INVESTIGATIONS INTO THE METABOLIC REQUIREMENTS FOR LIPOIC ACID AND LIPID SPECIES DURING THE LIFE CYCLE OF THE MALARIAL PARASITE *PLASMODIUM BERGHEI*

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ABSTRACT

Investigations into the metabolic requirements for lipoic acid and lipid species during the life cycle of the malarial parasite *Plasmodium berghei.*

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Plasmodium, like many other pathogenic organisms, relies on a balance of synthesis and scavenging of lipid species for replication. How the parasite creates this balance is particularly important to successfully intervene in transmission of the disease and to generate new chemotherapies to cure infections. This study focuses on two specific aspects in the field of lipid biology of *Plasmodium* parasites and their hosts. Lipoic acid is a short eight-carbon chain that serves a number of different functions with the cell. By disrupting a key enzyme in the lipoic acid synthesis pathway in the rodent species of malaria, *Plasmodium berghei*, we sought to investigate its role during the parasite life-cycle. Deletion of the lipoyl-octanoyl transferase enzyme, LipB in *P. berghei* parasites demonstrate a liver-stage specific need for this metabolic pathway. In order to explore the impact of the fatty acid and triglyceride content on the pathogenesis of *Plasmodium* parasites, this study tests two methods to reduce lipid content *in vivo* and test the propagation of *P. berghei* parasite in these environments. Results from this study set forth new avenues of research with implications for the development of novel antimalarials and vaccine candidates.

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LIST OFABBREVIATIONS

ACP	Acyl-Carrier Protein
ACS	Acyl-CoA Synthetase
ACT	Artemisinin Combination Therapy
BCDH	Branched Chain Dehydrogenase
BSA	Bovine Serum Albumin
ELO	Elongase Enzyme
ER	Endoplasmic Reticulum
ERAD	Endoplasmic-reticulum-associated protein degradation
EXP-1	Exported Protein 1 (Parasitophorous Vacuole protein)
FAS-II	Fatty Acid Synthesis-II
GAP	Genetically Attenuated Parasite
Hep-G2	Hepatocellular Carcinoma
HPI	Hours post inoculation
HSPG	Heparan Sulfate Proteoglycan
IP	Intraperitoneal
IV	Intravenous
LA	Lipoic Acid
LipA	Lipoic Acid Synthase
LipB	Octanoyl-acyl-carrier protei N-octanoyltransferase
MSP-1	Merozoite surface protein 1 (Parasite Plasma Membrane protein)
NEFA	Non-esterified fatty acids
PDH	Pyruvate Dehydrogenase
PPM	Parasite Plasma Membrane
PVM	Parasitophorous Vacuole Membrane
RBC	Red blood cell
SREBP	Sterol Regulatory Element-Binding Protein
TG	Triglyceride
UPR	Unfolded Protein Response
UTR	Untranslated Region
WHO	World Health Organization
XBP1	X-box binding protein 1

LIST OF WORK PUBLISHED OR IN PREPARATION

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STATEMENT OF EXPERIMENTAL CONTRIBUTION

I have contributed to this work through the generation of the genetically mutant parasite lines *ΔpbLipB* and PbBCDH-GFP. I characterized the *ΔpbLipB* in sporozoite infections of mice. I also completed the growth assay data and did the imaging staining of blood-stage parasites. I completed the 8-BOA treatment studies for blood-stage parasites and did the quantification of liver stage sizes for the *ΔpbLipB* and wild-type controls. I completed the studies of the clofibrate mice, the determination of their lipid levels and the inoculations with blood stage parasites and sporozoites. I completed the study of the XBP1 KO mice, also to determine their lipid levels, and the progression of blood and liver stage infections.

Krista Mathews (Johns Hopkins University) completed the Western blot for detection of lipoylated proteins from parasite extracts.

Christina Deschermeier and Leonie-Sophie Hecht (Bernhard-Nocht-Institute for Tropical Medicine) completed the staining of *ΔpbLipB* parasites in the liver stage time course. They did the staining of the apicoplast lipoylated proteins, the morphology of the apicoplast, the liver stage treatments with 8-BOA and counted the numbers of detached cells.

Santha Kumar helped with the generation of the knock-out parasites and transfection experiments and Andrea Ecker helped with mosquito dissections.

SPECIFIC AIMS

I: Lipid metabolism is essential for the pathogenesis of malaria parasites, yet there is a limited understanding of the role of enzymes outside the canonical fatty acid synthesis pathway (Fatty Acid Synthase-II). Lipoic acid is an eight carbon chain that is a required cofactor of multi-subunit complexes and has both synthesis and salvage pathways in *Plasmodium*. In parallel, a novel approach to malaria vaccine development is the generation of genetically attenuated parasites that arrest within the liver-stage of development and confers protection to subsequent challenges. Our specific aims include determining the essentiality of the lipoic acid synthesis pathway within the life-cycle of rodent malaria parasites, *Plasmodium berghei*. Additionally, we sought to test the hypothesis that lipoic acid synthesis is required for the completion of liver-stage development and parasites disrupted for *PbLipB* could serve as genetically attenuated parasite (GAP) vaccine candidates.

II: The nutrient conditions of the host are likely to play an important role in the pathogenesis of *Plasmodium* parasites and the severity of malaria as a disease. Our aim is to test how lowering the levels of fatty acid and triglycerides in mice will affect the rates of blood-stage and liver-stage growth of *Plasmodium berghei* parasites. We are testing for any differences between two methods of lowering lipid species in mice. In our first method, we use clofibrate, a chemical agent that induces beta-oxidation of fatty acid species in the liver. In the second, we take advantage of the ΔXBP1 knock-out mouse that has a lower level of lipid synthesis in the liver. We seek to demonstrate that these model systems have reproducibly lower lipid species i*n vivo* and to monitor their impact on the replication of *Plasmodium berghei* parasite

1. INTRODUCTION

MALARIA: ORIGINS

Malaria has been noted in human history for more than 10,000 years. The first evidence of malaria parasites in mosquitoes is from preserved amber from the Palaeogene period, approximately 30 million years ago [1]. Human malaria likely originated in Africa and has coevolved with the mosquito vector and non-human primates, enabling its pathogenesis as a vector borne disease. The impact malaria on the human species can be traced back as early as the introduction of agriculture in human history. One consequence of its presence is the evolution of blood disorders that confer an advantage against infection, in particular sickle-cell disease and thalassemias [2].

The name "malaria" comes from the Medieval Italian for "bad air," because to the Ancient Romans this disease originated in the horrible fumes from the swamps. However the disease also affected populations beyond Europe. Medical writings dating from 2700 BC from ancient China describe characteristics of the disease. They noted the clinical symptoms as periodic fevers in addition to enlarged spleens and a pattern of epidemics. In a Sanskrit medical treatise dating back to the 6th century BC, the symptoms of malarial fever were described and attributed to the bites of certain insects. By the 4th century, malaria became widely recognized in Greece and was responsible for the decline of many of the city-state populations (http://www.cdc.gov/malaria/about/history/).

However the identification of the causative agent of malaria, *Plasmodium* parasites, wasn't made until 1880 by Charles Louis Alphonse Laveran, a French army surgeon stationed in Algeria. He

made the discovery from the blood of a patient suffering from the disease and his work was awarded the Nobel Prize for Medicine in 1907 [2]. Applying the principles of Louis Pasteur, that most infectious diseases are caused by microbial germs, Laveran studied the disease in order to identify its casual agent. Laveran called the organism he found, *Oscillaria malariae,* and hypothesized that it is the causal agent for the disease of malaria [3].

PHYLOGENY

Malaria is caused by *Plasmodia* protozoans, belonging to the phylum of Apicomplexa that consists of a large group of protists. The name refers to their apical complex that enables the parasite to invade host cells. The group of protists also contain a unique organelle called the apicoplast, a non-photosynthetic, plastid. The apicoplast organelle is essential for parasite survival and is thought to originate from a secondary endosymbiotic event that gave rise to its four-membrane structure [4]. In the mosquito-specific infectious stage, sporozoite, the parasite contains three distinct structures for the apical complex. The apical complex consists of microtubules, a secretory body (the rhoptry) and one or more polar rings. Slender electron dense secretory bodies known as micronemes are also present. Structures pertaining to motility, such as the flagella, are only present in gamete stages of the disease [5]. The literature annotates over 200 species of *Plasmodia* that infect a variety of hosts.

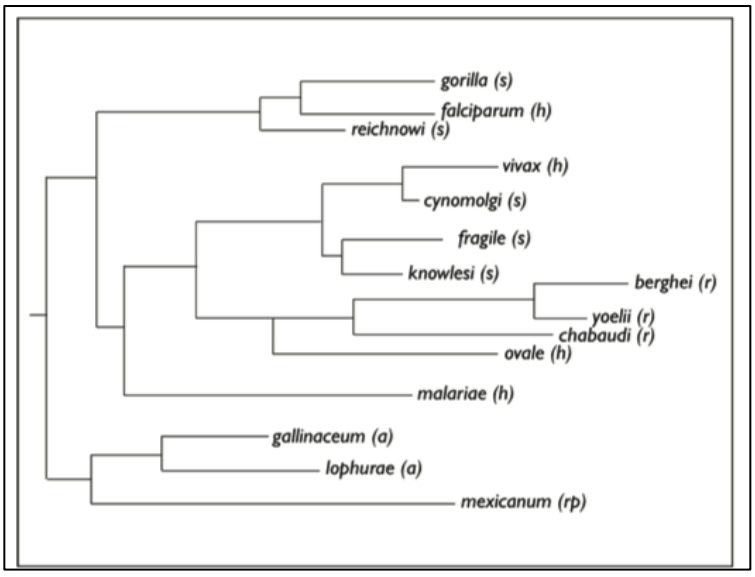


Figure 1.1. The phylogenic lineage of *Plasmodium* species
Schematic representation of the common lineage of *Plasmodium* parasite strains. (r) Refers to rodent infective malaria species; (h) human infective malaria species; (s) simian infective malaria species; (rp) reptile infective malaria species; (a) avian infective malaria species. The figure is adapted from [6].

