in TCA metabolism, glutamate utilization is higher than glucose utilization, the opposite is occurring in gametocytes, where the parasites seem to switch to TCA catabolism of pyruvate[32].

All the data above suggest that although the TCA cycle is not imperative for parasite growth inside the red blood cell (blood stages), its disruption causes developmental issues when the parasites leave the host cell (in mosquito stages) or even earlier, during gametocytogenesis. It is thought that the parasites either switch their energy source from glycolysis to oxidative phosphorylation because glucose is no longer abundant during the mosquito stages, or they use both metabolic pathways in order to accumulate certain molecules in anticipation of the energy requirements of the sexual stages.

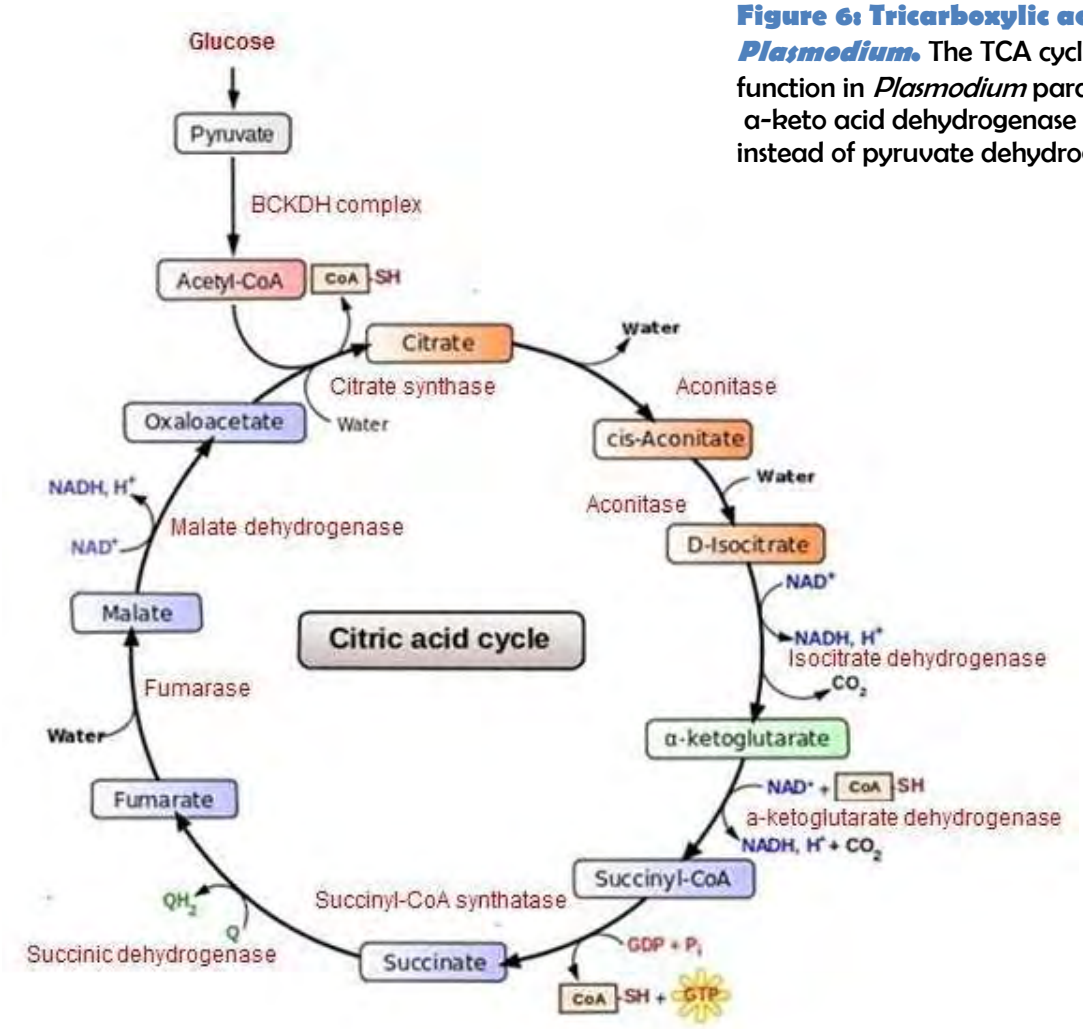


Figure 6: Tricarboxylic acid cycle in *Plasmodium*. The TCA cycle is proposed to function in *Plasmodium* parasites with chain α-keto acid dehydrogenase (BCKDH) complex instead of pyruvate dehydrogenase.

1.6 Gamma-Aminobutyric acid (GABA)

Gamma-Aminobutyric acid is an amino acid known since the 1960s as the main inhibitory neurotransmitter in the mammalian central nervous system. GABA is produced by nearly all prokaryotes and eukaryotes, from bacteria to humans. Although for years research was focused on its role as a neurotransmitter and its effect on the nervous system in vertebrates, it has been recently implied that GABA participates in the regulation of a wide range of developmental stages, from cell proliferation to circuit refinement[34].

GABA is formed by a metabolic pathway called the GABA shunt. The first step is the formation of glutamate (Glu). Glutamate, the precursor of GABA, can be a product of either glucose or glutamine metabolism. Transamination of α-ketoglutarate, which is an intermediate of the TCA metabolism of glucose, by GABA α-oxoglutarate transaminase (GABA-T) results in the formation of glutamate. Glutamate can also be synthesized via the glutamate/glutamine cycle by a phosphate-activated glutaminase. Following its production, glutamate is decarboxylated to form GABA by an enzyme called glutamate decarboxylase (GAB).GABA can be used as an energy source feeding into the TCA cycle. GABA-T metabolizes GABA to succinic semialdehyde (SSA). SSA is subsequently oxidized by succinic semialdehyde dehydrogenase (SSADH) into succinate which can enter the TCA cycle[35] (Figure 7).

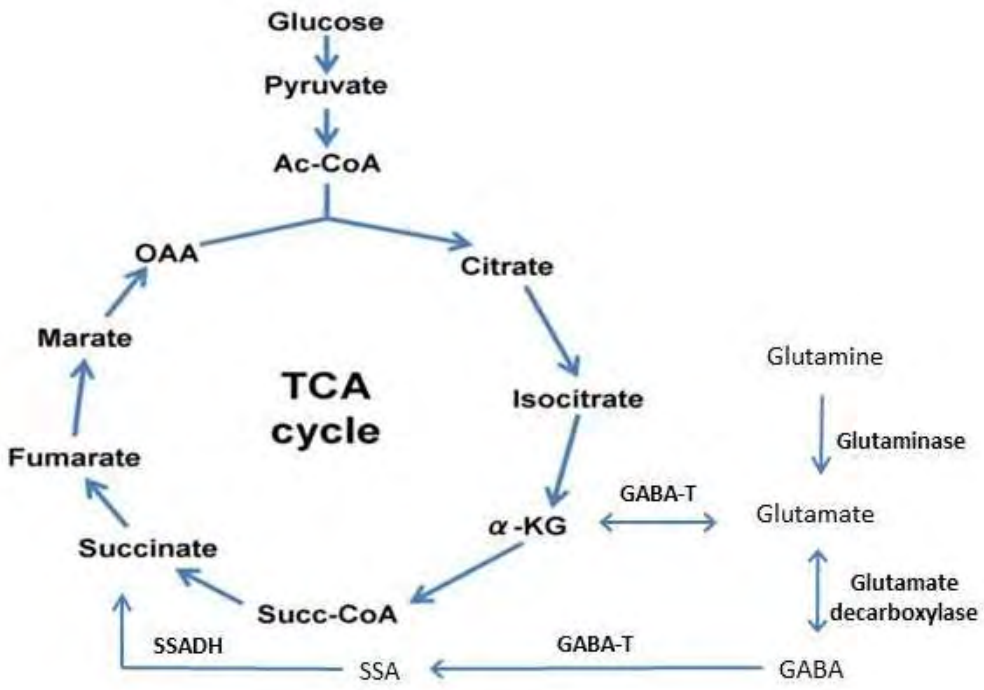


Figure 7: GABA shunt.

Gamma-Aminobutyric acid in *Plasmodium* spp.

According to recent findings the GABA shunt is functional in *Toxoplasma gondii*[36]. However, things differ in *Plasmodium* spp. because the genome does not encode for the enzyme succinate semialdehyde dehydrogenase (SSADH), which is required for the conversion of GABA to succinate. Despite the lack of such an enzyme, when labelled glucose and glutamine where used in the media for cultures of *P. falciparum* parasites high levels of labelled GABA were detected only in infected red blood cells, thus suggesting the presence of a partial GABA shunt. In *P. falciparum* gametocytes GABA and glutamate were strongly labelled from ^{13}C-glucose, whereas in schizonts the labelling was mostly coming from^{13}C-glutamine. This could mean that the TCA cycle operates in asexuals but primarily in gametocytes and that TCA cycle intermediates are used for synthesis of glutamate and GABA[32]. *P. berghei's* gametocytes seem to be accumulating GABA and the highest level of GABA was observed during ookinete development (unpublished data). As it was previously stated, disruption of the TCA cycle leads to non-functional ookinetes which fail to form oocysts in *P. berghei*[33], also perturbation of the TCA cycle in *P. falciparum* parasites resulted in loss of viability of gametocytes. It is still unknown whether these defects during the parasite's sexual stages are in any way connected with variations in GABA levels.

Gamma-Aminobutyric acid in signalling pathways

Research about GABA in invertebrates was focused for several years on its role as a metabolite. However, recently there have been suggestions that GABA can also act as a signalling molecule. It was demonstrated that GABA accumulates rapidly in response to biotic and abiotic stresses in plants[37]. Furthermore, modifications in GABA metabolism pathways, e.g. mutants lacking enzymes necessary for GABA production or enzymes responsible for its degradation, usually result in growth inhibition and developmental problems[38]. Supporting the existence of GABA-mediated signalling pathways were the alterations in gene expression due to GABA. In *Arabidopsis thaliana* plants grown in the presence of GABA 71 genes were down-regulated and 36 genes were up-regulated, as compared to plants grown under standard conditions[39]. Furthermore, disruption of succinic semialdehyde dehydrogenase (SSADH) in *A. thaliana* resulted in the aggregation of reactive oxygen intermediates (ROS) when the plants were exposed to environmental stresses[40].It was also proposed that GABA can function as a signal in the plant-*Agrobacterium tumefaciens* interaction, affecting the extent to which *Agrobacterium* can spread inside the plant[41].

AIMS AND OBJECTIVES

In this project we aimed to determine the impact that lack of the MRP1 gene would have on *Plasmodium berghei* parasites. Firstly, we performed phenotypic analysis of the mrp1 knock out compared to wild type parasites that revealed differences in the number of gametocytes produced by the two lines. The mutant parasites produce 70-80% less gametocytes than wild type parasites. The gametocytes produced by the mutant line, though fewer in number, are capable of normal exflagellation and ookinete conversion. Further on, we used metabolomics to reveal the differences between wild type and the MRP1 knock out lines with differential gametocyte conversion rates. The metabolomics analysis revealed that a unique metabolite, GABA, was involved in the sexual stages and we began to elucidate the role of GABA in the sexual stages of *P. berghei*. We aimed to disrupt GABA metabolism by knocking out genes that are possibly involved in it. Deletion of five out of the six genes we targeted was successful and cloning of three knock out lines was possible within the limited time we had. Finally, we checked whether loss of these genes would have any effect on the production of gametocytes, so we performed phenotypic analysis on the cloned mutant lines we created.

2a.1 Molecular Biology

Buffers, Solutions and Media

5x TBE electrophoresis buffer:

445mM Tris, 445mM Boric acid, 10mM EDTA and ddH_2O

LB agar:

1% pepton, 0.5% yeast extract, 1% NaCl and 1.5% agar

LB broth:

1% pepton, 0.5% yeast extract, 1% NaCl

Ampicillin:

Stock: 100mg/ml

Final concentration in LB broth/agar: 100µg/ml

TNE buffer:

10mM Tris pH 8.0, 5mM EDTA pH 8.0, 100mM NaCl and demineralized water

2a.2 *Plasmodium berghei* methods

Buffers, Solutions and Media

Phosphate buffered saline (PBS):

The 10x PBS contains: 0.01M KH_2PO_4, 1.37M NaCl and 0.027M KCl, pH 7.0

Erythrocyte-lysis buffer:

The components of the 10x solution are: 1.5M NH_4Cl (80.23g/l), 0.1M$KHCO_3$ (10.012g/l) and 0.01M EDTA (3.725g/l)

Giemsa solution and staining buffer:

The stock Giemsa solution is diluted in staining buffer. 40ml of Giemsa dye (12%) contain 5ml of stock Giemsa solution and 35ml of Giemsa staining buffer

For 500ml of staining buffer 0.25gr KH_2PO_4, 0.05gr Na_2HPO_4 $.2H_2O$ are added and the pH is adjusted to 7.2 with NaOH

Complete culture medium:

RPMI 1640 pH 7.3 containing 25% v/v AlbuMAX provided by Invitrogen

RPMI 1640 with L-glutamine and 25mM HEPES is provided by Invitrogen

Ookinete culture medium:

RPMI 1640 pH 7.3containing 10% v/v foetal calf serum and 10-100 mM of xanthurenicacid

Minimal medium:

For 10L of 10x stock:

1gr $Ca(NO_3)_2$ $.4H_2O$, 4gr KCl, 488mg $MgSO_4$(anhydrous), 53gr NaCl, 20gr $NaHCO_3$, 8gr $NaHPO_4$(anhydrous), 41mg Hypoxanthine, 20gr D-Glucose, 10mg Glutathione, 59,58gr HEPES, 50mg Phenol Red, 650mg L-Cystine.2HCl, 200mg L-Glutamic Acid, 3gr L-Glutamine, 500mg L-Isoleucine, 150mg L-Methionine, 200mg L-Proline, 300mg L-Tyrosine.2Na.$2H_2O$ and 2.5mg Ca-Pantothenate

All components were mixed and Pen-Strep (1ml/l) was added before use. pH was adjusted to 7.3, the medium was sterilized using a 0.22u filter and AlbuMAX (5g/L) was added.

Nycodenz:

138g of Nycodenz powder (LucronBioproduct BV) is dissolved in 500ml of Buffered Medium containing 302.85mg Tris/HCl (5mmol/l), 111.85mg KCl (3mmol/l) and 56.15mg Ca Na EDTA (0.3mmol/l)

A 55% Nycodenz-normal PBS solution is used for purification of schizonts from an asexual culture.

Pyrimethamine:

Stock solution: dissolved pyrimethamine in DMSO to a final concentration of 7mg/ml

100 times dilution of the stock solution in added in the drinking water

Sulfadiazine:

Mice are treated with 25mg/l sulfadiazine in the drinking water

Phenylhydrazine-HCl (phz):

125mg phz (12.5mg/ml) are dissolved in 10ml 0.9% NaCl

100µl of stock solution are injected to each mouse i.p.

Gas mix:

5% O_2, 5% CO_2 and 90% N_2

5-fluorocytocine:

Mice are treated with 1.5mg/ml 5-fc in drinking water

2a.3 Flow cytometry

Buffers, Solutions and Media

Hoechst dye:

Hoechst dye is provided by Invitrogen

The solution used in FACS protocol consists of 10μM Hoechst made up in enriched PBS

Enriched PBS:

Normal PBS enriches with 20mM Hepes, 20mM Glucose, 4mM $NaHCO_3$ and 0.1% BSA

FACS buffer:

PBS and 3-5mM of EDTA

2a.4 Metabolomics methods

Buffers, Solutions and Media

Internal standards:

1μM 5-fluoroudine, 1μM Cl-phenyl-cAMP, 1μM N-methyl glucamine, 1μM Canavanine, 1μM Piperazine

Extraction solvent:

Chloroform: methanol: water::1: 3: 1 with internal standards (all at 1μM concentration each)

2b METHODS

2b.1 Molecular Biology

Polymerase Chain Reaction (PCR)

In a 1.5ml Eppendorf tube the following solutions were added: genomic DNA from *Plasmodium berghei* parasites of the strain HPTBB (template), PCR buffer (-$MgCl_2$), MgCl2, dNTPs, forward primer, reverse primer, Taq Polymerase and ddH2O.

Table 1: PCR reagents for a 50µl reaction.

Reagent	Volume (µl)	Initial Concentration	Final Concentration
HP DNA	1	50ng/µl	50ng
PCR Buffer	5	10x	1x
$MgCl_2$	2	50mM	2mM
dNTPs	5	2.5µM	0.05µM
Primer F	2	10pmol/µl	20pmol
Primer R	2	10pmol/µl	20pmol
Taq	0.4	5units/ µl	2units
ddH_2O	32.6	-	-

The PCR was performed with the following cycling protocol:

1. Initial denaturation for 3 minutes at 94°C
2. Denaturation at 94°C for 30 seconds
3. Annealing at 55°C for 30 seconds
4. Extension at 68°C for 2 minutes
5. Final extension 68°C for 10 minutes

} x29

The annealing temperature of 55°C worked for all primer pairs, except for primers GU2930+GU2971. The LDC upstream region was acquired with an annealing temperature of 60°C.