There are four species of *Plasmodium* that are known to be specific to humans, *Plasmodium malariae*, *Plasmodium ovale, Plasmodium vivax* and *Plasmodium falciparum*. *Plasmodium vivax* and *Plasmodium ovale* are distinguished by the formation of hypozoites, a dormant stage of the parasite that can remain in the liver for months to a year after the initial infection [7]. Recently, *Plasmodium knowlesi*, a species known to infect macaques, has also been shown to infect human hosts [8]. The human-infective species are remotely related to each other, suggesting that adaptation to humans has occurred several independent times [9]. The last common ancestor of *P. falciparum* and the chimpanzee parasite *P. reichenowi* occurred around the time of the human-chimpanzee divergence [10]. However, recent studies place *P. falciparum* within the clade of gorilla-derived sequences, providing evidence that *P. falciparum* is of gorilla origin [11]. Phylogenetic analyses of full-length mitochondrial sequences demonstrated that the human pathogen *P. falciparum* forms a monophyletic lineage with *P. falciparum* species that infect

gorillas, indicating that both parasite strains are descendants from a single common ancestor. It remains to be determined when *P. falciparum* entered the human population and whether present-day ape populations represent a source for recurring human infection [12].

CLINICAL MANIFESTATIONS OF DISEASE

As mentioned previously, the recurring clinical symptoms of regular cycles of fevers and chills has been documented in many civilizations, during infection with different *Plasmodium* strains. The key clinical symptoms of malaria are regular cycles of fevers and chills, anemia and an enlarged spleen. Mortality occurs in children under 18 months due to severe malarial anemia. These clinical manifestations of the disease typically begin approximately two weeks after the bite of an infectious mosquito, when the parasite has completed liver stage development. Synchronized invasion, development and lysis of the red blood cell, releasing daughter parasites, trigger the immune response to generate the cycles of fevers and chills.

The clinical outcome of infection is dependent on the species of parasite, the host and geographic and social factors. These factors include the host age, previous exposures to the parasites and the general health of the patient. Access to health care and medications also greatly influences the clinical outcomes of an infection. An infection can develop into sever malaria or cerebral malaria depending on the patient. Severe malaria is considered to be much more complicated than originally thought [13]. The clinical pattern of severe malaria differs between non-immune adults and semi-immune children. Cerebral malaria is a form of severe malaria, typically involving sequestration of the infected erythrocytes in the brain tissue [14]. Cerebral malaria is the primary

cause of death in 1.5 - 5 year old children and *P. falciparum* remains the deadliest form of the parasite [15].

LIFE CYCLE

Malarial infection of the mammalian host begins with a bite from an infected Anopheles mosquito, injecting sporozoites into the dermis [16] (Figure 1.2). The number of sporozoites that are inoculated by a mosquito on average is relatively low. The range of sporozoite inoculated is between 0 and 1,297, with a mean number of 123 and a median of 18 [17]. Sporozoites can remain in the skin for hours before entering the blood stream or being cleared by the local draining lymph node [18]. Only a third of the transmitted sporozoites penetrate a blood vessel and potentially reach the liver [19]. The parasites that end up in the draining lymph node induce a strong cell-mediated immune response. This immune response may contribute substantially to immune protection against subsequent challenges [18]. Sporozoites that enter the blood stream will migrate to the liver where they can establish an infection in hepatocytes. At this point of development, the parasite loses its apical complex and surface coat and transforms into a trophozoite.

Inside the hepatocyte, a single sporozoite can develop into tens of thousands of daughter cells known as merozoites. Development in the liver takes approximately 5 - 7 days for *P. falciparum* infections. Additional discussion of the process of sporozoite invasion of hepatocytes and liver stage development is provided below in the section entitled “Liver Stage.” Merozoites are released into the blood stream upon completion of liver-stage. Parasites begin the asexual blood stages of development following the initial invasion of erythrocytes. All Apicomplexa adopt a

common mode of host-cell entry, utilizing a specific set of ligand-receptor interactions. The adhesins connect to a parasite actin-based motor, enabling the parasite to invade the host cell [20]. Inside the red blood cells (RBC), the parasites develop from the ring stage to the trophozoite and finally mature into a schizont. The eventual membrane rupture of the infected erythrocytes, achieved using orchestrated events of proteolysis, releases the daughter cells as well as erythrocyte debris and hemozoin pigment in the circulatory system of the human host. Erythrocyte lysis can lead to anemia and in cases of higher parasitemia (>4% red cells infected) leads to severe malarial anemia. The blood stage of development takes 48 hours for *Plasmodium falciparum*, *P. vivax* and *P. ovale.* The erythrocytic cycle last 72 hours for *P. malariae*.

The life cycle of *Plasmodium* parasites inside the mosquito host begins with the ingestion of gametocytes from the mammalian host. Gametocytes, the sexual form of the blood-stage infection, develop as a subset of the asexually replicating population in the blood stream. These forms are ingested during a mosquito bite and therein begin a process of sexual reproduction within the mosquito host [21].

After ingestion by the mosquito, the male and female gametocytes are released from their red blood cells in response to environmental changes including temperature and pH [22]. Gametocytes quickly give rise to sexual gametes, which fertilize in the midget lumen to form zygotes. The zygote form develops into motile ookinetes, which invade and traverse the midget epithelium between 12 to 24 hours after blood ingestion. Ookinetes are diploid and undergo meiosis upon reaching the basal side of the midgut. Losing their elongated shape they transform into oocysts,

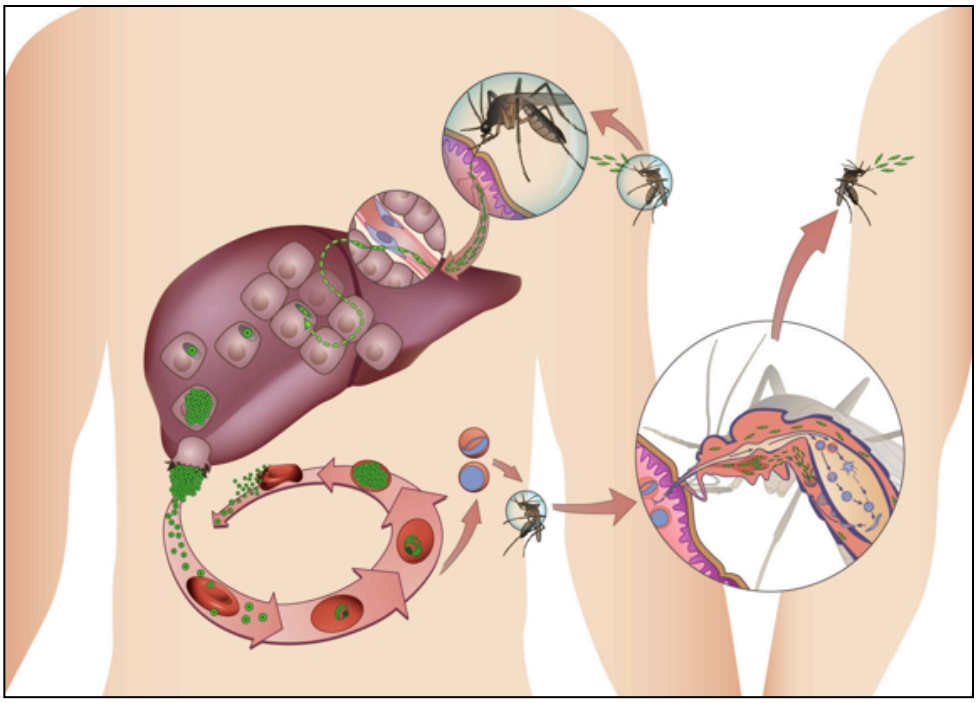


Figure 1.2. The *Plasmodium* life cycle
Sporozoites are inoculated into a mammalian host through the bite of an infected mosquito. Sporozoites migrate into the liver of the host and begin to rapidly multiply and replicate, generating thousands of daughter cells. These merozoites escape the infected hepatocyte when the parasitophorous vacuole membrane begins to break down and are released into the circulation. Blood stage asexual replication begins when a merozoite invades a red blood cell. The parasite develops into the ring, trophozoite and schizont forms in the infected erythrocyte. A subset of the blood-stage parasites switch into the sexual gametocytes forms which when ingested by a mosquito develop inside that host. The gametes generate oocysts in the midget and sporozoites in the salivary glands. Image taken from [23].

which undergo several rounds of mitosis as they mature. Each oocyst releases thousands of haploid sporozoites into the mosquito hemocoel. These sporozoites travel through the hemolymph and invade the salivary glands [24]. The mosquito immune responses are important factors in determining sporozoite development [25]. Sporozoites are transmitted to a new vertebrate host during an infective bite. One recent study predicted that if the approximately 10,000 gametocytes were ingested in a blood meal, then on average only 1,000 ookinetes would successfully develop, and fewer than 5 would survive to develop into oocysts [25]. Therefore this

stage of the life-cycle offers a bottle-neck during parasite replication when its numbers are relatively small.

PARASITE SEQUESTRATION

A key feature of the pathogenesis of *Plasmodium falciparum* is its ability to sequester the infected red blood cells in the microvasculature of the infected host, thereby preventing clearance into the spleen. The parasite adheres to tissues through the generation of electron-dense protuberances or 'knobs' that protrude from the infected red blood-cells and enable the infected cell to adhere to essential organs [26]. Several complications occur due to the sequestration of infected erythrocytes in the microvasculature, such as cerebral malaria, acute renal failure and placental malaria [17]. The obstruction caused by sequestered parasites causes the release of cytokines, nitric oxide and other host mediators [20].

IMMUNE EVASION

In addition to the pathogenic feature of sequestration in the human host, malaria parasites also have the ability to evade the immune system by their ability to undergo antigenic variation. Antigenic variation enables parasites to display a diversity of antigenically distinct Pf Erythrocyte Membrane Protein 1 (PfEMP1) proteins (encoded by the *var* gene family) on the surface of the infected red blood cell. The parasite will only express one *var* gene at any time. This process is known as antigenic or clonal variation. Switching its surface protein leads to evasion the immune response and subsequent expansion of the clone, causing a new wave of parasitemia [24]. The large family of *var* genes is primarily located in subtelomeric regions of the *P.f.* chromosomes. Strains are thought to typically encode 60 *var* genes [27]. PfEMP1 proteins are

trafficked from the parasite to Maurer's clefts that enable expression on the surface of the erythrocyte membrane. Expression of the other variant gene families, including rifin, steavor and Pfmc-2Tm which all have stage specific expression. Rif genes are expressed during the trophozoite stage and steavor and Pfmc-2tm are expressed during the mature trophozoite stages [27]. Adhesion between infected and uninfected erythrocytes can form "rosettes" in the microvasculature [28]. Expression of var, rifin and steavors also occurs in gametocytes [21]. Gametocytes can also express variant genes other than those expressed by their asexual progenitors. Human antibodies that block binding of infected erythrocytes to host cells are important determinants of protective immunity to severe malaria.

Parasitized red cell surface proteins bind to a set of host receptors. Host receptors CD36 and ICAM-1 are the major receptors in the adhesion of most *P. falciparum*-infected cells [29]. These glycoproteins are expressed on vascular endothelial cells and are bound by certain forms of PfEMP1 [30]. PfEMP1 adhesion to chondroitin sulfate A (CSA), a glycosaminoglycan on the placenta, is the likely interaction enabling the accumulation of infected erythrocytes in the placenta [31]. Placental malaria is a severe condition that can cause substantial morbidity in women and will increase the risk of low birth weight or still-birth of the developing fetus.

EPIDEMIOLOGY

There are currently close to 3 billion people at risk of malaria infection, an entirely preventable and treatable disease. In 2010, there were an estimated 655,000 deaths due to malaria and 216 million cases, the majority of are children under the age of 5 [32]. Most malaria cases and deaths occur in sub-Saharan Africa, yet many regions of Asia, Latin America, and to a lesser extent the

Middle East are also affected. In 2010, malaria was present in 106 countries and territories around the world. Of the five species of *Plasmodium* parasites that can cause malaria in humans, *P. falciparum* is the most predominant in Africa [33]. 86% of deaths globally were children.

The geographic and social factors that impact the clinical outcome of the disease include access to treatment, political stability of the country, transmission intensity of the *Anopheles* mosquito, including the season of the transmission and the number of infectious bites per year [12]. In particular, the mosquito determinants of disease progression in the host include the mosquito lifespan, behavior, genetic susceptibility and degree of Victoria competence [34]. Environmental factors, in particular the recent effect of climate change, have dramatically altered the landscape of malarial endemicity [35]. Temperature, rainfall and humidity affect the replication, maturation and viability of *Plasmodium* in mosquitoes. Social factors that influence the health outcomes include attitudes and behaviors that affect whether they seek treatment or follow through with the treatment regime [36].

The species of parasite also has an enormous impact on the clinical outcome of the disease. *P. falciparum* and *P. vivax* can cause severe anemia, but only *P. falciparum* can cause complications including cerebral malaria, hypoglycemia, metabolic acidosis and respiratory distress [23]. *P. falciparum* can invade a large percentage of red blood cells, more likely to create anemia and is generally more lethal. *P. vivax* replication is limited to reticulocytes, immature red blood cells, that leads to lower levels of parasitemia. *P. vivax* is limited to cells that express Duffy antigens because of the way it invades red blood cells. In regions of the world where the

population is largely Duffy blood group negative, for instance, West Africa, *P. vivax* has essentially disappeared [37].

There is also a close relationship between malnutrition and malaria. Because many malarial-endemic areas suffer food shortages, the resulting malnutrition increases the risk of death from infectious diseases due to deficiencies in the immune system. Malnutrition is arguably the most common cause of immunodeficiency world-wide [38]. Malnutrition leads to dysfunctions in the immune system, leaving the population very vulnerable to infections. Children with severe malnutrition (<70% normal weight/ height) have a markedly increased risk of mortality, often due to infection. Malnourished children with high parasitemias may show no common symptoms of infection, can develop severe malaria very quickly and may die without access treatment.

In the case of Africa, the fact of a country being in a malarial endemic area lowers its gross domestic product (GDP) by an average of 1.3% annually [32]. The economic costs of malaria endemicity to the growth of developing country are enormous. In addition to medical costs of treatment, loss of productivity including time away from school for children's education and work for adults significantly contribute to the reduction in GDP [39].

CONTROL, ELIMINATION, ERADICATION

In 2007, the Bill and Melinda Gates Foundation called for a renewed effort at achieving global malaria eradication [40]. Control of the disease implies that the number of malaria cases is manageable within the context of the local and regional population. Elimination of malaria is

particular to the country and population, however global eradication of malaria would mean permanent elimination of the parasite.

The Roll Back Malaria partnership launched its goals for achieving malaria eradication as the Global Malaria Action Plan in 2010 (http://www.rollbackmalaria.org/gmap/index.html). Its main objective was the reduction of the number of preventable malaria deaths to nearly zero by 2015 (http://www.who.int/malaria/world_malaria_report_2011/en/). These goals require the combination of several different strategies to reduce malaria infection and transmission.

The major approaches for malaria control include the prevention of new infection and successful case management. This includes methods to block the transmission of parasites and prevent new infections. Intervention of malaria transmission is a combined effort, hitting several of the points of the *Plasmodium* life cycle. Vector control would both protect individual people against infective malaria mosquito bites and to reduce the intensity of local malaria transmission at the community level [32].

The most powerful interventions are long-lasting insecticide-treated nets and indoor residual spraying using DDT [32]. Indoor residual spraying involves the application of residual insecticides into the inner surfaces of dwellings, to effectively control malaria transmission, reducing the local burden of morbidity and mortality. Protection from people getting bitten by infected mosquitoes operates by preventing contact through the use of the insecticide-treated bed net. The spread of insecticide resistance is a major threat for vector control programs.

Proper diagnosis and effective treatment are also important long-term strategies to reduce the burden of disease in communities. The use of diagnostics reduces the frequency and duration of malaria infection and can block mosquito transmission by reducing the human parasite reservoir [41]. Reliable diagnostic measures lead to improved care of parasite-positive patients and prevent the overuse of anti-malarials on parasite-negative patients.

Preventive treatments aim to block malaria transmission by chemoprevention for vulnerable populations. These include the intermittent treatment for malaria in pregnancy through the administration of sulphadoxine/ pyrimethamine (SP) during the second and third trimesters. Women who are HIV-positive may require more frequent treatment [42]. This treatment is also recommended for intermittent preventive treatment in infants; in countries in sub-Saharan Africa with moderate to high malaria transmission it is recommended that infants receive 3 doses of SP at the same time as their measles immunizations [32].

CHEMOTHERAPIES FOR MALARIA INFECTION

Chemotherapies are a critical component in reducing malaria morbidity and mortality. Drugs to kill the parasite target the various stages of its parasite life cycle. Drug treatments to malaria have been discovered throughout the course of human development. Quinine was discovered nearly 400 years ago. It is found within the class of quinolones that has been a major focus for the treatment of malaria. Chloroquine for decades was the first line of treatment for malaria worldwide. This weak base concentrates in the highly acidic digestive vacuoles of sensitive *Plasmodium* parasites, where it binds to heme and disrupts its sequestration [43]. However, drug-resistant forms of *P. falciparum* are now common to most parts of the world [44].

Antimalarial drugs can be classified based on their activities during the life cycle. They can also be classified by the way they are absorbed into the body, which can impact their effectiveness in treating the disease. There are three main classes of drugs. The first can act against the primary or latent liver stages. A second class is defined as drugs that are directed against the blood stages. A third class is effective against the liver stage and gametocytes.

Quinolines are a class of drugs that are active against the blood stages of malaria. The 4-aminoquinoline chloroquine was discovered in Germany in the 1930s and introduced in the United States after World War II. Chloroquine is inexpensive, safe and highly effective against the blood stages of malaria species. This drug was the foundation for the first campaign of malaria eradication in the 1950's. The spread of chloroquine-resistant strains world-wide has diminished its use [45]. Chloroquine is highly effective against erythrocytic forms of *P. vivax, P. ovale*, *P. malariae*, *P. knowlesi* and sensitive forms of *P. falciparum*. The spread of chloroquine drug-resistant *P. falciparum* species has led to this drug being replaced by artemisinin-based combination therapies [46].

Chloroquine is still widely used to treat *P. vivax* although the drug has no activity against latent hyponozoite forms of *P. vivax* or *P. ovale* [47]. Mefloquine is also a highly effective quinoline for the treatment for blood stage infections. It is an arylaminoalcohol analogue of quinine that was synthesized in the 1960s. Early work showed that mefloquine associates with intraerythrocytic hemozoin, suggesting similarities to the mode of action of chloroquine [48]. The drug is rapidly absorbed, however with marked variability between individuals [49]. The drug displays highly

potent schizonticidal activity against blood stage parasites. Mefloquine is no longer the first-line treatment of malaria in most clinical contexts [49]. Primaquine acts on exoerythrocytic tissues in the liver to prevent and cure relapsing *P. vivax*. This drug belongs to the family of the 8-aminoquinolines. Although primaquine does not exhibit any inhibitory activity against the blood stages, it is thought to be a powerful gametocytocidal compound. Tafenoquine has a longer plasma half-life and is thought to be less toxic than primaquine [50]. Quinine is the main alkaloid of cinochona, the bark of the South American cinchona tree [49]. Quinine acts against blood stage forms and has no significant effect on hepatic forms. This drug is more toxic and less effective than chloroquine.

Artemisinin is a sesquiterpene (15-carbon) lactone endoperoxide derived from wormwood. Chinese have ascribed medicinal value to this plant for >2000 years. Artemisinins are very potent, fast-acting antimalarials, inducing a very rapid parasite clearance and fever resolution. Artemisinins caused a significant reduction in parasite burden. Three to four cycles (6-8 days) of treatment can remove essentially all the parasites from the blood [51]. Artemisinins also possess gametocytocidal activity, leading to a decrease in malarial parasite transmission [52]. These drugs are valuable for the treatment of severe *P. falciparum* malaria. However, they cannot be used alone because of their very short half-life and limited ability to eradicate infection completely [53]. Artemisinins should not be used for chemoprophylaxis because of their short half-life. Several derivatives of artemisinin have been synthesized and characterized. Dihydroartemisinin (DHA) is a reduced product and active metabolite of artemisinin, artemether is the lipophilic methyl ether of dihydroartemisinin, and artesunate is the water-soluble hemisuccinate ether of DHA [51]. Artemisinins are active against nearly all the parasite blood stages, resulting in rapid parasite

clearance. They are also active against immature *P. falciparum* gametocytes [54]. Their mode of action remains uncertain, although it has been reported that the integrity of the endoperoxide bridge is necessary for antimalarial activity [55].

The short plasma half-life of artemisinin (1-3 hours) and its derivatives translates into treatment failure rates when artemisinins are used as monotherapy [56]. They are now combined with partner drugs with a much longer half-life to ensure a continued antimalarial activity. The use of combination therapy also is a method of protecting artemisinins against the emergence of parasite resistance. Amodiaquine is a 4-aminoquinoline that is used as a partner drug in the ACTs. *In vivo*, amodiaquine is rapidly converted into its metabolite, monodesethyl amodiaquine, which is characterized by a long plasma half-life of 9- 18 days [57]. Piperaquine is a bisquinoline that is structurally related to chloroquine and other 4-aminoquinolines. It became the primary antimalarial in China during the 1970s and 1980s. Piperaquine has a large volume of distribution and reduced rates of excretion after multiple doses. The drug is rapidly absorbed, reaching its maximal concentration 2 hours after a single dose [58]. Piperaquine has the longest plasma half-life (approx. 35 days) of all ACT partner drugs, suggesting that piperaquine-dihydroartemisinin might be effective in reducing rates of reinfection following treatment [59]. Pyronaridine is structurally related to amodiaqine, developed in the 1970s in China. The compound is highly effective against both *P. falciparum* and *P. vivax* blood stages [49].