Table 2: Primers used for PCR amplification of downstream and upstream regions

	Primer Sequence	Number	Restriction Enzyme
GDH1 upstream forward	actAAGCTTCTATGTATATATTTTTCGACATTTAACTGC	GU2943	HindIII
GDH1 upstream reverse	catCCGCGGCAATGTAACAAATTATATAAGTATTTATTTGTTTGTAT	GU2944	SacII
GDH1 downstream forward	tcaGGTACCCGAGGATGCTTCGTATGATCTTAGCCAC	GU2945	KpnI
GDH1 downstream reverse	ctaCTCGAGCCGAGATGTTGTAGAGCATCATATGTATGAC	GU2946	XhoI
GDH3 upstream forward	actAAGCTTGGTTATGCAATATACTGACAAATATGTATAGCC	GU2951	HindIII
GDH3 upstream reverse	catCCGCGGTAAGGGCGGGGGAGTAATATATATGTAATGT	GU2952	SacII
GDH3 downstream forward	tcaGGTACCGAGTGTGCATGTTTTACTACATGTTTTGTATTAC	GU2953	KpnI
GDH3 downstream reverse	ctaCTCGAGGGGAAATCAATCACTTCCGTTATACTACAA	GU2954	XhoI
OAT upstream forward	atcGGGCCCGCGTAAGGCATGTATACATGATGTTATGTA	GU2955	ApaI
OAT upstream reverse	ctaCCGCGGAGTACAAATGCAGGACAGAACACATTGCAA	GU2956	SacII
OAT downstream forward	tcaGGTACCTAGAGGCATGTTGATGTTTACTAGTATATGC	GU2957	KpnI
OAT downstream reverse	ctaCTCGAGATATGTCGTTTGTGTTAAGAATGATATGTACTG	GU2958	XhoI
Putative transporter upstream forward	atcGGGCCCCGGAACTTTTCGAGCTATTATCATTATTTAATG	GU2959	ApaI
Putative transporter upstream reverse	ctaCCGCGGTTGTAGTTGTCTTTGCTTGGGTCTATATGT	GU2960	SacII
Putative transporter downstream forward	tcaGGTACCGAGCCAGGTGTTTATGTGTGTATATAAATGAT	GU2961	KpnI
Putative transporter downstream reverse	ctaCTCGAGATCAGAACTTATCTCATACATAAATCTGGTGG	GU2962	XhoI
LDC upstream forward	attGGGCCCGCTTTGATAATGTGATTAGCCCAGTGTG	GU2930	ApaI
LDC upstream reverse	ctaCCGCGGCAACAAAGTGATCGTAGTACCCATATATATAG	GU2871	SacII

High-Fidelity Polymerase Chain Reaction

Amplification of GDH2 upstream and downstream regions as well as LDC downstream region was performed using the KAPA HiFi PCR Kit, which contains a proofreading DNA polymerase, to avoid any mutations in the adjacent genes. The kit is provided by KAPA Biosystems.

Table 3: Primers used for Hi Fidelity PCR amplification of downstream and upstream regions

	Primer Sequence	Number	Restriction Enzyme
GDH2 upstream forward	atcGGGCCCCCTGAAGCATATGCTGCACATGCAA	GU3065	ApaI
GDH2 upstream reverse	ctaCCGCGGGCACTATTTCCACACTATTTGTTACTACATTC	GU3066	SacII
GDH2 downstream forward	tcaGGTACCCAAATGGAACCGAACCCTCAATTATATTTCC	GU2949	KpnI
GDH2 downstream reverse	ctaCTCGAGGAGAGCCAAACATGTCAAATATCACGAG	GU3067	XhoI
LDC downstream forward	tcaGGTACCGAAATGGCTGAGGATAAACATTACGAAAC	GU3069	KpnI
LDC downstream reverse	ctaCTCGAGGGGTATGTCGCTAATGATAAGCATTGT	GU3070	XhoI

Table 4: HiFi PCR reagents for a 50µl.

Component	Volume (µl)	Initial Concentration	Final Concentration
HP genomic DNA	1	50 ng/µl	50 ng
KAPA FidelityHiFi Buffer	10	5x	1x
dNTP mix	1.5	10 mM	0.3 mM each
Primer F	1.5	10 µM	0.3 µM
Primer R	1.5	10 µM	0.3 µM
HiFi DNA Polymerase	1	1 U/µl	1 U
ddH_2O	33.3	-	-

The PCR was performed with the following cycling protocol:

6. Initial denaturation for 3 minutes at 95°C
7. Denaturation at 98°C for 20 seconds
8. Annealing at 62°C for 15 seconds
9. Extension at 72°C for 1 minutes
10. Final extension 72°C for 2 minutes

X25

Electrophoresis

For the electrophoresis of all PCR products 1% agarose gels were used; for a 100ml gel, 1gr of agarose was added to 100ml of 1x TBE buffer and staining was done by using 1x SYBR® Safe DNA Stain (Catalogue no. S33102, Life Technologies) in the gel.

Preparation of DNA constructs

Following their amplification, the upstream and downstream regions of the genes of interest were inserted into a vector-plasmid. The vector used for the preparation of DNA constructs was plasmid pL0035 (Figure 8), which contains regions of multiple restriction sites, a resistance gene for ampicillin (amp), two 3' UTRs of *P. berghei's dhfr* gene (3'pbdhfr/ts), a human *dhfr* gene which makes the cells resistant to pyrimethamine (*hdhfr*), gene *yfcu* which produces an enzyme that catalyzes the transformation of prodrug 5-FC (5-Fluorocytosine) to a substance that is toxic to the cells and is used for negative selection (yfcu)[42]. The expression of the selection cassette is under the regulation of a constitutive promoter which is active all through the cell cycle of *P. berghei* (pbeef1aa).

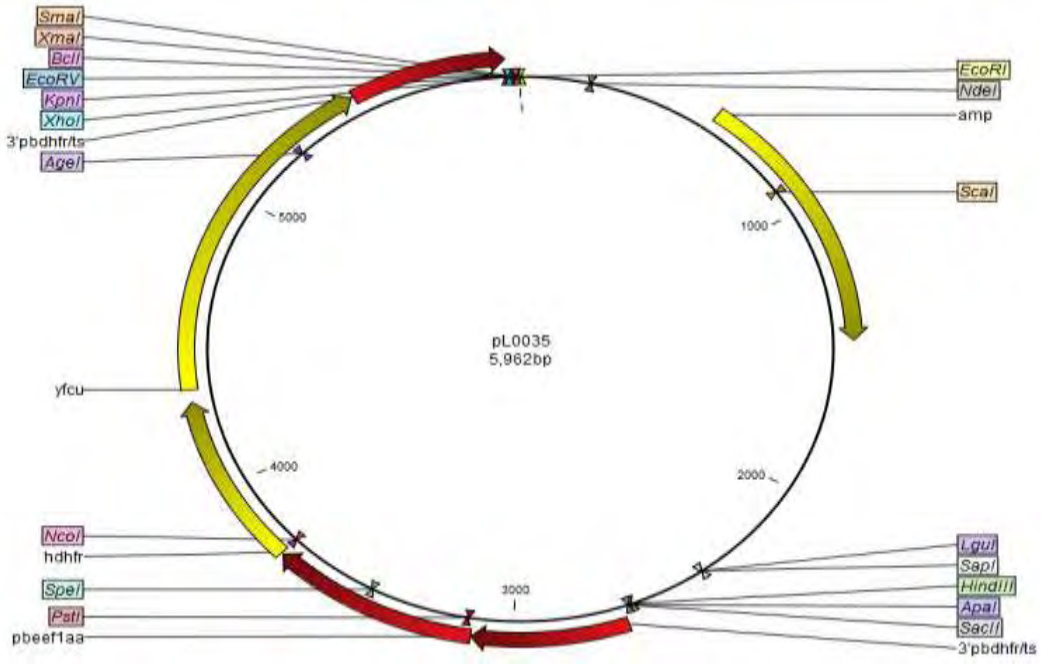


Figure 8: Plasmid pL0035.

Digestion of insert and vector

The enzymes (acquired from New England Biolabs) used for the digestion of the upstream regions were HindIII (20u/μl) or ApaI (50u/μl) and SacII (20u/μl). ApaI was used for upstream regions in which the HindIII restriction site was present more than once. All downstream regions were cut with KpnI (20u/μl) and XhoI (20u/μl).
For the digestion of the insert the following are added in a 1.5ml Eppendorf tube: 50μl of the PCR product, 6μl Buffer 4, 0.6μl BSA, 1μl Enzyme 1, 1μl Enzyme 2 and 1.4μl ddH_2O. The tubes are incubated for two hours at 37°C*.

For the digestion of the vector the following are added in a 1.5ml Eppendorf tube: 10μl of the vector, 6μl Buffer 4, 0.6μl BSA, 1μl Enzyme 1, 1μl Enzyme 2 and 41.4μl ddH_2O. The tubes are incubated at 37°C for two hours*. After the vector islinearized, 1.0μl of CIP is added and the vector is incubated for 30 minutes at 37°C. Alkaline Phosphatase, Calf Intestinal (CIP) nonspecifically catalyzes the dephosphorylation of 5' and 3' ends of DNA and it prevents religation of linearized plasmid DNA.

* ApaI is the only enzyme which functions at 25°C, so in the cases where it is used, ApaI is added in the beginning and the reaction mix is incubated at 25°C for two hours. Afterwards, SacII is added and the tubes are incubated at 37°C for two hours as usual.

Ligation of the insert in vector

All the ligation reactions were performed with 25ng of vector DNA and a vector to insert ratio of 1 to 3. For the ligation of any insert into a plasmid, 25ng of vector DNA were added to the needed amount of insert DNA (as calculated by ligation calculator[43]), 2μl of 5x dilution buffer* and ddH_2O up to 10μl. Afterwards, 10μl of 2x ligation buffer* and 1μl of DNA ligase* were added. The reaction mix was incubated at room temperature for 15 minutes and used for transformation.

*These reagents were obtained from the Roche rapid DNA ligation kit (Cat. No. 11 635 379 001).

Transformation of bacteria with plasmid DNA

E. coli competent cells were used for chemical transformation. 2μl of the ligation mix were added to 100μl of *E. coli* cells and incubated on ice for 30 minutes. The cells were heat-shocked at 42°C for 45 seconds and transferred on ice for 1 minute. 500μl of LB broth were added and the cells were incubated for 1 hour at 37°C at approximately 200rpm. The cells were subsequently spun down at maximum rpm for 20 seconds, 500μl of the supernatant were removed and the pellet was resuspended in the remaining 100μl. The resuspended pellet was plated in LB agar plates containing ampicillin (100μg/ml) and incubated overnight at 37°C.

Diagnostic digestion

The presence of the insert in the pL0035 vector was confirmed with diagnostic digestions of plasmid DNA extracted from the ampicillin resistant colonies. For that purpose the colonies were picked and incubated overnight in 3ml LB broth with ampicillin (100μg/ml) at 37°C and 200rpm. The plasmid DNA was then extracted from the cultures* and the diagnostic digestions were performed using the same enzymes that were used for the digestion of the insert and vector.

Note: Once the insertion of the upstream region of a gene in the vector is confirmed, the same process is carried out for the downstream region of the gene (digestion, ligation, transformation and diagnostic digestion). For the downstream region the vector used is plasmid pL0035 with the upstream region of the gene which was constructed previously.

*Plasmid DNA was extracted from the cultures with the use of Qiagen plasmid mini preparation kit.

Final digestion

When both the upstream and the downstream regions are present in the plasmid, the vector is completed. A large quantity of plasmid DNA* is obtained from 100ml cultures, done in LB broth with ampicillin, of *E. coli* cells transformed with the construct. The digestion was performed to remove the plasmid backbone from the required fragment of the vector containing the homologous arms and selectable marker.
The final digestions were set up as follows: 80μgof vector, 1x buffer 4 (10x stock), 1x BSA (100x stock), 0.4U/μl ApaI or HindIII (depending on the vector), 0.4U/μl XhoI, 0.4U/μl BsaI and ddH_2O.All reactions had a final volume of 400μl.
*Plasmid DNA was extracted from the cultures with the use of Qiagen plasmid midi preparation kit (catalogue number 12145).

DNA precipitation

To precipitate DNA, 1/10 of the sample's final volume 3M sodium acetate (NaAc) and 2x of the sample's final volume 100% Ethanol are added. The samples are incubated at -20°C from 3 hours to overnight. Afterwards, the samples are centrifuged at maximum rpm for 30 minutes at 4°C. The supernatant is discarded and the pellet is washed with 500μl of 70% Ethanol. The samples are centrifuged at maximum rpm for 10 minutes at 4°C, the supernatant is discarded and the pellet is air dried to avoid ethanol carry over before being resuspended in 50-100μl of ddH_2O.

Diagnostic PCR

Diagnostic PCR is performed for the confirmation of 5' and 3' integration in the parasite's genome. To confirm the 5' integration, a specific primer binding to a sequence in the 3'UTR of the *dhfr* gene (GU0205), present only in the construct, is used paired with a primer which binds to a sequence located upstream of the 5' homology arm, present only in the parasite's genome. To confirm 3' integration, a primer binding to the 3'UTR of the dhfr (GU0204), present only in the construct, is paired with a primer binding to a sequence downstream of the 3' homology arm, present only in the parasite's genome. Another pair

of primers is also used to amplify the open reading frame of the gene (present in wild type parasites).
The reagents used for diagnostic PCRs are the same as mentioned previously for the regular PCR reaction with a final volume of 25µl. The template used for diagnostic PCRs is DNA extracted from parasites that have potentially integrated the 5' and 3' UTRs.

Table 5: Primers used for 3' and 5' UTR integration.

	Primer sequence	Number
GDH2 5' integration	GTCTTATTTTATCAATCGATATATCATTATACGG	GU3005
GDH2 3' integration	CATATTTACCATTGTTTTTAACATCGCCACAAC	GU3006
GDH2 orf forward	AAATATTGTGCAACATTGCCACTTCCACTA	GU3007
GDH2 orf reverse	GATGATATTTTGTCACAACTCAATGTTAAGTG	GU3008
GDH1 5' integration	GTATGTGTGCATATATGCTTATTCACCA	GU3009
GDH1 3' integration	TTGGGAACATATTGTCTTTACAAGAGATG	GU3010
GDH1 orf reverse	CTATTGTTGATGGCATATTAGCACCTTCAC	GU3011
GDH1 orf forward	AAATGACGCAATGCCGAGATAGTACCG	GU3012
GDH3 5' integration	GTTCAATTACTTTCTTATTCTGTGAAAACAACC	GU3013
GDH3 3' integration	CAAGAACGAAACATGTATAAGAAGCGAAC	GU3014
GDH3 orf forward	ATGTCCATCCGTAACATTATCACCTGCA	GU3015
GDH3 orf reverse	CATTATTCCAGTCCCTAAATTACTAGATCCAG	GU3016
OAT 5' integration	CACAAGCAAAACGATACACATTCACTTGTT	GU3017
OAT 3' integration	GTAATAATAAATGTACTAATATCTTCACCGTC	GU3018
OAT orf forward	GGAGCTCACAATTATGATCCCATTCCC	GU3019
OAT orf reverse	GTTTTGTCATGAACATCCCTAGTAATTAAACC	GU3020
put. transporter 5' integration	TCAATCGTTTCGTTAGCTAAATTGTCGTTTC	GU3021
put. transporter 3' integration	GCTTATACAAGACTTAAGTAGTTAGACAG	GU3022
putative transporter orf	CTAAAATGGCAAAAACAACCCATCCATAATG	GU3023
putative transporter orf	TCTTGAATTTTCAAGGAGGGATTGTTCG	GU3024
LDC 5' integration	ACAGTATGTTGCTAAAGAACACACGGAA	GU3025
LDC 3' integration	CTTTATTTAGGCCAATACTTGTCTTACTC	GU3026
LDC orf forward	ATCCGCCTGGATTTCCTGTATTAGTG	GU3027
LDC orf reverse	TGTTGGTAGAAGTATTCTGAACGCTTGC	GU3028

PCR for negative selection

The reagents used for a PCR reaction to confirm the presence of negatively selected parasites are the same used for a normal PCR reaction except for the template and the primers. In this case DNA isolated from parasites that have possibly looped out the cassette following the 5-FC treatment is used as template.

Table 6: Primers used for confirmation of negative selection.

	Primer Sequence	Number
GDH1 upstream forward	actAAGCTTCTATGTATATATTTTTCGACATTTAACTGC	GU2943
GDH1 downstream	ctaCTCGAGCCGAGATGTTGTAGAGCATCATATGTATGAC	GU2946
hDHFR exon forward	ATGGTTGGTTCGCTAAACTG	GU0051
Yfcu exon reverse	TGATAGCACTACCACCGGTG	GU0054

2b.2 *Plasmodium berghei* methods

Monitoring the parasitemia from Giemsa stained blood smears

Blood smears are made from infected mice or from *in vitro* parasite cultures. Smears from infected mice are made with one drop of tail blood. For the preparation of smears from parasite cultures, 1ml of the culture is transferred to an Eppendorf tube, centrifuged at maximum rpm for 20 seconds and most of the supernatant is removed. The smear is made from the resuspension of the cells in the remaining 5-10µl of medium.
Once the slides are prepared, the cells are fixed in methanol for 5 seconds and the slides are left to dry for 5 minutes. Afterwards, they are stained in 12% Giemsa solution and staining buffer for 15 minutes, rinsed in water and air dried.

Calculation of parasitemia

Parasitemia of infected mice is monitored by observation of Giemsa stained blood smears in the microscope.

$$\text{Parasitemia} = \frac{\text{Number of iRBCs}}{\text{Number of total RBCs}} \text{ x100}$$

Treatment with phenylhydrazine-HCl

P. berghei parasites prefer to invade reticulocytes rather than mature red blood cells. To induce the production of more reticulocytes, mice are injected with 0.1ml of phz-

HCl stock solution (12.5mg/ml) made up in 0.9% NaCl usually 2 days prior to their infection with parasites. Phz causes haemolysis, resulting in anemia and subsequently, increased erythropoiesis and production of more reticulocytes.