Lumefantrine is structurally related to quinine and mefloquine. It is thought to have the same mechanism of action, in which it is targeted to the parasite digestive vacuole in the blood stages [60]. Lumefantrine is paired with artemether in the Coartem® formulation [61].

Other anti-malarial agents include atovaquone and sulfadoxine-pyrimethamine and methylene blue. Atovaquone was developed as a synthetic derivative with activity against multiple *Plasmodium* species. It is highly active against *P. falciparum* blood stages, and against the liver stages of *P. falciparum* but not *P. vivax* hypnozoites. This drug targets the parasite mitochondrial cytochrome bcl complex and inhibits electron transport, collapsing the mitochondrial membrane potential [62]. Atovaquone is now paired with the antifolate proguanil, to prevent the development of parasite resistance [63]. The latter compound targets the parasite bifunctional enzyme dihydrofolate reductase-thymidylate synthase essential to parasite *de novo* synthesis of purines [64].

Sulfadoxine-pyrimethamine is a combination therapy of two antifolates: a sulfonamide (a *p*-aminobenzoic acid analogue) that targets the *Plasmodium* dihydropteroate synthase involved in the synthesis of dihydrofolic acid, and diaminopyrimidine that inhibits the parasite dihydrofolate reductase. These slow-acting blood schizonticides are more active against *P. falciparum* than *P. vivax.* This drug combination is facing increased development of resistance due to mutations in both target enzymes [65].

Methylene blue is a thiazine dye and was first introduced in 1891. The compound was earlier reported to inhibit the enzymatic activity of *P. falciparum* glutathione reductase *in vitro* [66]. However, a recent study demonstrated that glutathione reductase-null blood stage parasites exhibited the same susceptibility to methylene blue as wild-type parasites, implying this agent has a different target [67]. Clinical studies are ongoing to evaluate methylene blue as a new partner drug for the treatment of malaria.

The currently recommended treatment for malaria is Artemisinin-Based Combination Therapy (ACT). However, the 2008 World Malaria Report showed that only 3% of children suspected of having malaria were treated with an ACT. Artemisinins are effective against early-stage gametocytes and the symptomatic asexual blood stages [68]. Recent evidence from the field indicates a reduced clearance time of Arteminisin in Western Cambodia [69]. The emergence of artemisinin-resistant malaria on the western border of Thailand has brought the forefront a need for development of a malaria vaccine to prevent infections in the face of new drug resistant strains [70].

The process of absorption depends on how it will pass through a barrier to enter a cell. If a process of lipid diffusion absorbs drugs, then the drug dissolves in the lipid components of the cell membrane. Certain antimalarials are classified as lipophilic drugs, such as atovaquone, and lumefantrine. It has been recently demonstrated that a doubled concentration of plasma triglycerides modifies the effectiveness of the drug. Studies with *P. falciparum* parasites *in vitro* required higher concentrations of both atovaquone and lumefantrine to kill the same percentage of parasites [71]. Other drugs, such as chloroquine, which is hydrophilic, did not respond to changes in the triglyceride concentrations. This is an important finding, revealing a close relationship between the anti-malarial activity of lipophilic drugs and the nutritional status of the host. This area requires further investigation to optimize the use of these therapies in clinical settings. Also, proper administration of drug concentrations will prevent their overuse and protect drug resistance from emerging with the effective compounds that we currently have.

VACCINE DEVELOPMENT

As mentioned previously in the introduction, malaria vaccines have an important role to play in the long-term goals of malaria elimination and eradication [72]. The different stages of the life-cycle of *Plasmodium* parasites each provide a new opportunity in which to develop a vaccine. In addition, natural immunity to malaria infection occurs after many years of repeated exposure [73]. However, maintenance of immunity requires a persistent subclinical infection because of immune evasion mechanisms such as antigenic variation [74]. Natural immunity is only partial, strain specific and short-lived unless reinforced through frequent exposures. An ideal vaccine would protect the semi-immune as well as people with little or no immunity.

Immunity to *P. falciparum* involves cellular and antibody mediated responses [75]. The human host develops a repertoire of antibodies against the antigenically variable proteins expressed on the surface of infected erythrocytes. Cellular responses to malaria antigens include CD4+ T cells that proliferate and secrete interferon (IFN)- γ when stimulated [76]. IFN-γ responses associate with protection against malaria amongst volunteers undergoing experimentally induced infections and naturally exposed human populations [77]. Adaptive and innate lymphocytes contribute to the generation of IFN-γ [75]. It is uncertain whether the clinically silent sporozoite and liver stages play a role in the development of natural immunity [78]. However, targeting this stage of the life-cycle, to generate protective immunity to preventing the onset of a blood-stage infection, remains attractive. A broad range of vaccine strategies have been developed to try to utilize the principles of naturally occurring immunity through exposure to different types of *Plasmodium* antigens. The most currently tested malarial vaccine is the RTS,S/AS01 vaccine. The RTS,S/AS01 vaccine is based on the fusion of the *P. falciparum* circumsporozoite (CSP) protein with the hepatitis B

virus surface antigen. RTS,S induces the production of antibodies and T cells that diminish the malaria parasite's ability to infect, develop and survive in the human liver [75].

In 2011, results from a large-scale phase 3 clinical trial of RTS,S showed 55% reduction in the frequency of malaria episodes after 12 months of follow-up [79]. However, earlier reports for this vaccine were far less encouraging [80]. Furthermore, RTS,S reduces the development of naturally acquired antibody responses to blood-stage antigens, leading many researchers in the field to continue searching for a vaccine with better efficacy [81].

The majority of asexual blood-stage candidate vaccines that have been developed are based on a small number of antigens expressed on merozoites [82]. Many of these vaccine candidates are based on just a few antigens, Merozoite Surface Protein-1 (MSP-1) and Apical Membrane Antigen-1 (AMA-1) in particular. Candidate vaccines have contained a protein given with an adjuvant designed to induce protective antibodies. Some approaches have focused on inducing antibodies that impair parasite invasion, while others have aimed to induce antibodies that impact parasite development in collaboration with effector cells [82]. Although protection has been achieved in experimental animals, little clinical success has so far been achieved in humans with blood-stage vaccines [83].

There are also major challenges to the development of blood-stage vaccines. A major hurdle to the development of a blood-stage vaccine is the antigenic variation of the surface proteins of *Plasmodium* parasites. The constant switching of epitopes, encoded by the *var* gene family, during blood-stage growth means every 48 hours the host needs to produce a new antibody to

combat the infection. Another challenge is the difficulty in expressing conformationally correct large antigens and scaling up the methods needed to do this to the extent that would allow large-scale manufacture. Another challenge is the modest amount of antibody responses produced to the antigens currently tested [82]. Another challenge is the allelic polymorphism observed in proteins, for example in the AMA-1 protein [84]. AMA-1 is a polymorphic protein that contains different amino acid substitutions observed in *P. falciparum* isolates from various regions of the world.

Vaccines that target the pre-erythrocytic stages of infection, encompassing the sporozoite stage and liver stages, constitute an attractive strategy. These pre-erythrocytic stages contain low numbers of parasites in comparison to blood stages, are completely asymptomatic, yet provide an opportunity for an effective immune response to eliminate the parasites (5-7 days for *P. falciparum*) [85].

Vaccines that target the mosquito stages focus on antigens that are expressed during replication in the mosquito but not in the human host [86]. Strategies to prevent transmission will greatly reduce the chance that the parasite will invade erythrocytes and be able to produce gametocytes for transmission of the infection. Immunization of mosquitoes with gametocyte or ookinete antigens could reduce or ablate oocyst development in the mosquito. This principle has been well established for decades [86]. Efforts have been made to standardize membrane-feeding assays that allow sera from vaccinated animals or humans to be evaluated for their ability to reduce or prevent transmission. This approach has also been supplemented by the *in vivo* use of transgenic parasites to assess the efficacy of antibodies induced by *P. falciparum* and *P. vivax* antigens [87].

Another recent development has been the findings that antigens from the *Anopheles* midgut wall, in particular aminopeptidase APN1, appear to be a receptor for ookinetes and may act as suitable transmission-blocking vaccine components [87]. The mosquito-stage vaccine also has the particular advantage that it may be effective against more than one species of *Plasmodium*.

Another approach for vaccine development is the development of whole-cell attenuated forms of the parasite. Radiation-attentuated sporozoites have been used to inoculate mice, revealing that the ability of sporozoites to invade hepatocytes and arrest therein was correlated with the induction of protective immunity to subsequent challenge (Figure 1.3) [88]. Irradiated sporozoites delivered by mosquito bite in human trials can induce very high levels of protective efficacy [89].

More recent trials failed to achieve high levels of protection although they illustrated the feasibility of producing enough irradiated sporozoites for eventual use in humans as an optimized vaccine. However this recent study used a different method of inoculation of irradiated sporozoites; through needle inoculation of purified sporozoites [90]. This method of inoculation of sporozoites into human patients resulted in suboptimal immunogenic protection. It is currently unknown how much immunity to *Plasmodium* infection occurs when mosquitoes bite the dermis and also inoculate mosquito antigens into the host. Irradiation of sporozoites is thought to introduce lethal double stranded breaks in the parasite DNA. When dosed adequately, sporozoites can invade liver cells, begin development to produce defective trophozoites that cannot mature and rupture to release merozoites that would normally invade red blood cells and continue the infection [91].

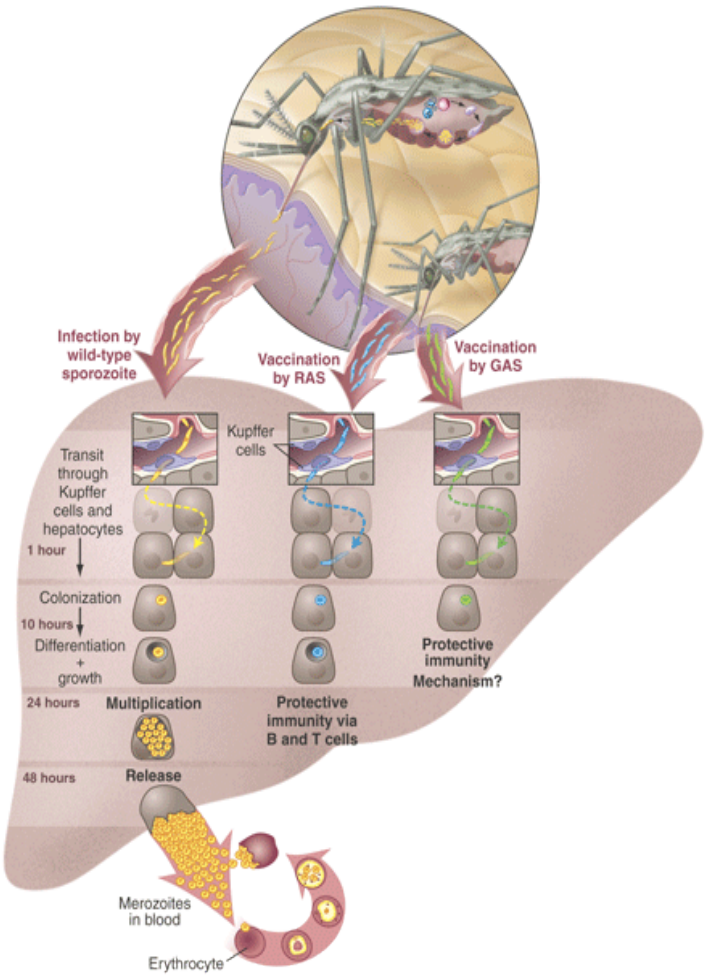


Figure 1.3. Arrest during liver stages confers protection to subsequent challenges
Sporozoites that are mutated by radiation (RAS) or genetic attenuation (GAS) arrest within the liver. Sporozoites that do not complete liver-stage development can provide protection to subsequent challenge. Schematic reproduced from [92].

Immune protection during the liver stages is likely to be achieved through the action of CD8+ T cells that target the parasitized hepatocytes [93]. Infected hepatocytes display parasite proteins on their surface through the Major Histone Compatibility Complex-II (MHC-II) protein, an antigen-presenting molecule important for the immune response. The presentation of foreign peptides generates a response in T-cells [94]. Antibodies to the sporozoites are thought to have a lesser function in conferring protection [95].

Attenuation of sporozoites by chemical treatment can also induce sterilizing immunity. Studies with a DNA-binding drug, centanamycin, showed attenuation of sporozoites and when inoculated into naïve mice, provided protection to subsequent challenge [96]. The protection was demonstrated across species: inoculation with *P. berghei* parasites and centanamycin treatment led to protection against challenge with *P. yoelii* parasites. High levels of CD8+ T cells and antibodies were generated in response to immunization with chemically-attenuated sporozoites (CAS), suggesting protection occurs through the same mechanisms as with radiation attenuated sporozoites.

Attenuation can also be obtained by the deletion of genes essential for liver stages [85]. These Genetically Attenuated Parasites (GAPs) therefore would not require irradiation to attenuate them and are potentially more efficacious if they are capable of progressing to a later liver stage of development [85]. Genes, including, highly Up-regulated in Infectious Sporozoites (UISs) have been demonstrated to be essential for the completion of liver stages in both *P. berghei* and *P. yoelii*. UIS3 and UIS4 are proteins of the PVM and act at the host-parasite interface during liver-stage development [97 98]. Deletion of UIS3 led to a complete arrest in liver stage development, however deletion of UIS4 had occasional break-through when high numbers are used for immunization [98]. Other genes that are critical for liver stage development have also demonstrated protection to challenge with wild-type parasites; including P52, SAP1 and SLARP. When P52 was deleted in *P. berghei* strains of rodent malaria, the knock-out parasites arrested in the trophozoite stage in the liver. P52 encodes a putative GPI-anchored protein and is a member of the 6-Cys-protein super-family [99]. This deletion strain was protective against subsequent challenge and no break-through occurred [100]. Deletion of SAP1 in *P. yoelii* also conferred

protection to subsequent challenge with no breakthrough [101]. SAP1 is critical for post-transcriptional regulation of infectivity during sporozoite stages. Deletion of SAP1 reduced expression of a number of sporozoite specific genes. A similar protein was disrupted in *P. berghei,* Sporozoite And Liver stage asparagine-Rich Protein, (SLARP) [16]. Deletion of SLARP resulted in complete arrest during early liver stages [16].

Double knock-outs can be highly effective experimental vaccines, with *P. falciparum* genetically attenuated parasites provide good protection in a humanized mouse model [102]. Generation of a blood stage infection was abolished in the generation of double-knockouts in the rodent malaria parasites. Simultaneous deletion of p52/p36 genes resulted in complete attenuation with no breakthrough infections [99]. P36 encodes a putative secreted protein. Also a double knock-out of UIS3 and UIS4 conferred protection with no break-through [103]. However, ensuring a complete arrest during liver stage development in *P. falciparum* might be difficult, even if more than one mutation is introduced.

GAPs involved in the synthesis of fatty acids have also demonstrated arrest in liver stages. Components of the FAS-II pathway were expressed the highest in liver stages when compared to other life cycle stages [104]. The block within liver stage development for the FAS-II deleted genes was confined to the later stages of development, with no defect during the initiation of liver stage [105] [106]. Since FAS-II knockouts develop significantly and form large liver-stage schizonts, they induce a more robust immune response [107].

Many of these findings were made through the study of mouse forms of malaria, *P. berghei, yoelii* and *chabaudi*. Mouse models have served as a great source of information about the protective mechanisms the mammalian host has for controlling the infection [95]. Additionally, the initial stage of vaccine evaluation takes place in the animal model. These studies can determine whether humoral or cellular immunogenicity is demonstrated with the vaccine candidate.

Various murine model systems have been developed with parasites originally isolated from African wild rodents [108]. These forms of malaria offer an advantage over simian models that they are more practical to study and the mouse immune system is well characterized. The four rodent species regularly used for scientific study are *Plasmodium berghei, P. chabaudi, P. vinckei* and *P. yoelii.* Although no one of the four species accurately reflects the infection in humans, together each rodent species offers a resource for studying the mechanisms of protective immunity. Even the host factors in eliminating parasites during blood-stage development have been discovered in mouse models [109]. *P. chabaudi chabaudi* is the best murine model to study immune responses to blood stage malaria since it is similar to *P. falciparum* in several aspects: the blood stages infect normocytes and show synchrony *in vivo.* These parasites also undergo peripheral withdrawal of older parasitized RBC and in some mouse strains, recovery from the acute primary parasitemia is followed by one or more patent recrudescences by parasites of variant antigenicity [110].

For liver stage examination, the use of human-infective *Plasmodium* species, *P. falciparum* and *P. vivax* sporozoites poses many safety concerns. Therefore more liver stage studies have been made in rodent and non-human primates. *P. berghei* and *P. yoelii* have been commonly used

rodent models for liver stages. The examination of liver stage development of sporozoites has been achieved during *in vivo* infections of rodents as well as *in vitro* studies in several cell lines. The advantage of studying *Plasmodium* in cell lines is that they are easy to maintain, have a well-defined genome and are amenable to genetic manipulation. The most commonly used cell lines are HepG2, Huh7 or Hepa1-6 cell lines for rodent *Plasmodium* parasites. *P. vivax* can infect HepG2 and HC04 cell lines [111]whereas *P. falciparum* can only grow within the HC04 cell line [112]. A caveat to the use of cell lines is that they are immortalized and may have lost some of the features of the cells they were derived from. The possibility of using primary hepatocyte cultures for infections is an alternative approach. Primary hepatocytes retain more, although not all the characteristics of liver cells, and are more likely to replicate the natural infection. However they are difficult and time-consuming to produce for experimental use [113].

Despite the knowledge gained through the study of rodent parasites, they are not natural mouse pathogens and much of the knowledge has been gained from infections initiated by unnatural routes. For instance, studies that determined the importance of CD8+ T cells in immunity to pre-erythrocytic stages occurred with sporozoites administered through intravenous injection (I.V.), which bypasses the natural intradermal route of inoculation. Sporozoites administered through I.V. injection are less available for elimination by antibodies than sporozoites inoculated into the dermis and the sporozoites take minutes instead of hours to enter the liver [114].As mentioned earlier, the immune response is likely affected by the contents of the mosquito bite itself [115], making these types of experimental procedures less valuable to study the mechanisms of immunity. Although these caveats result in a reduced level of host immunity, the fact that we still

see protection observed with irradiated sporozoites offers great potential for vaccine development.

An important tool to evaluate sporozoite vaccines in rodent models include humanized mouse models. Humanized mouse models that can support parasitized human erythrocytes or human liver stages provide a model that falls in between rodent models and human studies. The humanized mouse model for *P. falciparum* infection uses NOD/SCID mice inoculated intraperitoneally with human red blood cells [116]. This model can be used to study the blood-stage replication of *P. falciparum* infections *in vivo.* Liver stage development of *P. falciparum* has also been demonstrated in immunodeficient mice that allow survival of differentiated human hepatocytes. One example is the knock-out mouse for the albumin-urokinase-type plasminogen activator (Alb-uPA) that confers a growth advantage to transplanted hepatocytes [117]. By controlling nonadaptive immune defenses, macrophages and NK cells, there was increased survival of transplanted human hepatocytes to 3 months within the rodent host. Sporozoites injected intravenously into transplanted mice and showed *P. falciparum* liver forms by light microscopy.

Finally, it is important consider how attenuated sporozoites might be able foster cross-species protection against other strains of malaria. This is an important factor when considering which strain of *Plasmodium* to use for inducing immunity. Cross-species protection has been demonstrated for chemically attenuated rodent malaria strains [96]. The genetically attenuated P36-p strain in *Plasmodium berghei* conferred partial cross-species protection to challenge with *P. yoelii* sporozoites [118]. The genetically attenuated *P. berghei* strain demonstrated cross-species

protection to the *P. yoelii* challenge, although not as robustly as radiation attenuated *P. berghei* parasites. The cross-species protection had an impact on the level of liver infection, length of prepatent period and the peak parasitemia of challenge infections. This report demonstrates how cross-species protection is possible, although it was not complete. Although cross-species protection was not demonstrated with human malaria strains (immunization with radiation attenuated *P. falciparum* sporozoites with *P. vivax* challenge), this study only had a single volunteer [89].Further investigation into cross-species protection with human *Plasmodium* parasites is important for the practical value of translating these findings into an attenuated liver-stage sporozoite vaccine.