Schizont cultures

For the production of schizonts, 200µl of blood from mice infected with *P. berghei* parasites 2-3 days earlier are added to 10ml of media + AlbuMAX. The samples are centrifuged for 8 minutes at 1800rpm at room temperature, the supernatant is discarded and the pellet is resuspended in 5ml of media. The resuspended pellet is transferred into 150cm^2 tissue culture (TC) flasks and topped up with 35 ml of media (0.5-1% cell concentration). The TC flasks are gassed with special gas mix and incubated at 37°C overnight.

Isolation of schizonts from cultures

Parasite cultures are centrifuged and the pellets are resuspended in 50ml falcon tubes with 35ml of standard medium. 10ml of 55% Nycodenz solution is layered at the bottom of the tubes and they are centrifuged at 1800rpm for 20 minutes at room temperature (density-gradient centrifugation) without brake. The middle layer of cells, containing the schizonts, is transferred to another tube and centrifuged at 1800rpm for 8 minutes at room temperature. The supernatant is discarded and the pellet is resuspended with approximately 1ml of media.

Transfection of schizonts using electroporation

Typically, 10µg of the digested vector is used for transfection of schizonts using electroporation. The schizonts are then injected intravenously (i.v) to naïve mice.

Selection of transfected parasites

The mice are treated with pyrimethamine following the completion of one cell cycle after the transfection (one day). Any parasites that have not been transfected will not carry the selectable marker (*hdhfr*) which makes them resistant to pyrimethamine and will not survive.
Presence of the construct in the parasites is confirmed by Diagnostic PCR.

Genomic DNA isolation of malaria parasites

Parasitemia of the positive mice needs to be over 1% for collection of heart blood. The blood is collected with a 2ml syringe containing 100µl Heparine in a falcon tube containing 10ml PBS. The collected blood/PBS is centrifuged at 1800rpm for 8minutes and the supernatant is discarded. The pellet is resuspended in 30µl of 1x lysis buffer and incubated on ice for 5 minutes. The falcon tubes are centrifuged at 1800 rpm for 8 minutes and the supernatant is discarded. The parasite pellet is resuspended in 700µl of TNE buffer and 20µlRNase (10mg/ml) and 100µl 10% v/v SDS are added. The samples are incubated at 37°C for 10 minutes and then 20µl Proteinase K (10mg/ml) is added. The samples are incubated at 37°C for one hour. After the incubation, buffered phenol is added up to 1.5ml, the samples are mixed by inverting the tubes several times and centrifuged at maximum

rpm for 5 minutes. Subsequently, the upper phase is transferred to new Eppendorf tubes and buffered phenol: chloroform: isoamylalcohol (25: 24: 1) is added up to 1.5ml. The tubes are mixed, centrifuged at maximum rpm for 5 minutes and the upper phase is transferred to new Eppendorf tubes. Chloroform: isoamylalcohol (24: 1) is added up to 1.5ml, the tubes are mixed and centrifuged at maximum rpm for 5 minutes. The upper phase is transferred to new Eppendorf tubes and the parasite DNA is precipitated according to the protocol for precipitation of DNA.

Cloning of transfected parasites

The uncloned transfected parasites are injected i.pin mice treated with pyrimethamine. When the parasitemia is 0.1-1% a drop of blood is diluted in PBS and the solution is used to count the number of cells with the hemocytometer. Based on the parasitemia, counted in Giemsa stained smears, a solution of infected RBCs in PBS is made containing 0.8 infected RBCs in 200μl. 200μl of the solution is injected intravenously in each mouse with a total of 10 mice injected per cloning experiment. 7-10 days after the injections Giemsa stained smears are made from the mice to check the presence of parasites in their blood stream. If less than 8-9 mice are positive for parasites the cloning is successful. The positive mice are bled when parasitemia is 1% or over. DNA of the parasites is isolated and the presence of wild type and mutant parasites is checked by diagnostic PCRs.

Negative selection

A mouse is infected with parasites from the cloned line and if parasites are visible on Giemsa stained smears one day after the infection the mouse is treated with 5 fluorocytocine (5fc) in drinking water. The parasitemia initially reduces after treatment with 5 FC, but after 5-6 days the number of parasites increases again. When parasitemia reaches 1% or over, the mouse is bled and DNA is isolated from the parasites. The DNA is used as template in a PCR reaction to determine if negative selection in the parasites was successful.

Exflagellation of male gametocytes

Activation of male gametocytes is induced with the addition of 100μl of blood from an infected mouse to 3ml of ookinete media (3%). The blood-ookinete media solution is incubated at room temperature (21-23°C) for 20 minutes. 10μl of the culture is used to count the exflagellation centers using the hemocytometer.

Ookinete conversion of female gametocytes

Conversion of female gametocytes to ookinetes occurs after incubation of 3ml cultures of ookinete media containing 100μl of blood from infected mice (3%) at 21°C for 21-24 hours. Afterwards, smears from the cultures are made, stained with Giemsa and the conversion rate is calculated as follows.

$$\text{Conversion rate} = \frac{\text{Number of ookinetes}}{\text{Ookinetes + female gametocytes}} \text{ x100}$$

Growth assay

To examine the rate of growth of a mutant *P. berghei* line compared to wild type, equal number of parasites (~10^5) of line 733cl1 (wild type parasites expressing RFP in all stages) and line 1025cl2 (MRP1 mutant parasites expressing GFP in all stages) are injected into the same naïve mouse. The parasites' growth is monitored over a period of 10 days by flow cytometry (FACS) analysis. When the parasitemia reaches 3-5% the parasites are passaged to naïve mice to continue the growth assay.
A mouse injected with equal amount of parasites from line 733cl1 (RFP) and line 507 (wild type expressing GFP throughout the cell cycle) is used as a control.
Mice used for FACS analysis are not treated with phenylhydrazine due to problems with auto-fluorescence (background) during flow cytometry analysis.

Monitoring gametocyte production

To monitor the production of gametocytes by different cloned lines, mice are infected by intraperitoneal injection with individual parasite lines. The lines used for this purpose are made in an 820 background resulting in the expression of GFP from male gametocytes and RFP from female gametocytes[44]. The gametocyte production is monitored with flow cytometry (FACS) over a period of 3-5 days following the protocol described below. During that period of time when the parasitemia is higher than 6% the parasites are passaged to naïve mice to avoid multiply infected red blood cells.
Blood from an uninfected mouse is used as a control.
Mice used for FACS analysis are not treated with phenylhydrazine due to problems with auto-fluorescence (background) during the flow cytometry analyses.

Drug assay

The effect of dihydroartemisine (DHA), atovaquone and primaquine on schizonts of lines 507tbb and 1025cl1 was tested to check for difference in drug sensitivity profiles of the MRP1 mutant and wild type parasites. Schizont cultures of the wild type and the mutant line were prepared and the schizonts were isolated using density-gradient centrifugation as described previously. The schizonts were injected intravenously into naïve non-phzed mice and 2-3 hours after the injections Giemsa stained smears were made to confirm the presence of ring stage parasites. The mice were bled and equal amounts of blood (1%) were put into culture plates containing standard media with a drug. The plates were gassed using gas mix and incubated at 37°C for 24 hours. 1ml from each culture was transferred to Eppendorf tubes, the cells were fixed with 0.25% glutaraldehyde/PBS solution and the samples were prepared for FACS analysis according to the protocol described below.

Each culture plate contained one of the drugs mixed with the media in different concentrations and for each drug concentration there were two replicates. All the drugs were tested in the following concentrations: 100μM, 50μM, 10μM, 5μM, 1μM, 500nM, 100nM, 50nM, 10nM, 5nM and 1nM. Cultures containing no drug were used as controls.

GABA analogues test

The effect of GABA analogues on the production of gametocytes in lines 820tbb, 1512cl1 was tested following the same procedure as the drug assay, only in this test the

culture plates were incubated for 38 hours and the cells were not fixed for the purposes of FACS analysis.

Four analogues were tested, Baclofen, CGP55845, Isonipecotid acid and Bicuculine. The analogues were tested in the following concentrations: 1mM, 100μM, 10μM, 1μM, 100nM, 10nM and 0. There were two replicates for each concentration and non-producer line G401cl1 was used as control.

2b.3 Flow cytometry

Protocol for FACS analysis of parasites

The parasites can be acquired either from an infected mouse or from schizont cultures. In the latter case, 1ml of the culture is transferred into an Eppendorf tube and centrifuged for 20 seconds at maximum speed. The supernatant is discarded and the cells get fixed by resuspension of the pellet in 500μl of 0.25%glutaraldehyde in ePBS and incubation at 4°C for 10 minutes (if the cells do not need to be fixed, the last step is not carried out). The tubes are centrifuged for 20 seconds at maximum rpm, the supernatant is discarded and the cells are resuspended in 500μl of Hoechst solution.The samples are covered with aluminum foil, because Hoechst is photosensitive, and incubated at 37°C for 30-60 minutes. After the incubation the samples are centrifuged for 20 seconds at maximum speed, the supernatant is removed and the cells are resuspended in 1ml of FACS buffer. The cell suspension is passed through a nitex membrane into FACS tubes to avoid the presence of any red blood cell clusters in the samples that might blood the machine.
If the parasites are obtained from an infected mouse, a drop of blood from the tail is collected in an Eppendorf tube containing0.25% glutaraldehyde in ePBS and the same procedure is carried out.

2b.4 Metabolomics methods

Phzed (reticulocytosis induced) mice were infected with *P. berghei* parasites from lines 820TBB, 1512cl1 and G401cl1. An uninfected mouse was used as control. The mice were bled 3 days after their infection and the blood was used for schizont cultures overnight*. Schizonts were isolated from the cultures using density gradient centrifugation. Subsequently, the purified schizonts were injected intravenously to naïve mice that had been phzed 5 days before (to allow for maximum number of reticulocytes to be present at the time of parasite incubation). This procedure was performed for synchronization of the parasites in the blood stream. After the completion of one cell cycle, 24 hours after the infection of the mice, the parasitemia was calculated from Giemsa stained smears and was 18-20%. Blood was collected from the mice one hour later, filtered to remove white blood cells using Plasmodipur® filters and centrifuged at 1800rpm for 8 minutes at room temperature. The supernatant was discarded and the pellet was resuspended in minimal

media. The last two steps were repeated three times before the red blood cells were used for schizont cultures. Each culture contained $6x10^{e8}$ cells and was topped up with minimal media to 12ml. The cultures were incubated at 37°C.

*In all the schizont cultures of this experiment minimal media was used to avoid any unnecessary background during the metabolomics analysis.

Metabolite extraction and storage

Metabolite extractions were performed at three time points; 6, 14 and 26 hours after the injections of purified schizonts into naïve mice.

At each time point the flasks were submerged in dry ice and ethanol baths immediately after their removal from the incubator to quench the metabolism. A thermometer was inserted in the cultures to read the temperature and the flasks were removed from the dry ice-ethanol bath as soon as it reached 8°C, at this point the actual temperature of the cultures was around 0°C. The 12 ml cultures were split to 6 tubes, so that each tube contained 10^{e8} cells, and centrifuged at 5000rpm for 5 minutes at 4°C. 10µl of the supernatant were used for extractions in 150µl extraction solvent (supernatant suspensions) and the rest of the supernatant was removed. Subsequently, each pelletwas resuspended in 150µl extraction solvent (pellet suspensions). The suspensions, both pellet and supernatant, were mixed vigorously on shaker for 1 hour at 4°C and centrifuged at 12000rpm for 5 minutes at 4°C. 100µl of the supernatant was transferred into glass vials, topped with Argon gas and stored at -80°C.

3a Lack of MRP1 leads to reduction in gametocytogenesis in *Plasmodium berghei*

3a.1 Introduction

MRP1 belongs to the ABCC sub-family of the ATP-binding cassette (ABC) superfamily, one of the largest evolutionary conserved families of proteins. Acquired resistance to several drugs has been linked to mutations in proteins-members of the ABCC sub-family. It has been demonstrated that polymorphisms resulting in changes in just a few amino acids in the sequence of these transporters are sufficient for acquisition of resistance[45]. Polymorphisms in *P. falciparum* chloroquine resistant transporter (*pfcrt*) were associated with resistance to the drug [46]. Additionally, mutations in *pfmdr* encoding for the P-glycoprotein homologue 1 have been linked to resistance to several antimalarial drugs, such as mefloquine, lumefantrine, halofantrine, quinine, and artemisinin. MRP1 was first identified in human tumour cells when it was discovered that the resistant cells did not overexpress any other protein known to induce resistance to anticancer substances, for example MDR1. It has been demonstrated that in humans, the protein can transport several compounds and organic anions, glutathione and sulphate conjugates, glucuronides as well as oxidized glutathione (GSSG)[47].

Plasmodium berghei lines 1025cl1 and 1512cl1 were generated by knocking out the MRP1 gene for the purpose of determining its function (these lines were obtained from the Leiden University Malaria Research group). *P. berghei mrp1* (PBANKA_144380) is located on chromosome 14 and consists of one exon (Figure 9a). The gene encodes a protein of 1935 amino acids. *Pb*MRP1 possesses the usual MRP structure (Figure 9b) comprising two transmembrane domains (TMDs) and two nucleotide binding domains (NBDs). Within the *mrp1* sequence of *P. berghei* six characteristic motifs of the nucleotide binding domains of ABC proteins have been identified; Walker A and B, ABC motif, as well as Q-, D- and H-loop.

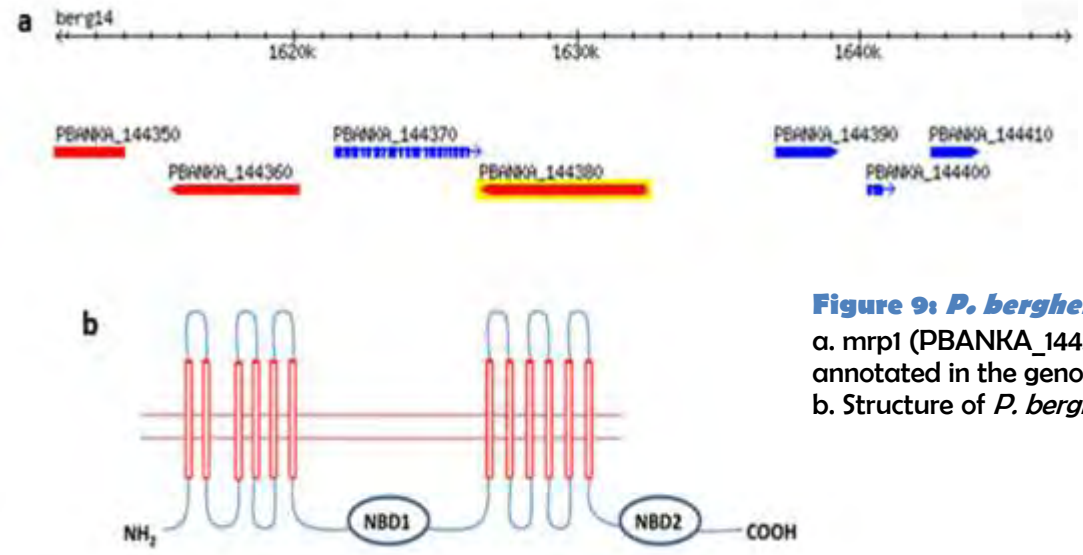


Figure 9: *P. berghei's* MRP1
a. mrp1 (PBANKA_144380) annotated in the genome
b. Structure of *P. berghei's* MRP1

3a.2 Results

The phenotypic characterization of the mutant as compared to the wild type was performed including asexual growth assay, gametocyte monitoring, exflagellation of male gametocytes and ookinete conversion *in vitro*. Characterization of the mutant lines revealed that lack of MRP1 had no effect on the asexual stages of the parasites but led to reduction in gametocytogenesis.

The phenotypic analysis demonstrated that the mutant parasites were able to grow and multiply normally inside red blood cells of the vertebrate host (Figure 10).

Figure 10: Asexual stages of parasites from lines 820tbb and 1512cl1. Ring (a), trophozoite (b) and schizont (c) stage parasites of the wild type and the mutant.

Asexual Growth assay:

An asexual growth assay was performed by FACS analysis to determine whether the mutant parasites had an advantage or a defect over wild type parasites (the experiment was only performed once). For this experiment lines 1025cl1 (mrp1 ko) and 733cl1 (wild type) were used. The experiment needs to be repeated for a longer period of time, as in this data set, it was observed that by day 9 and 10 the mutant line seemed to start to outgrow the wild type line (Figure 11).

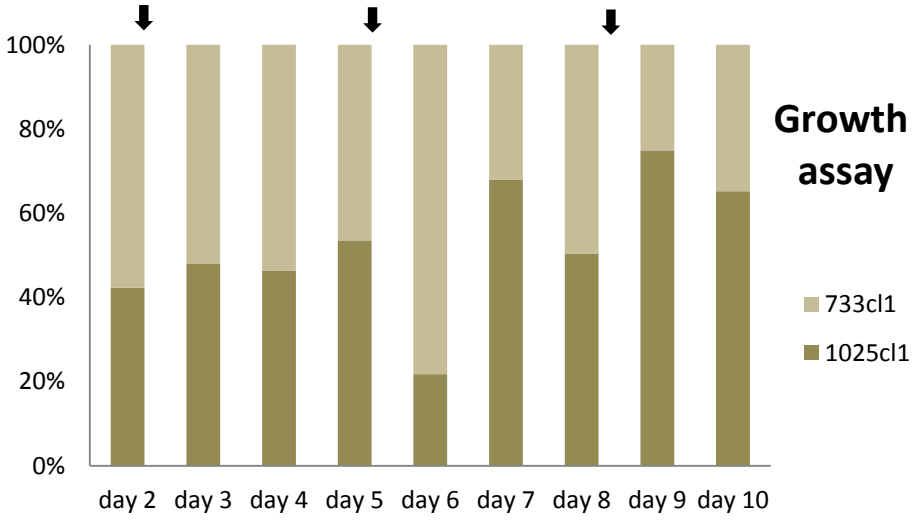


Figure 11: Growth assay for lines 1025cl1 and 733cl1.