LIVER-STAGE DEVELOPMENT

The liver stage is an interesting stage to target for vaccines development because it can elicit an immune response while remaining clinically silent. Liver-stage enables the parasite to transition from sporozoite infection to active blood-borne disease. The liver stage produces new parasite forms that can enter and live inside red blood cells. The knowledge of the liver stage is relatively limited because the complete liver stage development of human *Plasmodium* species can generally only be studied *in vitro* in primary human hepatocytes [119] and rarely *in vivo* in immunocompromised non-human primates [120, 121]. More recently, humanized mouse models have been developed that enable *P. falciparum* infections in the liver of rodents. Most of the knowledge about liver stages has been determined based on studies with rodent parasites. In the late 1940s, it was shown that sporozoites of mammal-infecting *Plasmodium* species initially invade hepatocytes, where they replicate asexually to form thousands of merozoites [122]. Surprisingly, at least in the case of *P. berghei, Plasmodium* sporozoites can infect a wider range

of cells than originally thought, in particular they can develop in the skin tissues [123]. However, it is generally thought that their main cell are hepatocytes types in which they complete development. To access them, the motile parasites need to cross the endothelium a second time after entering the bloodstream in the skin. In the liver sinusoids, the sporozoites bind to highly sulfated heparan sulfate proteoglycans (HSPGs) [124] present on the surface of the hepatocyte. Many cell types present hSPGs, but the sulfation level differs and is particularly high in liver tissue. Once inside the host cell, the parasite often localizes close to the host cell nucleus [125]. It is likely that the vacuole attaches to the cytoskeleton of the host cell and is passively transported near the nucleus. There it is often in close proximity to the ER and the Golgi apparatus [126]. After invasion of the host hepatocyte and formation of a PVM, the parasite undergoes an initial period of morphological changes. During the first 24 hours after infection, the parasite remodels its PVM and transforms from its elongated form to a small, round trophozoite. At 20 hours post invasion, the parasite nucleus begins dividing repeatedly, displaying one of the fastest replication rates known for eukaryotes. Up to 30,00 nuclei are generated, at the same time as the PV is expanding to accommodate the growing parasite. Apart from the host cell ER, which gathers around the parasite and a loose association with the Golgi apparatus, there appears to be no constant association between the PV and the host cell organelles [126]. Nutrient uptake from the host is speculated to involve parasite-encoded transport channels inserted into the PVM, allowing molecules up to 850 Da to freely cross the membrane [126]. Large molecules such as lipids and peptides need to be actively imported and again the parasite appears to be able to modify the PVM to allow this to occur. For the parasite, the supply of nutrients is essential, but it

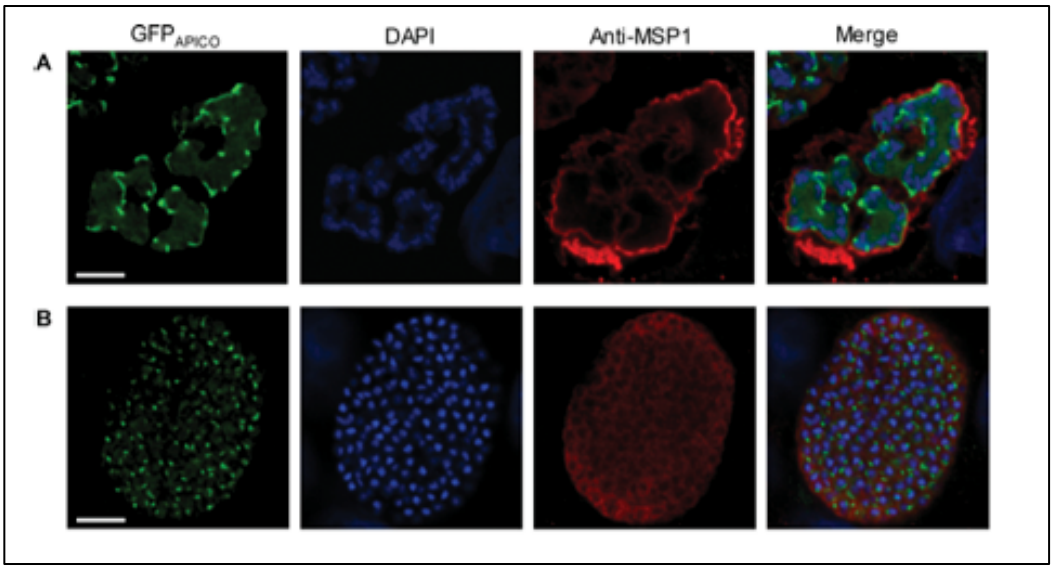


Figure 1.4. *Plasmodium berghei* liver-stage parasites.
Several different markers are used to visualize liver-stage development of *Plasmodium berghei* parasites. Apicoplast-targeted GFP is shown during mid and late-liver stages on the left-hand panel in green. DAPI is a DNA stain, indicating the individual daughter nuclei. Anti-MSP antibodies mark the Parasite Plasma Membrane (PPM). Merged images show during cytomere stage the individual nuclei and apicoplast organelles surrounded by parasite plasma membrane for the generation of infectious merozoites. Image reproduced from [127]

must also dispose of metabolic waste. For liver-stage parasites, so far no food vacuole has been described. Thus, the question remains of how metabolically active liver-stage parasites deal with waste products. The massive quantity of membrane required for replication during this period would explain the reliance of the parasite on *de novo* fatty acid biosynthesis during the liver stage.

Following the completion of nuclear division, at which point the single parasite can contain many thousands of nuclei, the parasite develops into a cytomere stage. The plasma membrane of the parasite invaginates to form what appear to be spheres of membrane, portioning the cytoplasm into between 5 to 20 units. During the cytomere stage, the individual units of plasma membrane are not fully separated and mitochondrial branches connect the segregating “clumps”

[128]. Once the formation of merozoites is complete, they must be transported into the bloodstream where they can infect red blood cells (RBCs) to continue the life cycle [128]. The release of merozoites is a well-orchestrated, multi-step process. During merozoite formation parasite proteins begin to leak into the host cell, demonstrating that the membrane of the PV becomes increasingly permeable before it is completely disrupted [129]. Parasite and host cell material mix freely in infected cells [130]. Not much is known about the ultimate breakdown of the PVM in *Plasmodium* liver stages. This takes place within a short time and can be inhibited by E64, an inhibitor of cysteine proteases [131]. LISP-1 in *P. berghei* is the only identified protein to be involved in PVM disruption. Deletion of LISP-1 prevents parasites from escaping the PV, however the protein has no functional protease domains. Once the PVM is dissolved, parasite-dependent host cell death is initiated [128]. It is unclear whether the host cell membrane remains intact after disintegration of the PVM, yet it has been demonstrated that the disruption of the PVM and the host cell membrane are differentially timed [125]. Egress of intra-erythrocytic merozoites is known to involve specific sets of proteases that are activated in cascades [132]. It is likely that similar mechanisms act during liver stage, although it remains unclear whether proteases are acting directly on the PVM or whether other effector molecules are involved. Merozoites remain surrounded by the hepatocyte-derived plasma membrane until they reach blood vessels where they can safely infect RBCs.

During the liver-stage of *Plasmodium* development, the apicoplast and the mitochondria appear to be in even less contact with each other [133]. In the liver stages of development, the apicoplast and the mitochondrion become extensively branched and intertwining structures. The organelles undergo impressive morphological and positional changes prior to cell division. The organelles

cannot be formed *de novo* by the parasites, so all daughter merozoites must contain a copy of these organelles and their genomes. Prior to the formation of daughter merozoites, fission of the apicoplast and mitochondrion occurs, with each new unit of apicoplast and mitochondrion containing one or more copy of the respective organellar genome [133]. The apicoplast always divides before the mitochondrion, with the latter dividing only very shortly before cytokinesis [133].

It has been demonstrated that liver stage development requires the production of *de novo* fatty acids by the *Plasmodium* parasites. The liver stage of parasite development, in contrast to the blood stage, requires a functional FAS-II pathway [105]. FAS-II is the biochemical pathway for the generation of fatty acids commonly found in bacterial and plant organisms. It is relatively highly expressed during this stage in *Plasmodium* parasites [104]. Targeting the genes related to biosynthesis pathway for genetic deletion can lead to potential genetically attenuated whole organism vaccines, since many of these genes are highly expressed. Drugs such as ethionamide are also known to target enzymes particular to fatty acid biosynthesis; these could potentially provide new leads for drugs that inhibit liver stage.

LIPIDS

Understanding the dynamic relationship between the lipids of *Plasmodium* parasites and its host is a major focus of this work. The term lipid is a broad definition of a group of molecules that include fats, sterols, fat-soluble vitamins, monoglycerides, diglycerides, triglycerides and phospholipids. A lipid contains a fatty chain of carbon atoms, usually of an even number and a polar head group. The fatty acid chain may be saturated or unsaturated. Fatty acids are unsaturated due to the presence of double bonds that are always configured in “cis.” The length

and degree of unsaturation of fatty acid chains significantly impacts membrane fluidity due to the kink that unsaturated fatty acids provide. Unsaturated fatty acids prevent the membrane from packing tightly together, therefore decreasing the melting temperature of cells.

There are many molecules that are defined as lipids. Variation in head-groups and aliphatic chains allows for the existence of >1,000 different lipid species in any eukaryotic cell. Lipids, in contrast to proteins, DNA and carbohydrates, do not form polymers. This is a possible explanation for the wide diversity in lipid molecules.

Lipids can be categorized into three different cellular functions. The first is their use in energy storage because of their relatively reduced state. Triacylglycerol and steryl esters in lipid droplets are primarily used for the function primarily as reservoirs for the efficient storage of caloric reserves as caches of fatty acid and sterol components needed for membrane biogenesis. Secondly, polar lipids form the matrix of cellular membranes. Using the bi-polar hydrophobic and hydrophilic nature of lipids in aqueous environments, drives the formation of membranes of lipid contents. The same principle also governs the formation of organelles within a cell, enabling the segregation of specific chemical reactions for increased efficiency and restricted dissemination of their products. Finally, lipids can act as first and second messengers in signal transduction and molecular recognition processes. A lipid messenger binds a protein target, such as a receptor, kinase or phosphatase, which in turn mediate the effects of these lipids on specific cellular responses. Lipid signaling is qualitatively different from other classical signaling paradigms because lipids can freely diffuse through membranes. A consequence of this is that

lipid messengers cannot be stored in vesicles prior to release and are often biosynthesized "on demand" at their intended site of action.

Lipid droplets are also organelles within the cell that contain varying amounts of lipids. They are dynamic cytoplasmic organelles ubiquitously found in cells [134]. They are linked to many cellular functions, including lipid storage for energy generation and membrane synthesis, viral replication and protein degradation.

LIPIDS IN CELL MEMBRANES

The solubility of a cell membrane is determined by its lipid components and these factors determine the ability of a substance to diffuse through membranes. It can determine the rate of absorption of chemical compounds and the extent of their potency.