Parasites were passaged to naïve mice on the days indicated by arrows (day 2, 5 and 8). Day 1 was not included in the analysis due to very low parasitemia.

Monitoring gametocyte production by FACS, revealed a significant reduction in the number of gametocytes produced from the mrp1 mutant compared to wild type production (Figure 12).

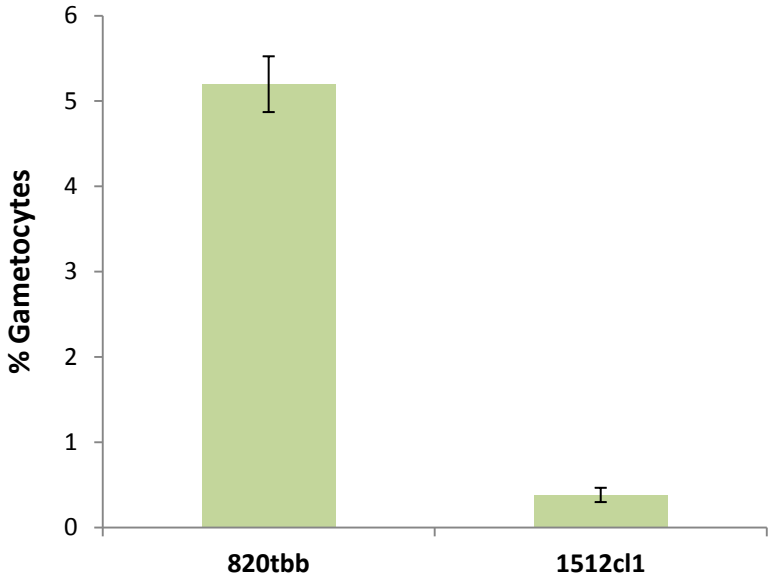


Figure 12: Gametocyte monitoring of wild type parasites (820tbb) and mrp1 mutant parasites (1512cl1).

Exflagellation and ookinete conversion:

The gametocyte monitoring clearly revealed a decline in gametocytogenesis in the mutant line. To examine whether the gametocytes that are produced by the mutant line are functional, we estimated the exflagellation (Figure 13a) and the ookinete conversion (Figure 13b) in lines 1512cl1 (mrp1 mutant) and 820tbb (wild type).

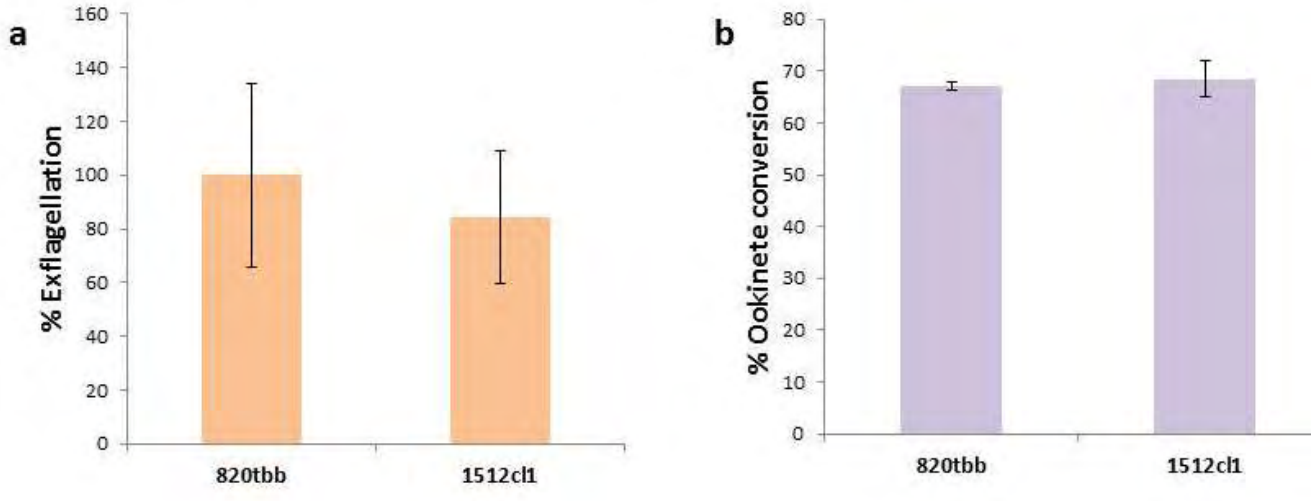


Figure 13: Exflagellation of male gametocytes (a) and ookinete conversion of female gametocytes (b).

In vitro drug assay:

MRP1 has an essential role in cell detoxification in many organisms since it can export several toxic compounds in a conjugated or unconjugated form. To test whether the lack of the *mrp1* gene would alter the parasite's sensitivity to antimalarials, a drug assay was performed. The mutant parasites' growth was compared to the development of wild type parasites in the presence of three different antimalarial drugs with flow cytometry. The drugs tested were dihydroartemisinine (DHA), atovaquone and primaquine. Lines 507tbb (wild type) and 1025cl1 (mrp1 mutant) were used; these lines are made in 507 background causing the parasites to express GFP in all stages.

The IC_{50} values calculated from the drug assay revealed that the mrp1 knock out parasites are approximately 20 times more sensitive to DHA than wild type parasites and 3 times more resistant to atovaquone and primaquine (Figure 14).

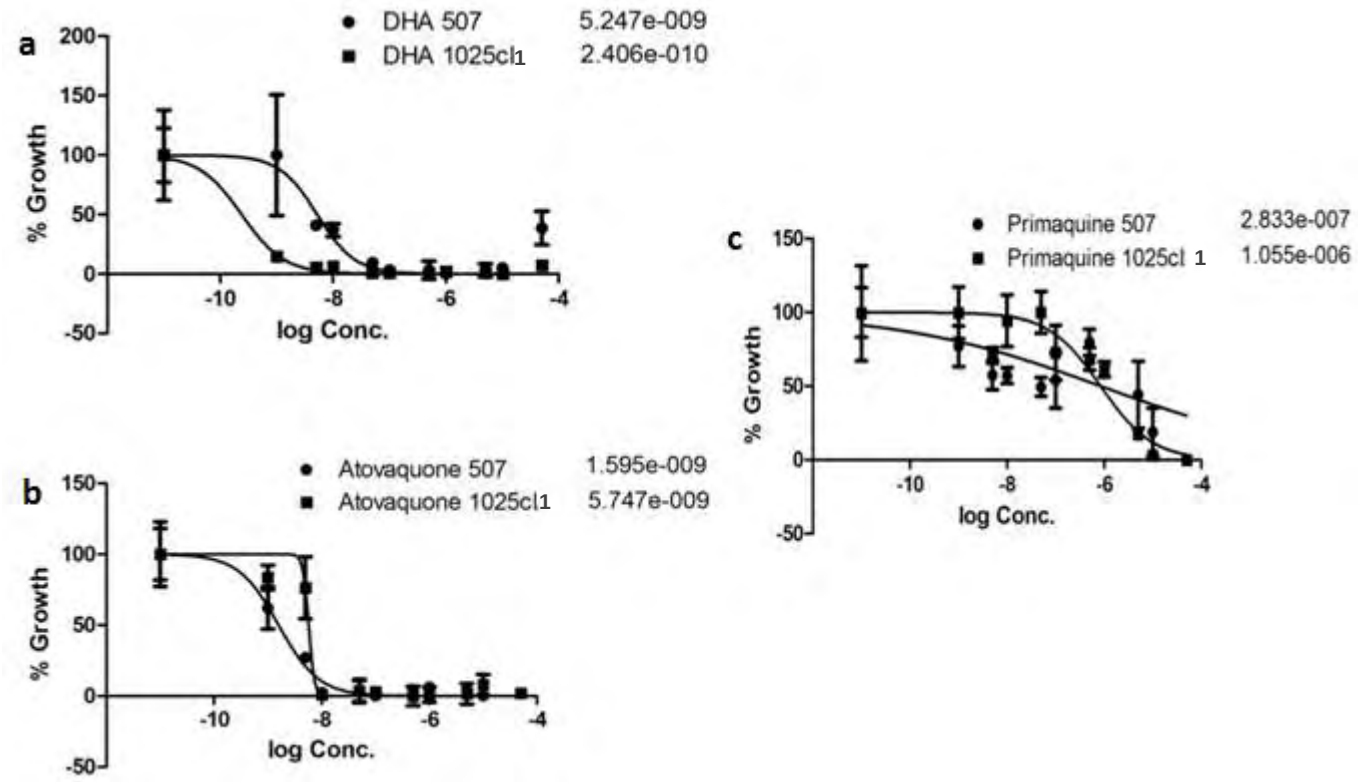


Figure 14: Growth curves and IC_{50} values. Parasites from lines 507tbb and 1025cl1 growing in the presence of a. dihydroartemisine (DHA), b. atovaquone and c. primaquine.

3a.3 Discussion

Artemisinin and its derivatives are amongst the most effective antimalarials to date. These endoperoxide-containing compounds interfere with the development of asexual blood parasites in the host, but also target the sexual stages of the parasite contributing to reduction of transmission. It was demonstrated that artemisinin derivatives lacking the endoperoxide bridge are devoid of antimalarial activity, indicating that the endoperoxide bridge is the key factor of the drug's pharmacological activity[48]. The mode of action of these drugs is not completely understood so far. One of the proposed mechanisms of action for artemisinin and its derivatives suggests that endoperoxide containing compounds are converted into free radicals in the presence of intraparasitic iron and subsequently alkylate *Plasmodium* proteins and other targets in the parasite[49,48]. This mode of action could provide an explanation of the mrp1 mutant's increased sensitivity to DHA. Detoxification from free radicals requires interaction with glutathione since it is a reducing agent and acts as an electron donor to free radicals. Lack of the MRP1 transporter could contribute to faster accumulation of free radicals inside the parasite, therefore the amount of the drug required for a toxic effect decreases and the parasites become more sensitive.

Mutant lines 1025cl1 and 1512cl1 are knock outs of the *mrp1* gene in *Plasmodium berghei*. Phenotypic analysis of the mutant lines revealed no difference in the asexual stages; parasites were able to develop and multiply normally. Gametocyte monitoring of the mutants however, dictated that lack of the *mrp1* gene is responsible for more than 80% reduction in the number of gametocytes produced compared to the wild type. Although fewer in number, the gametocytes produced by the mutant seem to be functioning normally as the percentages of exflagellation and ookinete conversion are within the normal range.

The substrates of MRP1 in *Plasmodium* are yet to be determined, but none of the substrates identified in other species has been known to be essential for gametocyte production. Since lack of the protein interferes with the parasite's ability to produce normal number of gametocytes and, to some extent, transmission of the disease, we attempted to identify the substrate(s) of MRP1 in *P. berghei* using metabolomics.

3b Metabolomics reveals differences in *Plasmodium berghei* lines with differential gametocyte conversion

3b.1 Introduction

Metabolomics involves study of all small molecule metabolites – products or intermediates of the metabolism of a single cell, a tissue or an entire organism. The metabolome is subjected to immediate changes as a result of ongoing biochemistry in the system studied. Any observed change in the metabolome, can be due to a number of conditions, such as treatment with a drug or an inhibitor or induced genetic alterations. Epigenetic regulation and post translational modifications can interfere with the study of the genome and the proteome, whereas the metabolome can provide a snapshot of the cells' biochemical activity and contribute in correlating the observed phenotype with biochemical changes[28].

When a set of metabolites or a specific pathway is under examination targeted metabolomics approaches are used. Otherwise, untargeted approaches are taken, when the entire metabolome is examined. An untargeted metabolomics analysis aims to identify and measure as many metabolites as possible[50].

In this study, an untargeted metabolomics approach was used to point out the metabolic pathway(s) affected by the lack of the *mrp1* gene in an attempt to identify the protein's substrates and define specific function. For metabolomics analysis, the separation method used was High Performance Liquid Chromatography (HPLC) and the detection method was Mass Spectrometry (MS).

High Performance Liquid Chromatography (HPLC) is a separation method in which the sample is introduced into the mobile phase and passed through a stationary phase column where the metabolites contained in the sample are separated based on their ability to be retained in the column which is determined by their inherent chemical properties. When a metabolite is separated from the sample its purified form can be eluted and the time required for a metabolite to elute off the column (retention time) is measured with HPLC. Following their separation, the mass of all the different metabolites is determined using Mass Spectrometry (MS). MS is an analytical technique in which the sample is introduced into the ionisation source, where it is ionized by electron bombardment. Based on the deflection of each ion, its mass is calculated by the detector; lighter ions are deflected more than heavier ions[50]. After a sample is analysed with LC/MS, the metabolites are identified based on their retention time and calculated mass using standards and information from existing databases (Figure 15).

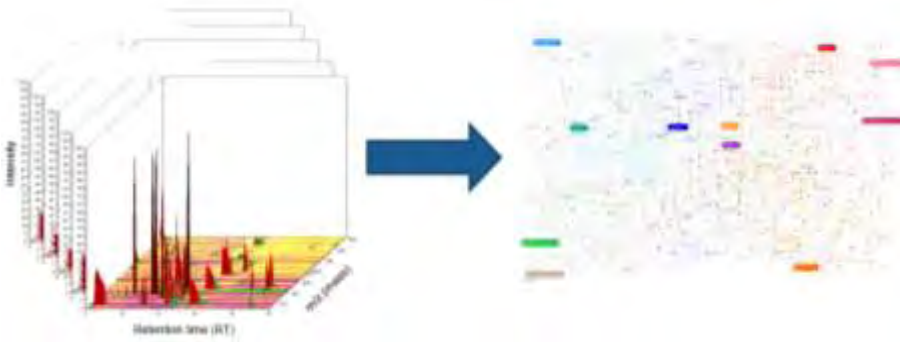


Figure 15: Metabolomics data analysis method. Compounds are matched to metabolic pathways based on their mass and retention time.

3b.2 Results

For the metabolomics analysis, purified schizonts of lines 1512cl1, 820tbb and G401cl1 were injected intravenously into naïve mice in order to synchronize the parasites in the bloodstream. The mice were bled when the parasites where at young ring stage and used for schizont cultures. Metabolite extractions from the schizont cultures were performed at three time points:

- 6 hours after the i.v injections at which point the parasites are at ring stage
- 14 hours post the i.v injections when the parasites are at trophozoite stage
- 26 hours after the i.v injections when the parasites are at schizont stage.

The extracts were sent for HPLC/MS analysis to the Glasgow Polyomics facility.

More than 700 putative metabolites were detected in the pellet and supernatant extracts. Analysis of the data from HPLC/MS revealed that changes in the abundance of many of the identified metabolites were noted mostly in just one or two of the three time points and were not consistent. In a PCA plot analysis each sample is compared to all the other samples. When two samples appear close on the PCA plot it means that their metabolic profiles are similar. Ring stage parasites from the wild type, the low producer and the non-producer all cluster together in a PCA plot, revealing their metabolic resemblance. The results were similar for the trophozoite and schizont stages. Therefore, the stage specific metabolism appears to be largely conserved between the lines (Figure 16).

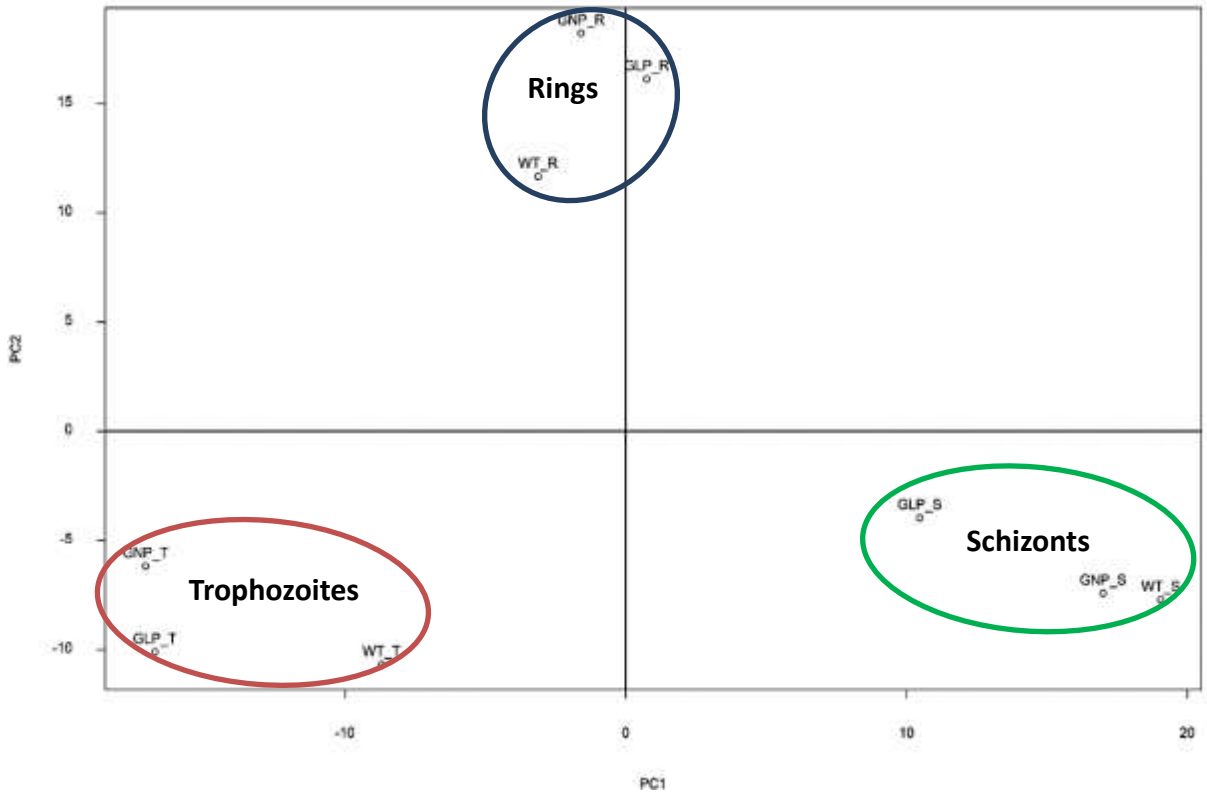


Figure 16: PCA plot analysis of pellet samples.

WT: wild type, GLP: gametocyte low producer, GNP: gametocyte non-producer.

In a volcano plot analysis two samples are compared with each other. A volcano plot analysis of the pellet suspensions revealed that only a few metabolites have a significant fold change between the wild type and the gametocyte low producer and thatthe changes were consistent in all time points only for three of these metabolites (Figure 17). The metabolites with consistent changes in all three time points are 3-Methylguanine, Pro-Ser-Ser and 4-Aminobutanoate (GABA) (Figure 18).

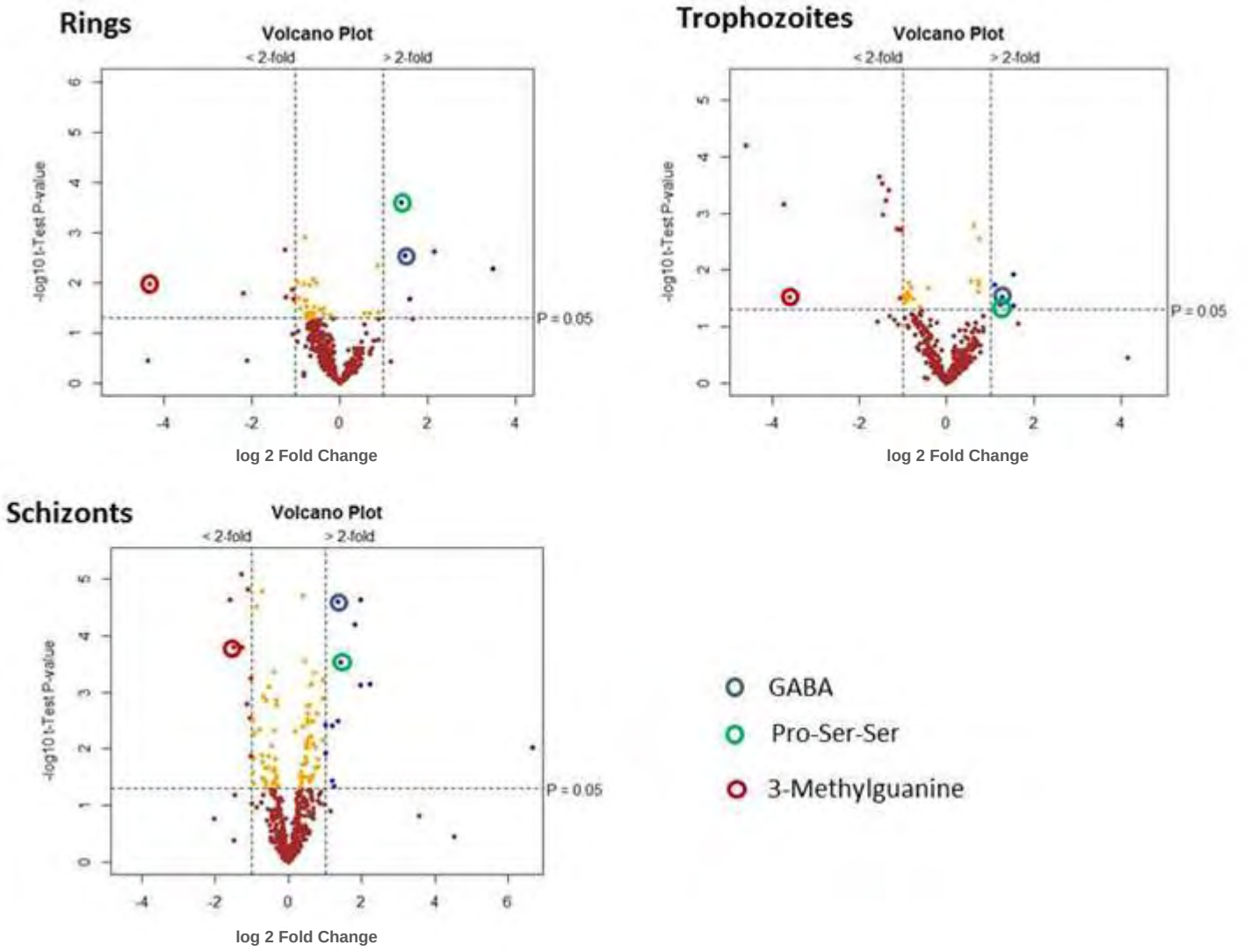


Figure 17: Volcano plot analysis. Wild type vs gametocyte low producer at ring, throphozoite and schizont stage.

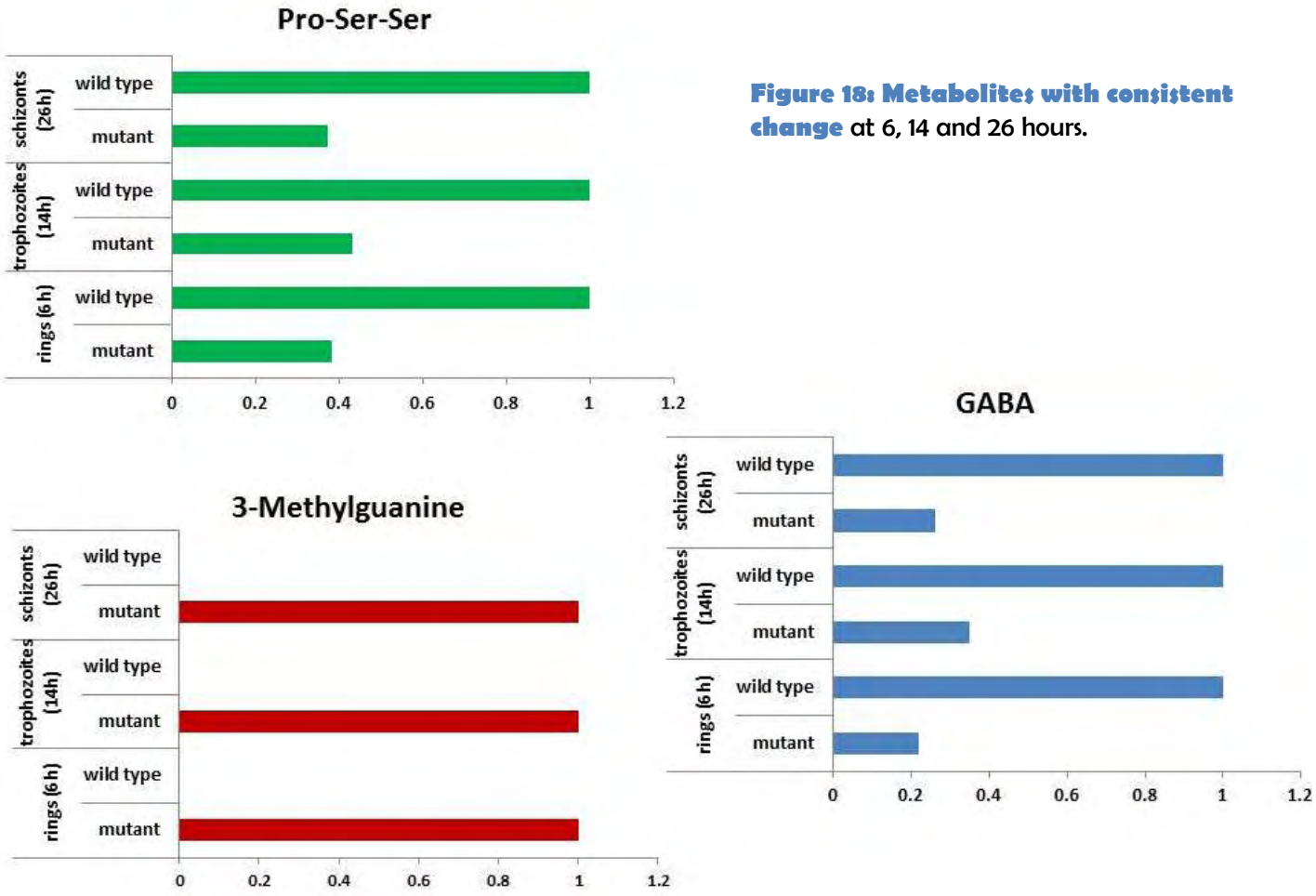


Figure 18: Metabolites with consistent change at 6, 14 and 26 hours.

3b.3 Discussion

The first reports of using metabolomics to monitor metabolites present in urine using gas chromatography coupled with mass spectrometry were published in the 1970s. Since then, metabolomics analyses have been used in a variety of studies, ranging from single cell to mammalian organisms. Metabolomics has been applied to add information to genomic, proteomic and transcriptomic data and contribute towards making a complete picture of the biological system studied, but it can also provide novel information. Metabolomics approaches have been used to study protozoan parasites in different aspects providing valuable information concerning parasite-host interactions, putative drug targets, metabolic networks and possible drug action mechanisms[51].

In this study, an untargeted metabolomics approach was used to study the metabolome of wild type, low producer and non-producer *P. berghei* parasites. LC/MS analysis of the samples resulted in the identification of more than 700 metabolites in the asexual stages. The PCA plot analysis revealed that stage specific metabolism appears to be largely conserved between the lines. In addition, comparison of wild type with low producer parasites in showed that the abundance of only a few metabolites differed significantly between the two lines. Loss of a gene is expected to have an impact on the metabolome of the parasites. Among the putative metabolites that where identified, only

three were found to be consistently changing across the studied time points; 3-Methylguanine, 4-Aminobutanoate (GABA) and Pro-Ser-Ser.

Pro-Ser-Ser is a tripeptide which is most probably a product of haemoglobin degradation in the parasite and is 60% less in the MRP1 mutant compared to wt. 3-Methylguanine is only present in the mutant parasites and is a metabolite that indicates DNA damage. Methylation of guanine could be a result of oxidative stress in the parasites lacking MRP1.

According to the phenotypic characterization of the mutant line the only difference between the wild type and the MRP1 mutant is the low production of gametocytes. Based on the literature and results of some experiments performed in *P. falciparum* [32] and *P. berghei* (personal communication A. Srivastava, unpublished data), we concluded that among the three metabolites GABA is the one that seems most likely to be involved in the sexual stage metabolism. We proceeded to investigate the role of GABA in the sexual stages of *P. berghei*.

3c Elucidating the role of GABA as an energy reserve in sexual stages in P. *berghei*

3c.1 Introduction

The TCA cycle has been shown to be closely linked with GABA metabolism. The TCA cycle was thought to be absent from *Plasmodium spp.* since it was believed that the parasites rely primarily on glycolysis for ATP synthesis[52] and that the enzyme pyruvate dehydrogenase is localised in the apicoplast[30]. Nevertheless, the presence of a single mitochondrion is essential for parasite survival and the genome encodes for all the genes necessary for the operation of the TCA cycle. Additionally, use of labelled glucose and glutamine in *P. falciparum* cultures resulted in the labelling of TCA cycle intermediates[32]. All the above provide evidence of a functioning TCA cycle in Plasmodium spp.

The GABA shunt, as described previously, was recently shown to be functioning in *T. gondii* [36]. However, as the *Plasmodium* genome does not encode for succinic semialdehyde dehydrogenase (SSADH), which is one of the enzymes necessary for the catabolism of GABA to succinate, or for glutamate decarboxylase, only a partial GABA shunt is possible in *Plasmodium* which does encode for the enzyme lysine decarboxylase PBANKA_100340 (LDC), which is thought to have the same function as glutamate decarboxylase[32].

It has been demonstrated that perturbation of the TCA cycle can interfere with the development of sexual stages. Knock out of succinate dehydrogenase resulted in the production of non-functional ookinetes in *P. berghei* [33] and inhibition of aconitase using NaFAc, led to reduction of gametocytogenesis in *P. falciparum*[32]. Furthermore, high levels of labelled GABA were detected in gametocytes and even higher in ookinetes when heavy labelled isotope of glutamine was used in *P. berghei* cultures (personal communication, A. Srivastava, unpublished data). GABA seems to be accumulated in gametocytes and even more so in ookinetes, possibly to serve as an energy reserve in mosquito stages, where glucose is no longer abundant. Therefore, we decided to test whether the disruption of GABA metabolism could provide evidence for its role in ookinete development.

Genes that are possibly involved in GABA metabolism and were targeted for deletion are lysine decarboxylase (*ldc*-PBANKA_100340) and putative transporters of GABA and/or glutamate (*gdh1*-PBANKA_102620, *gdh2*-PBANKA_101400, *gdh3*-PBANKA_122820 and a put. transporter-PBANKA_030670). Ornithine amino transferase (*oat*-PBANKA_010740), which functions as a transporter and possibly converts α-ketoglutarate to glutamate, was also targeted (Figure 19).

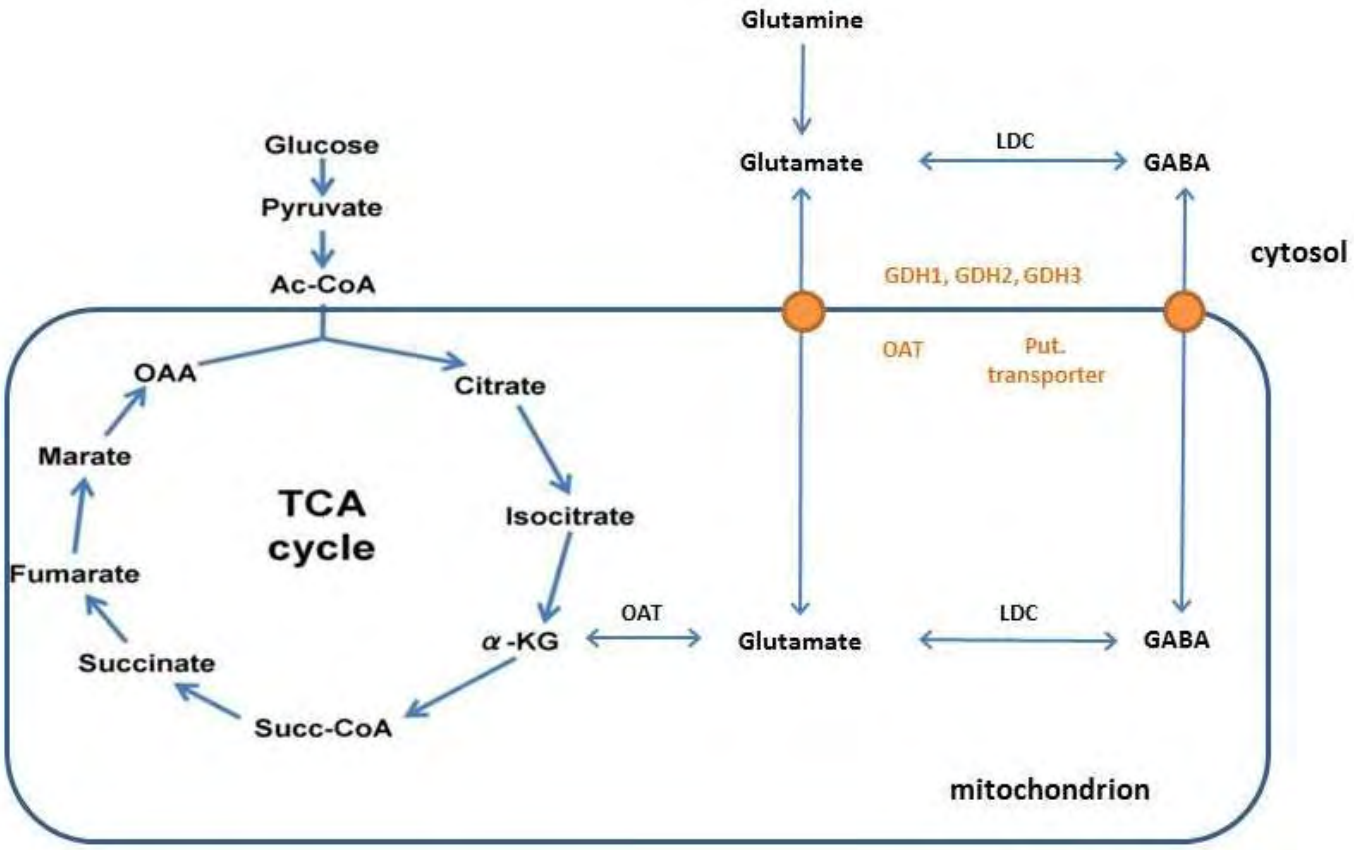


Figure 19: GABA metabolism in Plasmodium.