Lipids within the cell membrane provide the cell with many functions, including the potential for bidding, tabulation, fission and fusion. These processes enable the cell to divide, reproduce and allow intracellular membrane trafficking. The action of lipids can also define membrane domains that recruit proteins from the cytosol that subsequently organize secondary signaling or effector complexes. It has recently been described the presence of lipid rafts, that are defined as the organization of glycolipids and protein receptors organized in microdomains within the cellular membrane. These membrane microdomains enable to cell to compartmentalize cellular processes by serving as organizing centers for the assembly of signaling molecules, influencing membrane fluidity and membrane protein trafficking and regulating neurotransmission and receptor trafficking [135].Lipid rafts are more ordered and tightly packed than the surrounding bilayer but

float freely in the membrane bilayer [136]. Lipid rafts contain 3 to 5-fold the amount of cholesterol than in the surrounding bilayer that confers the rigidness of the raft. The distribution of phospholipids and sterols throughout the organelles and the plasma membrane within eukaryotic cells can create microdomains for the specialized function of enzymes or biochemical intermediates.

The major structural lipids in eukaryotic membranes are the glycerophospholipids, which contain a glycerol head group attached to a lipid base. Phosphatidylcholines (PtdCho) are the most abundant type of lipid species found within the plasma membrane of eukaryotes. Other components of eukaryotic membranes include the phosphatidylethanolamine, phosphatidylserine, phosphatidylinositol and phosphatidic acid. Their hydrophobic portion is a diacylglycerol (DAG) that contains saturated or cis-unsaturated fatty acyl chains of varying lengths. PtdCho accounts for over 50% of the phospholipids in most eukaryotic membranes. Most PtdCho molecules have on cis-unsaturated fatty acyl chain that enables them to be fluid in room temperature.

The amount of cholesterol in cellular membranes is critical for its rigidity. Cholesterol is synthesized in the cell by a regulated feed-back loop that controls its level. The balance is maintained by membrane-bound transcription factors called sterol regulatory element-binding proteins (SREBPs) that activate genes encoding enzymes of cholesterol and fatty acid biosynthesis. The three SREBP proteins are bound to membranes of the endoplasmic reticulum (ER) and nuclear envelope. SREBPs are released by two sequential cleavages; the first catalyzed by Site-1 protease (S1P) and the second by Site-2 requires the action of S2P. When sterols build up within cells, the proteolytic release of SREBPs from membranes is blocked and domains that

have already entered the nucleus are rapidly degraded [137].These mechanisms of regulation maintain tight control over the cellular level of cholesterol.

LIPIDS AND APICOMPLEXAN PARASITES

Lipids have emerged as important pathogenesis factors in a variety of infectious diseases. Lipid and fatty acid metabolism has been studied in many areas of apicomplexan biology; including parasite adaptation, host-parasite interaction and targets for drug therapy. Parasites have their own unique methods of fatty acid synthesis that have major implications for disease pathogenesis. However, the large amount of lipid scavenging from the host demonstrates auxotrophies in parasite species that can be used in the development of new therapeutics. Although Apicomplexan parasites have methods for *de novo* fatty acid biosynthesis, they must balance the processes of synthesis and scavenge to remain pathogenic.

Host lipids can have major impacts on disease pathogenesis. For example, intestinal infections with Giardia, a parasite that causes diarrhea, vomiting and malabsorpotion, host intestinal lipids and fatty acids influence the growth and encystation of the parasite [138]Free fatty acids generated from phospholipids and triglycerides from the host are detrimental to the growth of Giardia [139].In particular, dodecanoic (C12:0) can accumulate inside the trophozoite and alter the membrane permeablility and integrity.

Toxoplasma gondii, another *Apicomplexan* parasite, is known for scavenging a large variety of lipid precursors from host cytoplasm and modify them to generate complex lipids [140].*Toxoplasma,* contains enzymes of the Fatty Acid Synthase-II (FAS-II) pathway within the

apicoplast organelle [141].However, unlike *Plasmodium*, *Toxoplasma* seems to express also the Fatty Acid Synthase type-I (FAS-I) enzymes within the parasite mitochondria [142].*Toxoplasma* parasites lacking the acyl carrier protein, a central component of FAS-II, are viable, suggesting that the FAS-I and FAS-II pathways serve complementary functions. The uptake of lipid and lipid precursors from its environment is also important for the pathogenesis of *Toxoplasma gondii*. Phosphatidylcholine is the most prevalent lipid of *Toxoplasma*. It is about 75% of the total phospholipids of the parasite is primarily scavenged from the host [143]. *Toxoplasma* parasites are also capable of the scavenging choline [144]. In order to scavenge cholesterol from the host, in *Toxoplasma* infected cells, cellular mechanism of cholesterol biogenesis (through the LDL receptors and the HMG-CoA reductase enzyme) are both augmented to increase the cell cholesterol levels [145].The particular need for *T. gondii* parasites for choline and cholesterol import may provide new avenues for intervention. This hypothesis is supported by an analogue for choline, N,N-dimethylethanolamine, when taken up by intracellular parasites demonstrates progressive arrest [143]. Like the other Apicompleans, *Plasmodium falciparum* also demonstrates sensitivity to choline analogues.

THE APICOPLAST ORGANELLE

The apicoplast is a plastid organelle found in apicomplexan parasites. This organelle was discovered by Araxie Kilejian, who identified a circular, extrachromosomal DNA molecule in *Plasmodium lophurae*, a species of malaria that infects ducks [146]. Similar circular DNA molecules were described in *Toxoplasma gondii* by Piet Borst [147]. Identification of RNA polymerase genes that were homologous to plastid sequences in other organisms provided evidence that the apicoplast was a unique organelle with ancestral links to plant and algal

plastids [148]. Sequencing of the apicoplast genome revealed a small 35kb genome principally dedicated to its own expression. Apicoplasts are found throughout the Apicomplexa and are an ancient feature of this group acquired by the process of endosymbiosis. The only exception is that of *Cryptosporidium* spp [149].

The apicoplast was determined through the use of electron microscopy techniques to have four membranes bounding the organelle [150]. The apicoplast is thought to be derived from an engulfed red alga and was acquired before dinoflagellates and Apicomplexa diverged [151]. The function of the apicoplast remained a mystery after its discovery, especially since the photosynthetic capacity was lost from the organelle [152]. Pharmacological or genetic perturbation of the apicoplast leads to parasite death [153].

Genes for fatty acid biosynthesis in *T. gondii* and *P. falciparum* are targeted to the apicoplast [154]. The apicoplast also contains the pathway to synthesize isopentenyl diphosphate, a precursor of isoprenoids, ubiquinone side chains and dolichols, and an essential component for the modification of tRNAs [155]. Through data mining of genes targeted to the apicoplast, it was determined that malaria parasites make iron sulphur complexes and also cooperate with the mitochondrion in the synthesis of heme [156]. The apicoplast thus harbors a collection of plastid-like metabolic pathways that provide the organism with carbon, energy and reducing power in a manner identical to a non-photosynthetic plant or algal plastid [157].

THE APICOPLAST IS AN ANTIMALARIAL DRUG TARGET

The apicoplast is an interesting target for the development of antimalarial compounds since it contains a range of metabolic pathways and housekeeping processes distinct from the host [156]. Many of the cellular processes of the apicoplast, namely DNA replication, transcription, translation, post-translational modification, catabolism and anabolism, are bacterial-like and are sensitive to antibiotics that target these housekeeping pathways. Drugs targeting the apicoplast have been categorized for their targets: transcription, translation and genome replication. Ciprofloxacin is a gyrase inhibitor, that blocks prokaryotic DNA transcription. Ciprofloxacin inhibits apicoplast DNA replication but not nuclear replication in *P. falciparum* [153]. Rifampin and thiostrepton-treated parasites display reduced expression of the apicoplast-specific RNA polymerase subunits and reveal an abnormal unbranched apicoplast morphology [158]. Drugs that block the prokaryotic translation machinery include, doxycycline, clindamycin and spiramycin [159]. Lincosamides (lincomycin and clindamycin) block protein synthesis by interfering with peptidyl-transferase activity, blocking translocation of the peptidyl tRNAs [158, 159]. Tetracyclines and doxycycline target the 70s ribosome, resulting in a lack of amino acid incorporation into cytosolic proteins [160]. Macrolides (erythromycin and azithromycin) block peptide exit, preventing elongation of nascent peptides [161]. The antibiotic fosmidomycin targets the isoprenoid synthesis pathway in the apicoplast [162]. Fosmidomycin was demonstrated to inhibit growth of *P. falciparum* in culture as well as cure malaria in a mouse model. Mupirocin inhibits isoleucyl-tRNA synthetase, which is required in the apicoplast but is encoded in the nucleus [163]. .

Parasites that are cured of their apicoplast do not die immediately. Instead they invade new host cells but develop poorly [164]. Certain drugs targeting the apicoplast also demonstrate this “delayed

death" phenotype. Delayed death occurs when drug treatment does not show an impact on parasite viability until the next cell cycle. These drugs impact the viability of the progeny of treated parasites since they are inheriting non-functional apicoplasts [158].

PROTEINS ARE SPECIFICALLY TARGETED TO THE APICOPLAST

The four-membrane apicoplast organelle requires a unique set of tools for translocation of proteins encoded and synthesized by the parasite nucleus, to be incorporated into the organelle. An estimated 480 genes in the *P. falciparum* nucleus are targeted to the apicoplast [155]. Similar to plant plastids, *Plasmodium* parasites used the translocons (Tic and Toc) residing in the inner and outer membranes to identify appropriately-tagged gene products synthesized and import them in an unfolded state across the membranes [165]. Two components from plants, Tic 20 and Tic 22 have been identified in the apicoplast with Tic22 residing in the innermost membrane [166]. All known apicoplast-targeted proteins bear an N-terminal signal peptide, which directs the nascent protein to a specific translocon in the endoplasmic reticulum (ER) [167]. Once within the lumen of the ER, the transit peptide is sufficient and necessary to direct proteins into the apicoplast organelle [168]. The peptides of the apicoplast-target protein have properties that enable their predictions using the enable their predictions using the PlasmoAP algorithm [169].

The apicoplast contains an ensemble of bacteria-like pathways to replicate and express its genome plus an anabolic capacity to generate fatty acids, heme and isoprenoid precursors. Apicoplasts are essential, making the components of apicoplast metabolism, attractive drug targets. Apicoplast type II fatty acid biosynthesis has been the focus of the development of chemotherapies since it was first discovered. Several putative inhibitors against the parasite have

focused on the fatty acid machinery. However gene knock-out studies revealed that the parasite can replicate without these enzymes in blood-stages[105].

MORPHOLOGY OF THE APICOPLAST

The morphology of the apicoplast organelle evolves extensively during the stages of asexual replication and liver-stage development (Figure 1.5). The apicoplast first appears as a small circular organelle during the early ring stages of development, which then develops into a ring structure, followed by transformation into an extensive reticulated network as the parasite enlarges [170].