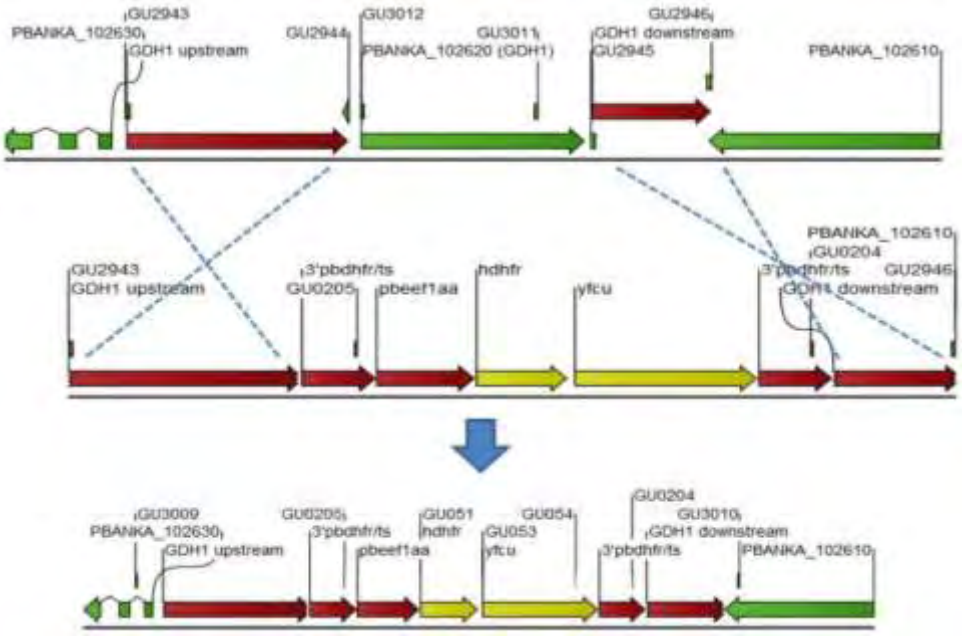
3c.2 Results

To generate the knock out lines, the upstream and downstream regions of the genes were inserted into plasmid pL0035 and the correct ligation and insertion was confirmed with diagnostic digestions. Plasmid pL0035 containing the homology regions was used as vector for the transfection of *P. berghei* parasites and with double homologous recombination the genes were knocked outand mutants were selected by pyrimethamine treatment. At this stage the parasite population is mixed, consisting of wild type and mutant parasites. In order to isolate the mutant parasites the lines are cloned (for more details see sections 2b.1 and 2b.2 of Methods).

Glutamate dehydrogenase 1 (PBANKA_102620)

GDH1 was successfully knocked out in *P. berghei* with double crossover homologous recombination (Figure 20a). Lines G1007 (GDH1 knock out in 507 background) and G1013 (GDH1 knock out in 820 background) were generated and successfully cloned (Figure 20b).

a



b

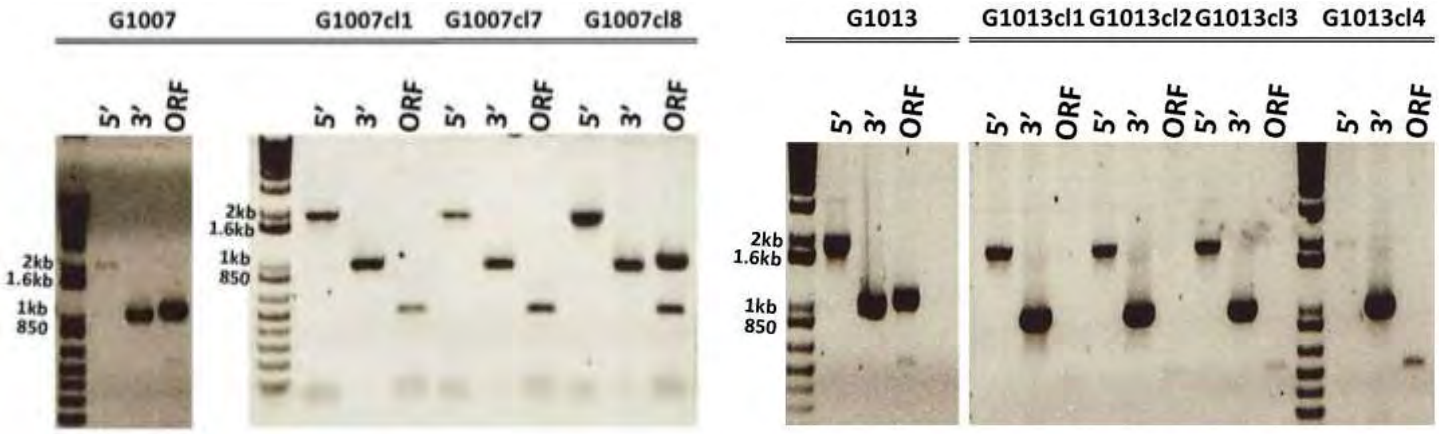


Figure 20: GDH1 knock out. a) Schematic representation of the double homologous recombination taking place in mutant parasites b) Uncloned (G1007, G1013) and cloned lines of GDH1 knock outs, in the cloned lines the open reading frame band is not present (G1007cl8 is not a cloned line, since the ORF band at 1.1kb is present). Abbreviations: 5' – 5' integration, 3' – 3' integration, ORF – open reading frame.

The GDH1 knock out lines were negatively selected in order to have the ability to create a double or triple knock out using the same selection cassette. For that purpose, mice are treated with 5-fc (see methods). The enzyme encoded by gene *yfcu* converts 5-fc into a substance that is toxic for the parasites, causing the single recombination of the two 3'pbdhfr/ts and removal of part of the cassette (Figure 21a). Afterwards, the same selectable marker can be used for another gene knock out. Negative selection was

successful for G1007cl1 and G1013cl1 but the lines were not cloned due to time restrictions (Figure 21b).

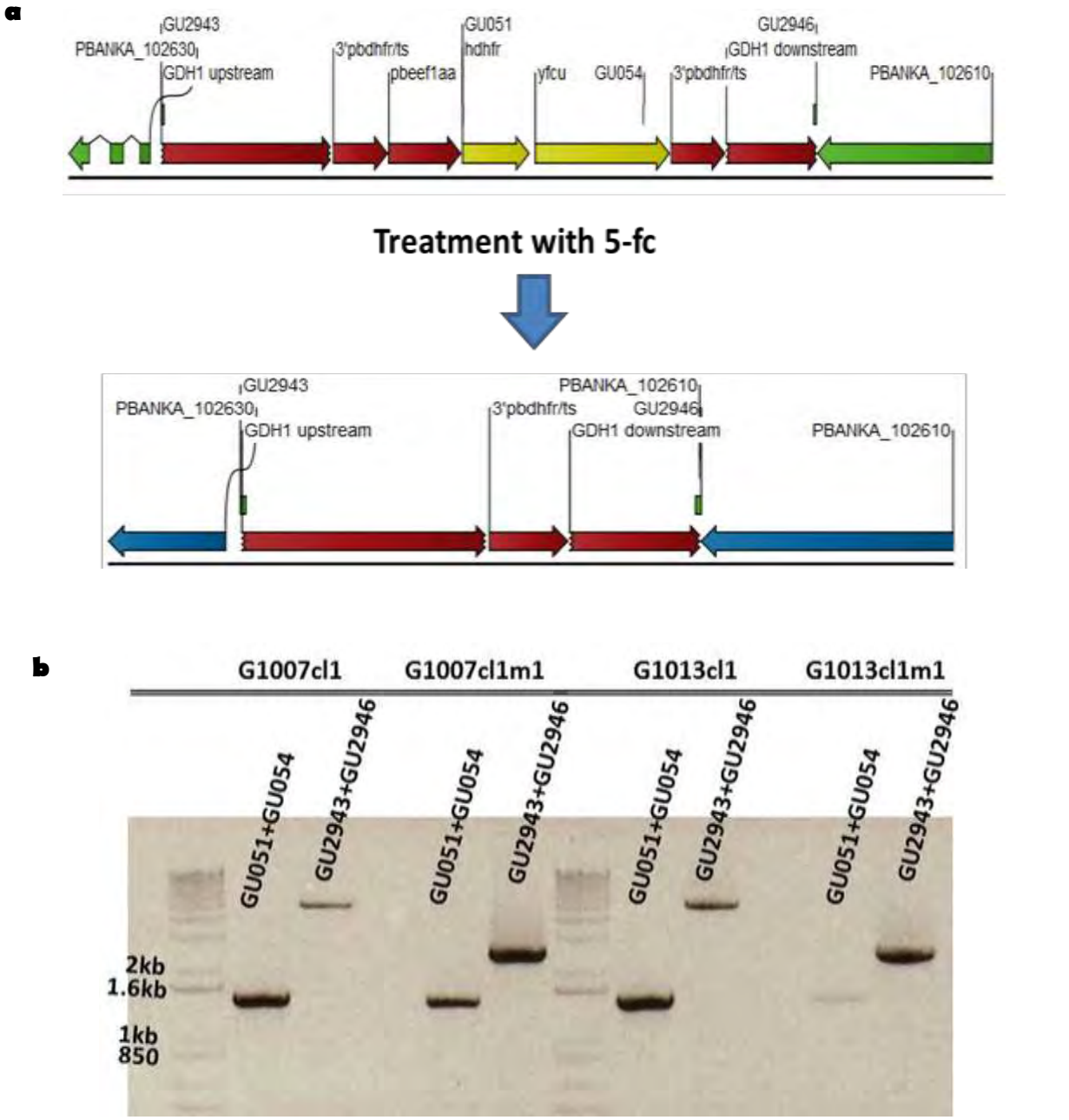


Figure 21: Negative selection. a) Single recombination of 3'pbdhfr/ts b) negatively selected G1007cl1 and G1013cl1, primers GU2943+GU2946 have a product of 5.5kb before negative selection and 2.6kb in negatively selected parasites (primers GU051+GU054 bind only if the cassette is present and were used as control). Abbreviations: 5' – 5' integration, 3' – 3' integration, ORF – open reading frame.

Glutamate dehydrogenase 2 (PBANKA_101400)

GDH2 was successfully knocked out in backgrounds 507 (line G1058) and 820 (line G1055). The lines need to be cloned for isolation of the mutant parasites (Figure 22).

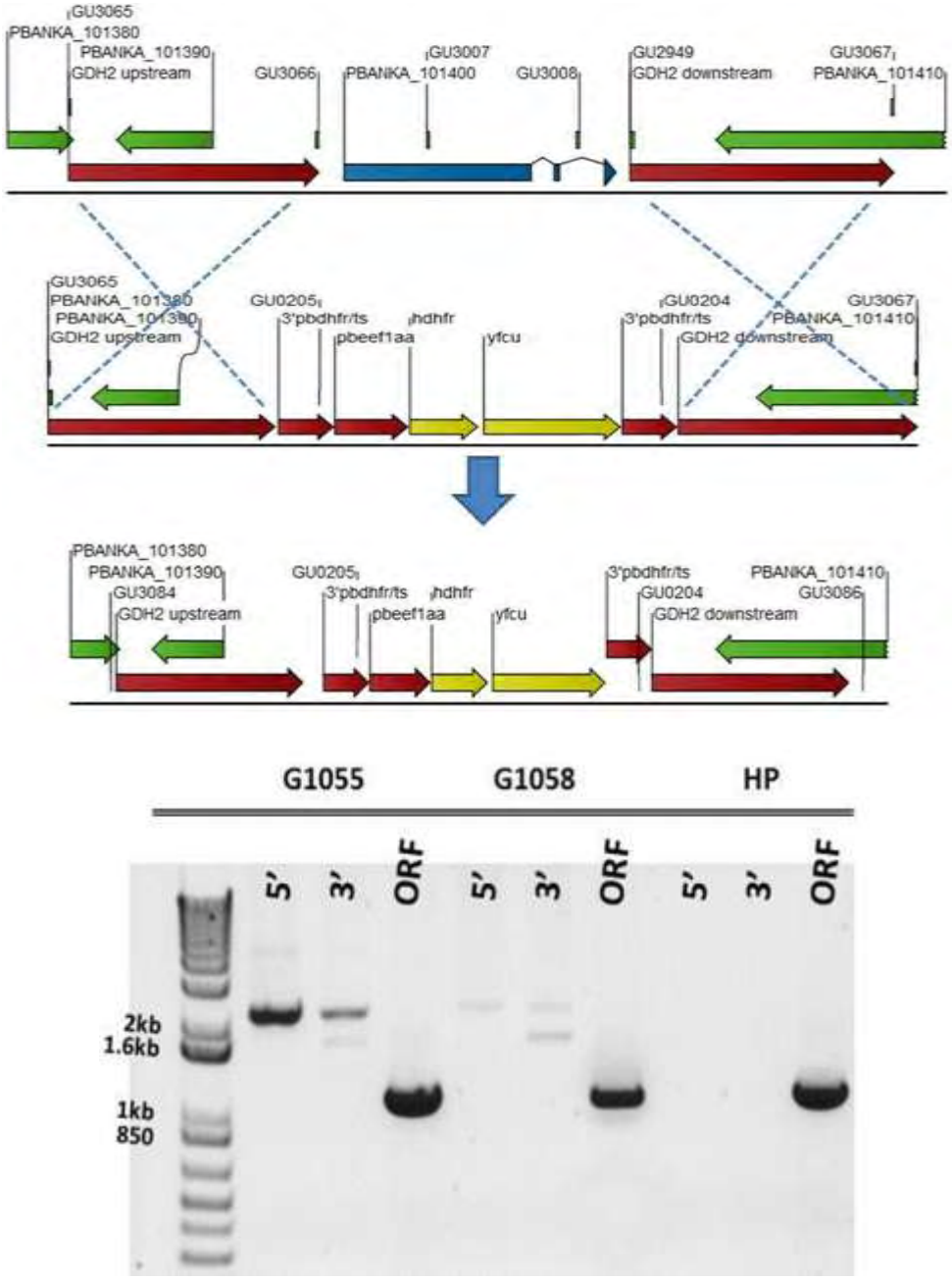


Figure 22: GDH2 knock out with double crossover homologous recombination; generation of lines G1055 and G1058. Abbreviations: 5' – 5' integration, 3' – 3' integration, ORF – open reading frame.

Glutamate dehydrogenase 3 (PBANKA_122820)

GDH3 was successfully knocked out with double homologous recombination and lines G1006 (GDH3 knock out in 507 background) and G1012 (GDH3 knock out in 820 background) were cloned (Figure 23).

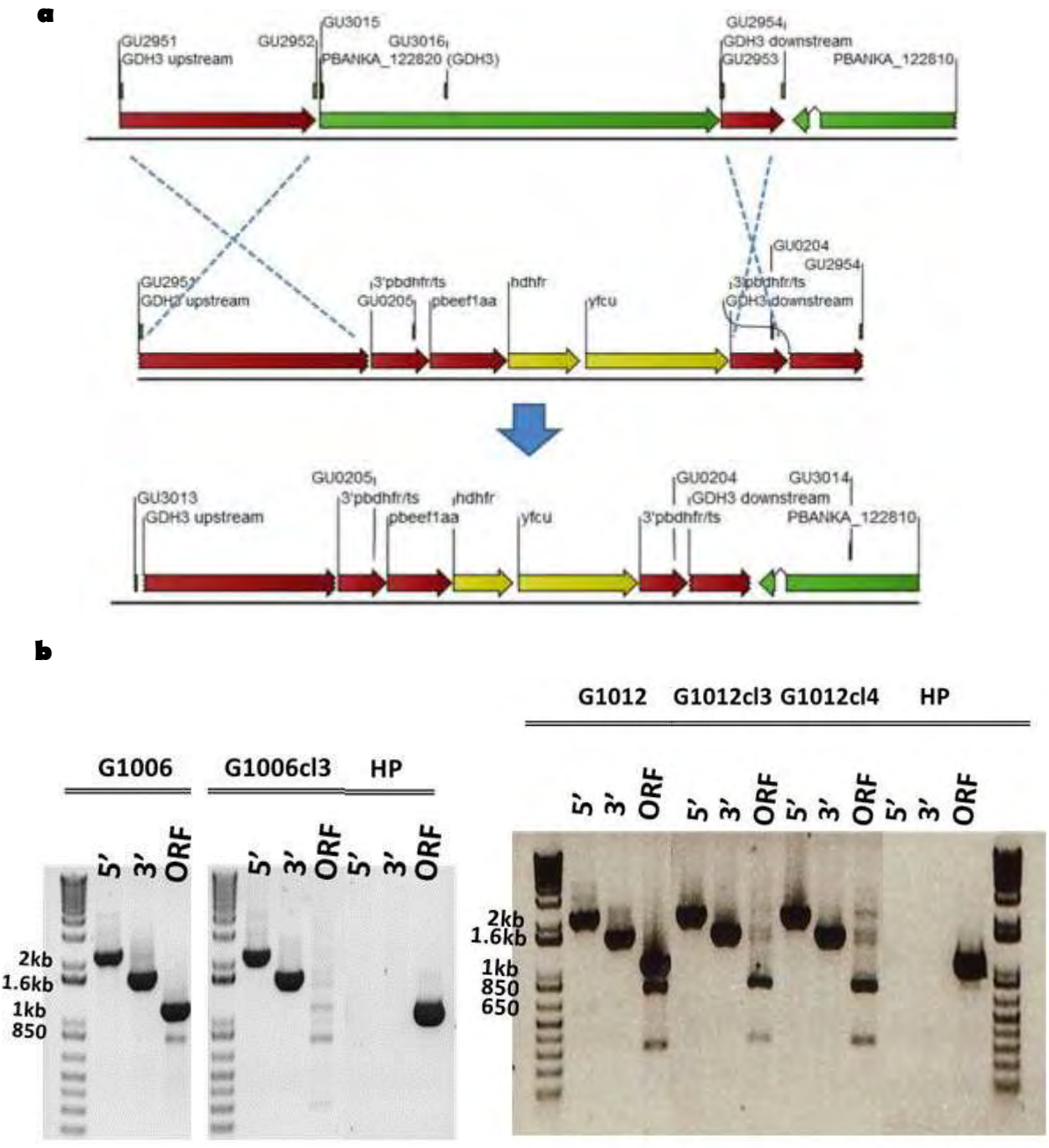


Figure 23: GDH3 knock out. a) Schematic representation of the double crossover homologous recombination resulting in deletion of GDH3 b) Successful cloning of lines G1006 and G1012. Abbreviations: 5' – 5' integration, 3' – 3' integration, ORF – open reading frame.

Ornithine amino transferase (PBANKA_010740)

Lines G1009 and G1015 were generated by deletion of OAT in the 507 and 820 background with double homologous recombination. The lines were successfully cloned (Figure 24).

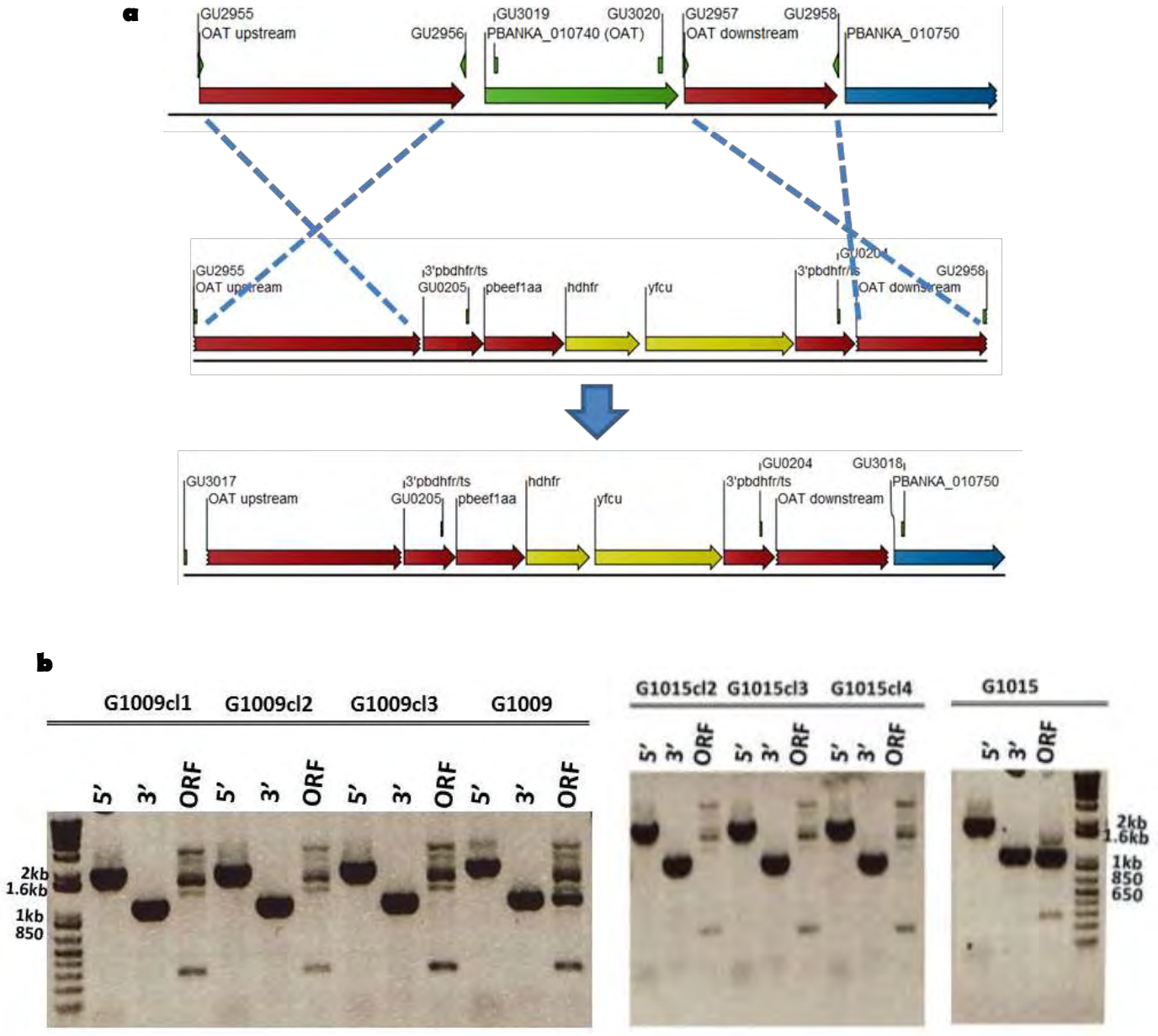


Figure 24: OAT knock out. a) Double crossover homologous recombination resulting in deletion of OAT b) Successful cloning of lines G1009 and G1015. Abbreviations: 5' – 5' integration, 3' – 3' integration, ORF – open reading frame.

Putative transporter (PBANKA_030670)

The putative transporter of GABA and/or glutamate was knocked out in the 507 (G1010) and the 820 (G1016) background. The generated lines have not been cloned yet (Figure 25).

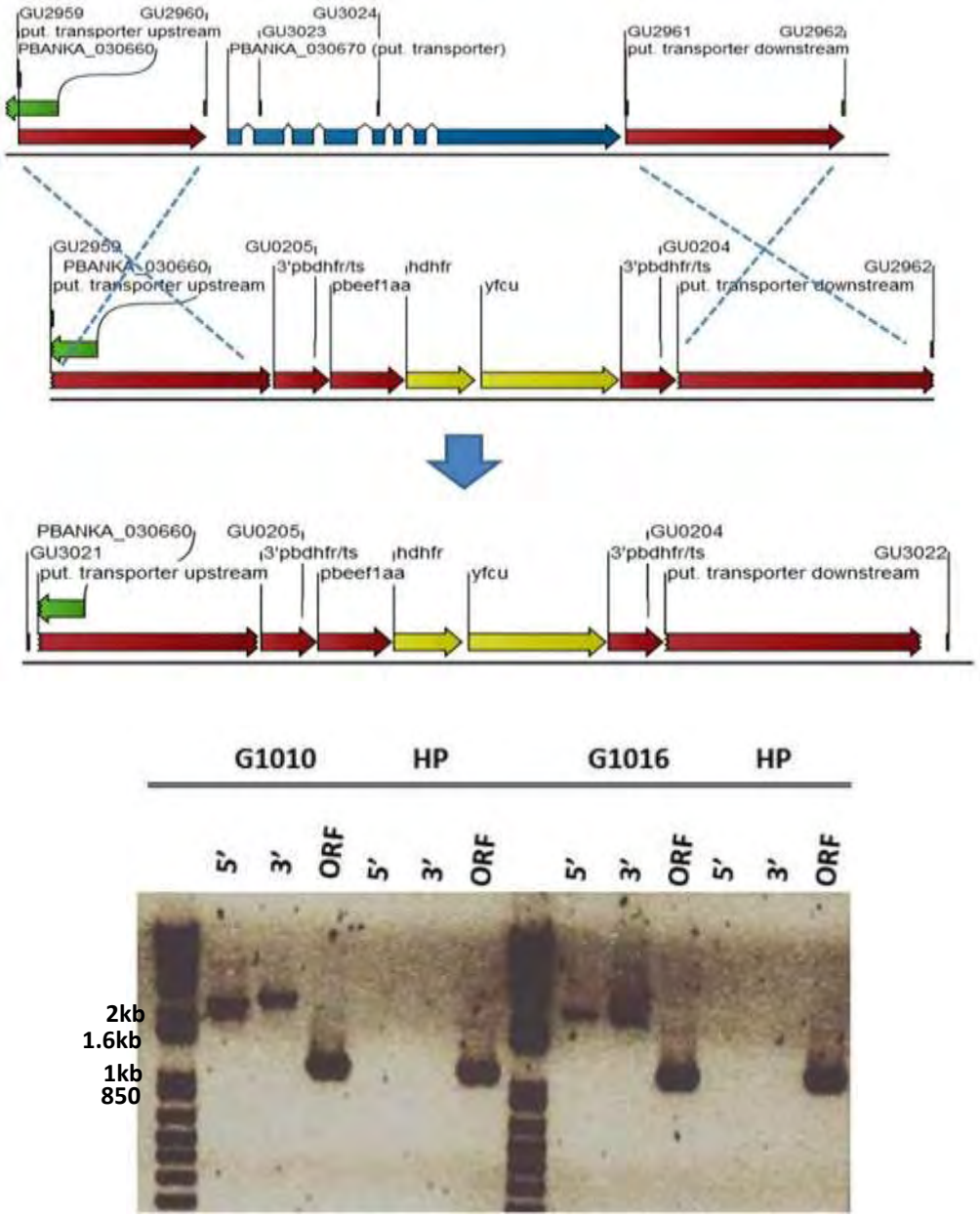


Figure 25: Putative transporter knock out with double crossover homologous recombination. Generation of uncloned lines G1010 and G1016. Abbreviations: 5' – 5' integration, 3' – 3' integration, ORF – open reading frame.

Lysine decarboxylase (PBANKA_100340)

Deletion of LDC was not possible even after six attempts with double crossover homologous recombination. Only 3' integration was detected in transfected parasites (Figure 26). Failure to knock out LDC could be due to the gene's size (~5.5kb), that area of the genome could be difficult to target (as we had difficulty in amplifying the upstream region) or it could mean that the gene is essential and cannot be knocked out in asexual stages.

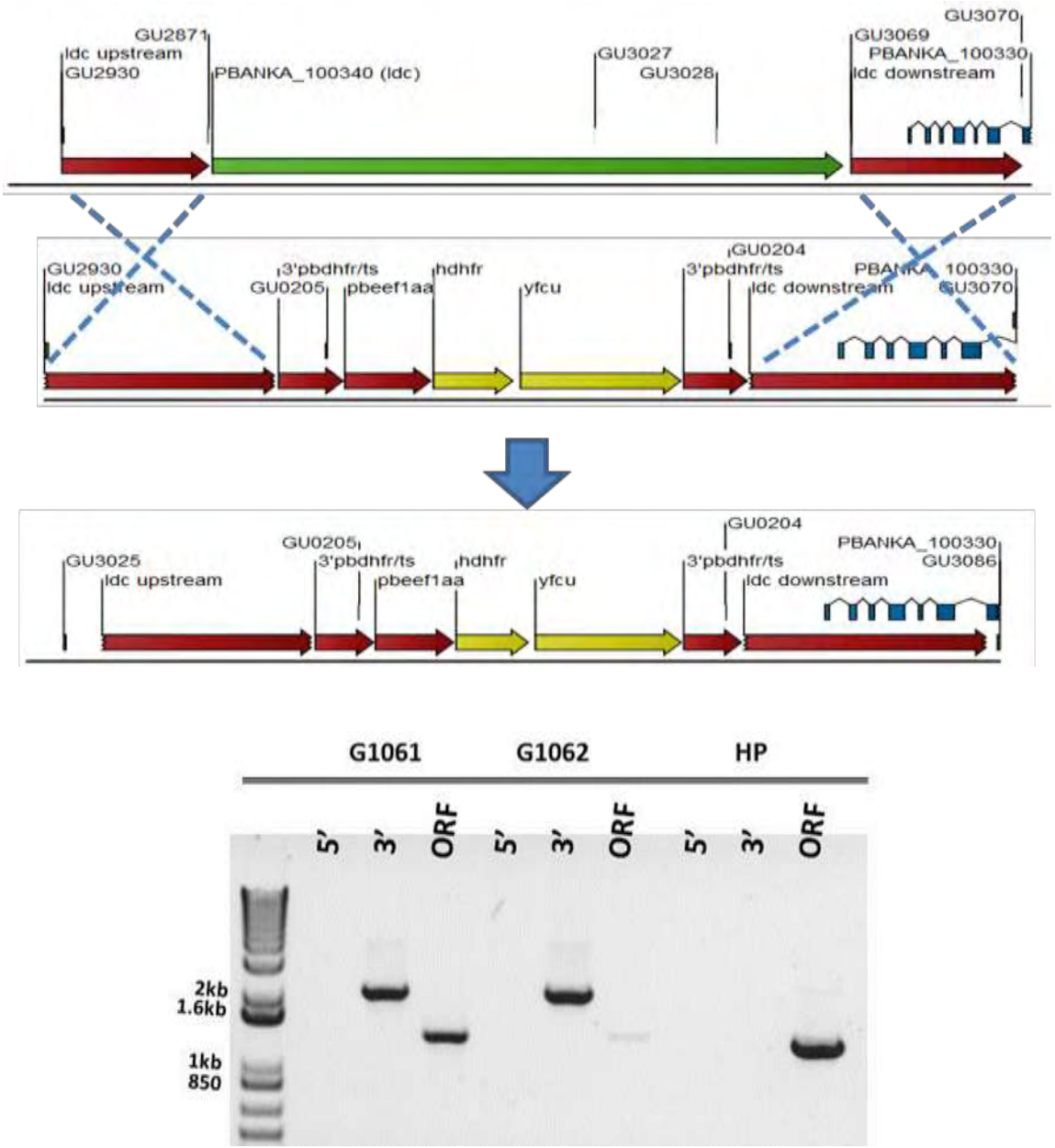


Figure 26: LDC knock out. Schematic representation of the double crossover homologous recombination that could lead to knock out of LDC. Deletion was not successful. Abbreviations: 5' – 5' integration, 3' – 3' integration, ORF – open reading frame.

❖ Phenotypic analysis of cloned lines

Following their generation, the cloned mutant lines were phenotypically characterized. The production of gametocytes was monitored and the exflagellation of male gametocytes and ookinete conversion were checked.

1. Glutamate Dehydrogenase 1 knock out (G1013cl1)

Gametocyte monitoring of line G1013cl1 (GDH1 knock out in the 820 background) revealed no significant difference from the number of gametocytes produced by the wild type (Figure 27a). However, there seems to be a decline in exflagellation (Figure 27b) and in the ookinete conversion (Figure 27c)of the GDH1 mutant parasites.

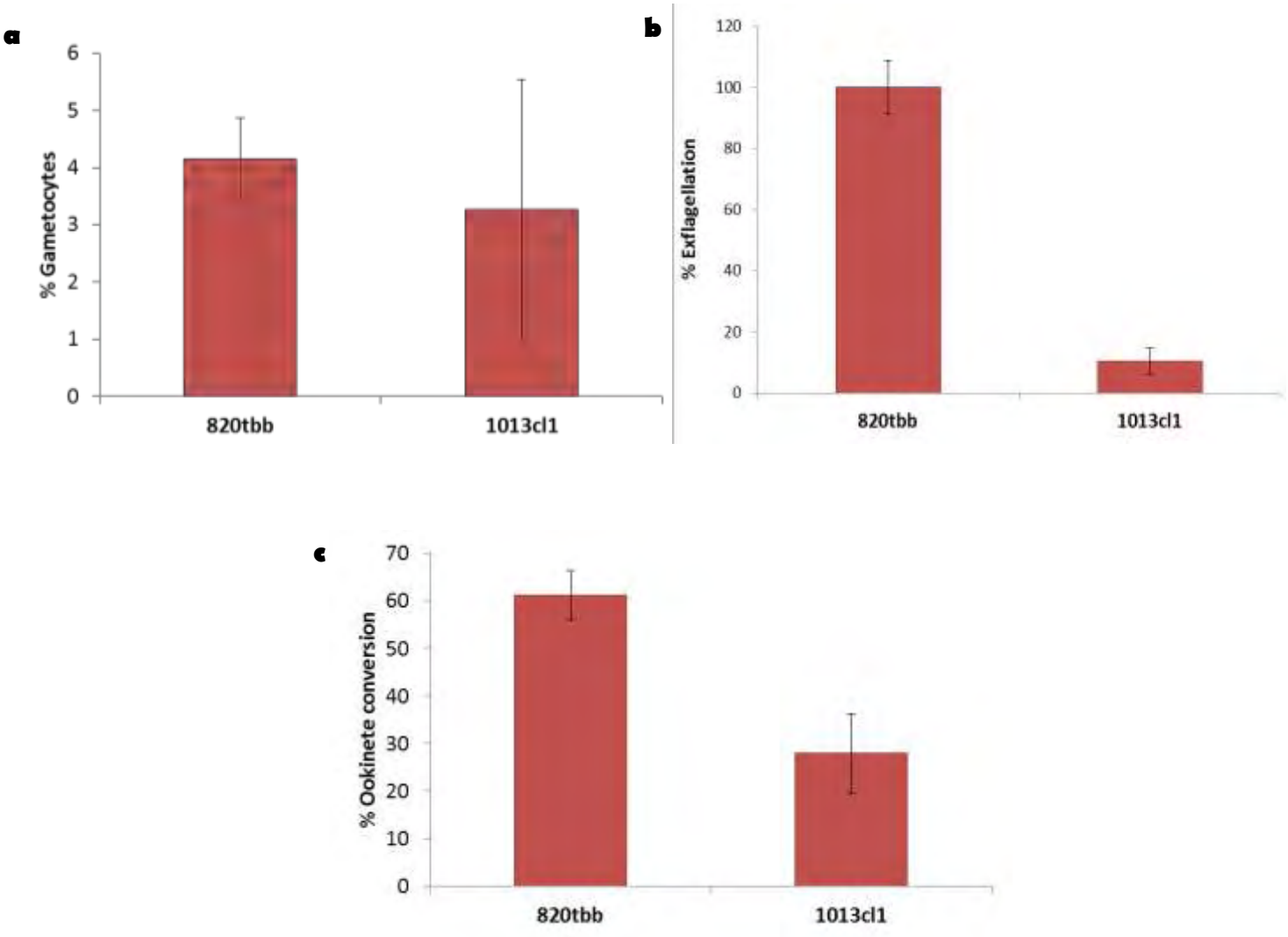


Figure 27: : Phenotypic analysis of the GDH1 knock out. a) gametocyte monitoring b) exflagellation percentage and c) ookinete conversion rates of the wild type and the GDH1 knock out(these experiments were performed twice and need to be repeated).

2. Glutamate Dehydrogenase 3 knock out (G1012cl1)

Phenotypic analysis of the GDH3 knock out line showed no significant difference in the production of gametocytes (Figure 28a), exflagellation of male gametocytes (Figure 28b) and ookinete conversion (Figure 28c) between the wild type and the mutant. These experiments were only performed once and need repetition.

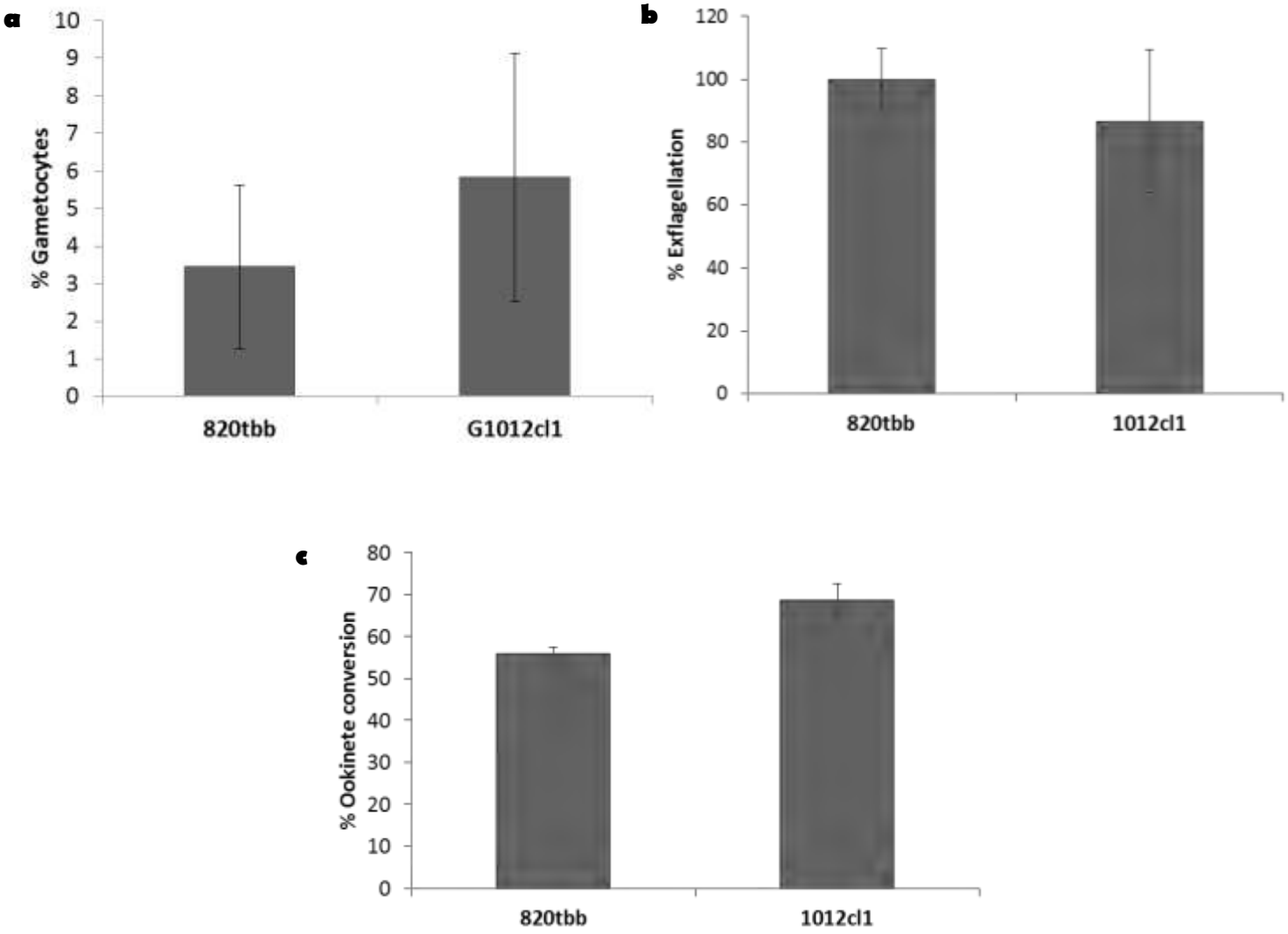


Figure 28: Phenotypic analysis of the GDH3 knock out. a) gametocyte monitoring b) exflagellation percentage and c) ookinete conversion rates of the wild type and the GDH3 knock out.

3. Ornithine Amino Transferase knock out (G1015cl2)

Gametocyte monitoring and calculation of exflagellation and ookinete conversion for the OAT knock out were only performed once and need to be repeated in order to get reliable results.It seems that gametocyte production (Figure 29a) and ookinete conversion rates(Figure 29c) of the OAT mutant line are within the normal range but the exflagellation of male gametocytes is reduced (Figure 29b). These experiments were only performed once and need repetition.

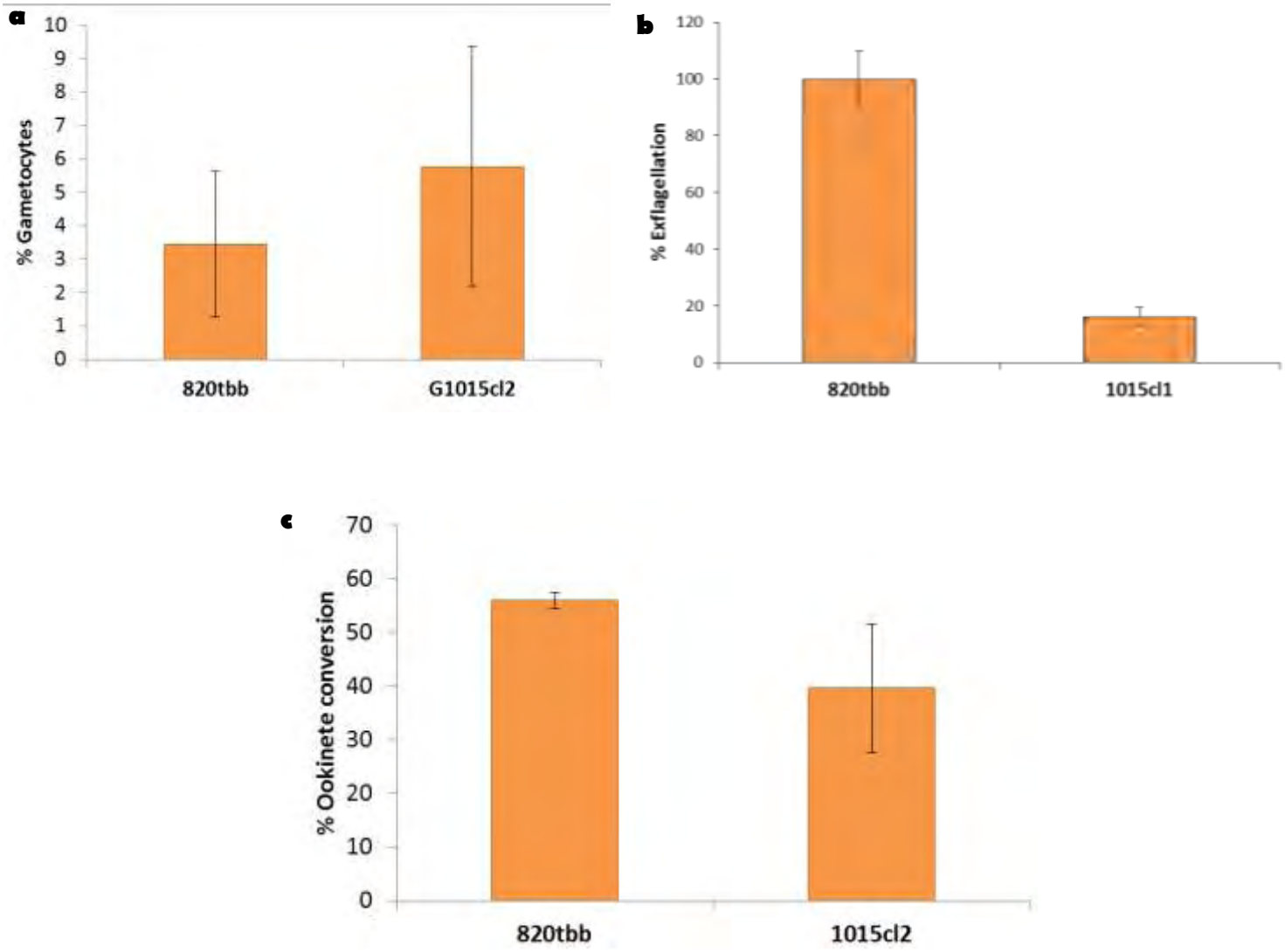


Figure 29: Phenotypic analysis of the OAT knock out. a) gametocyte monitoring b) exflagellation percentage and c) ookinete conversion rates of the wild type and the OAT knock out.

3c.3 Discussion

According to recent findings sexual stage parasites utilize the TCA cycle for energy generation. Also, gametocytes and especially ookinetes seem to accumulate GABA possibly to use as an energy reserve in the mosquito stages. Six genes that are likely to participate in the metabolism of GABA were targeted for making knock outs in *P. berghei* parasites. Glutamate decarboxylases (GDH1, 2, 3) as well as a putative transporter are all possible transporters of GABA and/or glutamate. Ornithine amino transferase (OAT)is also a putative transporter of GABA/glutamate but it could also have the same function as GABA-T, which converts the TCA cycle intermediate a-ketoglutarate into glutamate; the precursor of GABA. Finally, lysine decarboxylase (LDC) which is thought to catalyse the conversion of glutamate to GABA and GABA to glutamate was also targeted. The RNA sequencing data (A. Religa, unpublished data) show that RNA from all the targeted genes was detected in the sexual stages of *P. berghei*, but only *gdh1* is highly transcribed in the ookinete stage and *ldc* in gametocytes (Figure 30). It is expected that deletion of any of these genes can provide evidence for the role of GABA shunt for ookinete development.

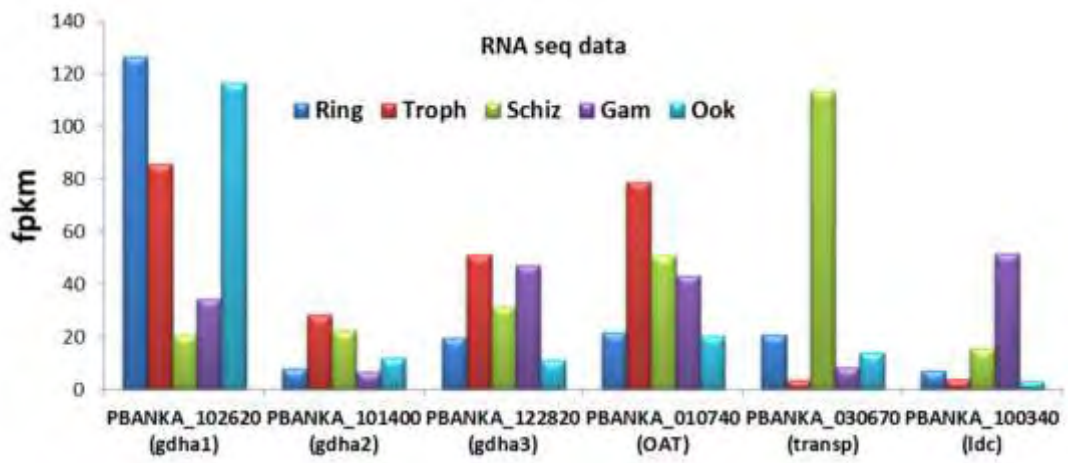


Figure 30: RNA sequencing data for *gdh1, 2, 3, oat, putative transporter* and *ldc*.

Deletion of five of the targeted genes was possible in asexual stages (*gdh1, 2, 3, oat* and *putative transporter*) in the 507 and 820 background. The GDH1, GDH3 and OAT knock out lines were successfully cloned in both backgrounds. These genes were targeted in order to disrupt the metabolism of GABA in *P. berghei* and show its role in parasite development. For this purpose, a phenotypic analysis of the cloned knock out lines was performed. The phenotypic characterization of the GDH1, GDH3 and OAT knock outs included gametocyte monitoring, exflagellation and ookinete conversion rates. The GDH3 knock out line (G1012cl1) shows no significant difference from the wild type in gametocyte production, exflagellation and ookinete conversion. In the GDH1 and OAT knock outs even though gametocyte production is normal, there seems to be a reduction in the exflagellation of male gametocytes. Ookinete conversion is not affected in the OAT knock out (G1015cl2), it is however slightly reduced in the GDH1 knock out (G1013cl1). These experiments need to be repeated before any conclusions about the role of GABA metabolism in the sexual stages can be drawn.

4 Final discussion

Transporter protein MRP1 has been identified in mammals and in a variety of non-mammalian organisms, such as protozoa, yeast, nematodes and plants. This particular protein can transport a wide range of substrates, including several drugs as well as glutathione and glucuronate conjugates which are products of Phase II metabolism in cells. It is therefore involved in detoxification mechanisms and protection of the cell from endo and xenobiotics[27]. We demonstrated that *P. berghei* parasites lacking only the MRP1 gene produce 80-90% less gametocytes than wild type parasites. These gametocytes, although fewer in number, seem to have normal exflagellation and ookinete conversion rates. The substrate(s) of MRP1 in *Plasmodium* is unknown, so we conducted a comparative untargeted metabolomics study of the MRP1 mutant and wild type lines aiming to find out which pathway(s) is affected by the lack of the transporter. Over 700 putative metabolites were identified with LC/MS analysis of samples. The samples contained extracts from synchronized parasites in all three stages (rings, trophozoites and schizonts). Analysis of the metabolomics data of the wild type (820tbb), the low producer (1512cl1) and the non-producer control (G401cl1) revealed that the metabolic profiles of these lines are largely conserved across the life cycle in asexual stages. When the wild type and the low producer were compared in a Volcano plot analysis it was clear that the metabolomes of the wild type and the low producer lines are highly similar in all three time points the extractions were performed. In fact, only three metabolites were altered in a consistent manner in all three time points; Pro-Ser-Ser, 3-Methylguanine and GABA.

The tripeptide Pro-Ser-Ser is probably a product of hemoglobin degradation in the parasites and is approximately 60% less in the mrp1 mutant. 3-Methylguanine is an indicator of DNA damage; methylated nucleotides are the origin of mutations due to mispairing and they can be identified in a variety of organisms ranging from bacteria to humans. Cells possess specific enzymes, called DNA glycosylases, which identify and excise methylated nucleotides from the DNA[53]. The presence of free 3-Methylguanine exclusively in the mutant parasites could indicate that the DNA is getting damaged, possibly by the accumulation of reactive oxygen species due to the lack of MRP1.

GABA is an amino acid known for its function as a neurotransmitter in mammalian cells. The abundance of GABA was found to be approximately 70% less in the mutant parasites. The reason that we focused on this metabolite was its participation in the TCA cycle and previous work (published[32] and unpublished) considering the relation between the sexual stages of the malaria parasites and the TCA cycle. Targeting genes that are possibly involved in GABA metabolism (*gdh1-2-3*, *oat*, *putative transporter* and *ldc*) was not entirely successful; we were able to delete *gdh1*, *gdh2*, *gdh3*, *oat* and the *putative transporter* but not *ldc*. Phenotypic analysis of the cloned knock out lines for *gdh1*, *gdh3* and *oat* revealed that disruption of GABA metabolism seems to affect exflagellation and mostly ookinete conversion rates (the experiments need to be repeated). Our results could be explained by the fact that GABA may serve as an energy reserve in the mosquito stages. Finally, the mutant parasites were tested against the antimalarials dihydroartemisinine (DHA), atovaquone and primaquine. There was no significant difference between the wild type and the mutantparasites against atovaquone and primaquine, but the mrp1 mutant was approximately 20 times more sensitive to DHA compared to wild type. Since the mode of action for artemisinin and its derivatives is not

entirely known, we cannot explain these results with certainty, but one of the modes of action for these drugs suggests that the endoperoxide containing drugs are converted to free radicals in the presence of intraparasitic iron and cause alkylation of proteins. As explained previously, free radicals could accumulate faster in parasites lacking MRP1 making them more sensitive to DHA.

5 Future work

Future work for this project includes metabolomics analysis of gametocytes in order to compare the metabolomes of the MRP1 knock out line and the wild type. Additionally, this comparison might reveal the substrate of the MRP1 protein, which is also one of the future objectives of this project. Furthermore, we need to tag MRP1 and the genes involved in GABA metabolism, using a fluorescent tag, for localisation. Concerning the genes we targeted for deletion, we need to create a triple knock out of *gdh1, gdh2* and *gdh3* in case there is functional redundancy. Phenotypic analysis of the triple knock out could help to determine the function of glutamate dehydrogenase in *Plasmodium berghei*. Future plans also include knock out or disruption of the *ldc* gene or conditional degradation of its protein in gametocytes and ookinetes to study its function in mosquito stages in case it is indeed essential in asexual stages. The reason that deletion of the *ldc* gene was not possible using a regular vector could be that it is a big gene (almost 10kb), so the vector used for the knock out needs to have long arms. This knock out might be successful with the use of PlasmoGen recombineering vectors. Also, disrupting *ldc* by removing part of the gene could give some indication of its function. Moreover, we need to do phenotypic analysis of all the generated mutant lines; including oocyst and sporozoite development as well as transmission to naïve mice. Finally, we aim to do targeted metabolomics using heavy labeled isotopes of glucose and glutamine of the GABA metabolism mutants in order to examine the effect of disrupting GABA metabolism on major metabolic pathways.

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