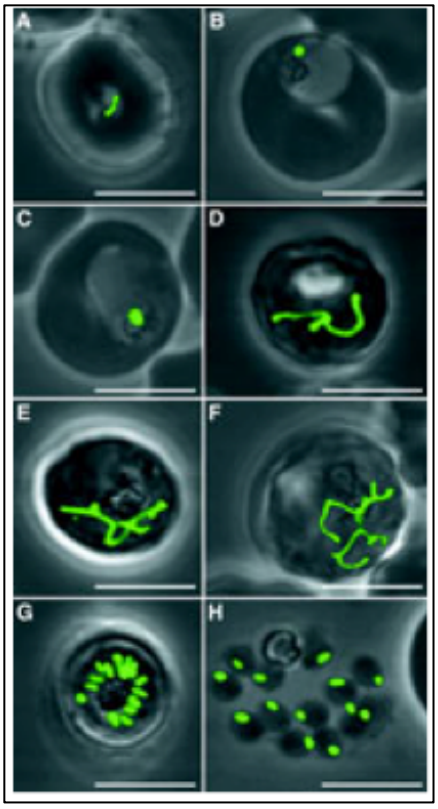


Figure 1.5. Apicoplast morphology during *P. falciparum* blood-stage replication
The apicoplast has a noticeable branched structure during the blood-stage of replication, which ensures segregation to daughter parasites during cell division. Image is reproduce from [171].

Both the apicoplast and mitochondrial organelles form highly branched structures in late-stage parasites, which undergo an ordered fission, first of the apicoplast and then by the mitochondria. These organelles are paired for packaging into daughter cells [170]. The two organelles share some points of contact but they are not consistent throughout the stage of asexual development (Figure 1.6). In general, the mitochondrion is slightly elongated in respect to the apicoplast. In the ring-stage cells, apposition between mitochondria and apicoplast is maintained. In late trophozoite and early schizont stages, there is little association between the organelles.

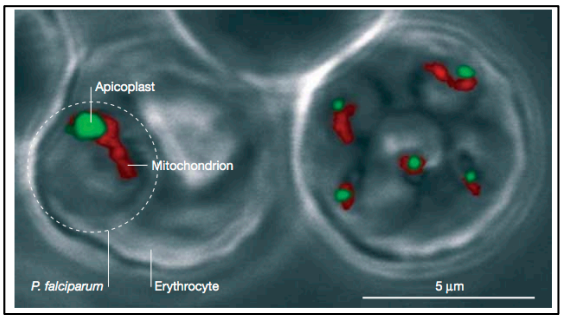


Figure 1.6. Contact between apicoplast and mitochondria organelles during *P. falciparum* blood-stage
Morphology of the apicoplast and the mitochondria organelles in during blood-stage development shows close contact between the organelles. Image is replicated from [155].

FATTY ACID METABOLISM IN PLASMODIUM

Rapid growth and multiplication that occurs during both the blood and liver stages requires the synthesis of membranes to encapsulate all of the daughter cells. In addition, infection of a red blood cell by *Plasmodium* results in a marked increase in the phospholipid content and a significant change in the lipid composition of the infected erythrocyte [172].As mentioned previously, the machinery for fatty acid synthesis is contained within the apicoplast organelle. A major question in the field of parasite biology remains as to how these lipids are generated, through the process of *de novo* synthesis or scavenge from the host. It appears that both of these

pathways are needed, although to different extends depending on the conditions of the host and the stage of the life-cycle. Understanding the balance between synthesis and salvage of fatty acids and other lipid species has been a central focus of this project.

Evidence for the uptake of scavenged fatty acids from the host has been shown with radio or fluorescence-labeled fatty acids that are incorporated into the parasite [173]. Lipids in the parasite are primarily fueled by precursors supplied by the host; including serine, ethanolamine and choline. These are the major building blocks used by the parasite in the synthesis of its structural and regulatory phospholipids [172]. Phosphatidylcholine and phosphatidylethanolamine are the predominant phospholipids in the *P. falciparum* membranes [174]. *P. falciparum* is capable of normal growth when cultured in the absence of exogenous choline, indicative of biosynthesis by the parasite [175] [176]. The minimal requirement for blood-stage replication of *P. falciparum* during *in vitro* cultures was determined as a combination of palmitic acid and oleic or steric acid. This combination of free fatty acids could fully replace the need for serum in culture [177]. Scavenged phospholipids can be incorporated without modification [178].

The cellular mechanisms of fatty acid import remain to be biochemically elucidated for *Plasmodium* parasites. However, Apicomplexan parasites encode a variety of acyl-CoA synthases (ACS) and acyl-CoA binding proteins (ACBP) that likely act in salvage and incorporation of host lipids [179]. In *P. falciparum*, these genes have been amplified by gene duplication and show considerable polymorphism, suggesting strong evolutionary selection [179]. The ACS genes in *P. falciparum* are located in epigenetically controlled regions of the genome that are usually associated with clonally variant gene families involved in immune evasion [180].

Plasmodium parasites modify fatty acids as needed by elongating or desaturating the lipids and incorporating them into phospholipids, such as diacylglycerols and tri-acylglycerols [177] [181]. In *Trypanosoma brucei,* elongase enzymes (ELO) are unique in generating the bulk of lipids for the parasite, since its ELO enzymes have adapted to use shorter carbon primers (C2) and elongate them to full length fatty acids (C10 or C14) [182]. Three ELO genes have been identified in *Plasmodium* species and remain to be characterized. Fatty acids, which are taken up from the host are converted to triglycerides by the acyl-CoA:diacylglycerol acyltransferase located within the parasite ER [183]. These triglycerides are stored in lipid bodies. *P. falciparum* generates oleic acid from radiolabeled stearic acid; this modification requires desaturation of scavenged fatty acids by the parasite. A delta-9 desaturase gene (PFE0555w) was recently identified in the *P. falciparum* genome and characterized in its expression during blood-stages, maximally in schizonts, and localizing to the ER [184]. *P. falciparum in vitro* cultures were sensitive to sterculic acid, a D9-desaturase inhibitor, implicating the essentiality of desaturation of fatty acids during blood-stage growth.

It was generally accepted in the scientific community that *Plasmodium* parasites do not synthesize their own fatty acids. However, analysis of the *P. falciparum* genome revealed the presence of the apicoplast-resident biosynthetic fatty acid synthesis type-II pathway, the biochemical pathway for the generation of fatty acids commonly found in bacterial and plant organisms (Figure 1.7). FAS-II enzymes are transcribed at a very low level in blood-stage parasites [185]. However a clinical *in vivo* transcriptional data-set provided evidence that the FAS-II pathway can be up-regulated during conditions of starvation [186].

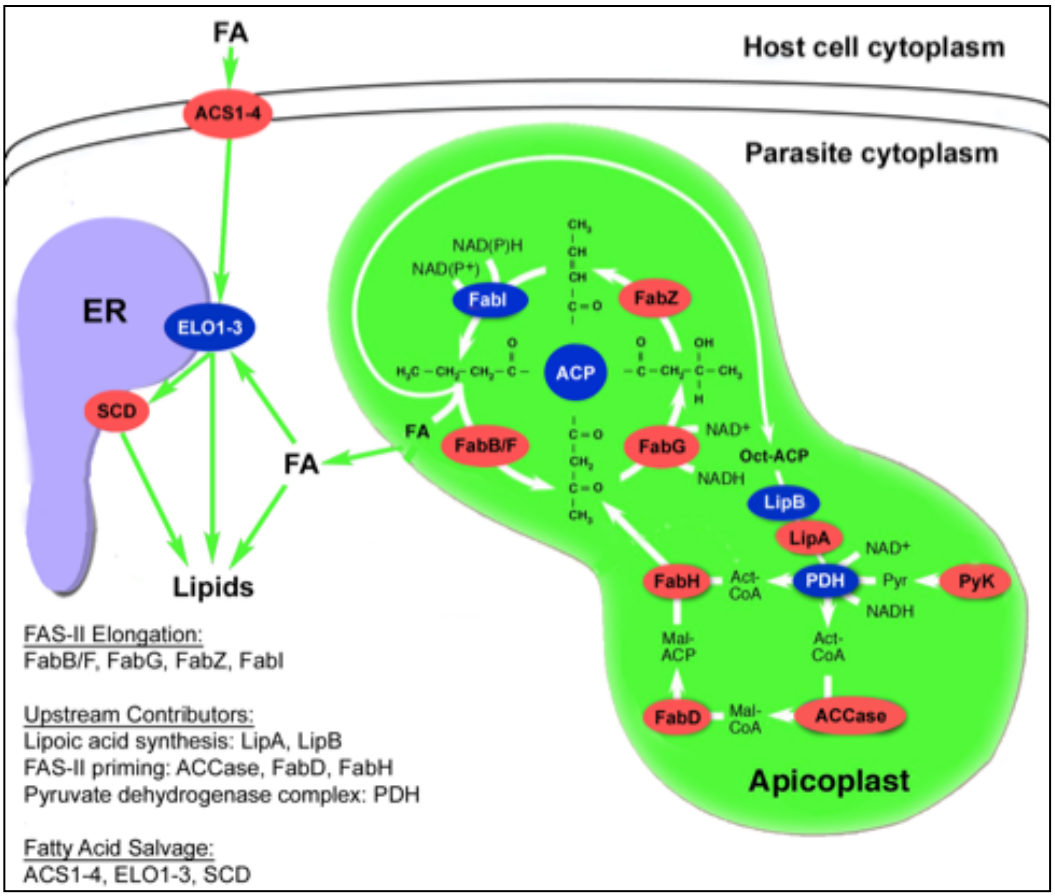


Figure 1.7. Genes hypothesized to be involved in lipid metabolism in *Plasmodium*
Genes involved in fatty acid biosynthesis include the ACP, FabI, FabZ, FabB/F and FabG. Lipoic acid synthesis in the apicoplast is generated by proteins LipA and LipB. Acyl-CoA Synthetase enzymes are involved the import of fatty acids from the environment. Elongation enzymes and desaturation enzymes, modify synthesized or scavenged fatty acids, are located in the endoplasmic reticulum. Adapted from [187].

Although the essential components for fatty acid biosynthesis have been demonstrated to be essential for liver-stage development, scavenge from the host is also important for development. Studies with obese Zucker rats, which had increased fatty acid synthesis and triglyceride content, supported the growth of four times the number of liver-stage schizonts than their leaner counterparts [188]. Import of lipids must occur through the PVM, which encloses the developing parasite after invasion. The PVM itself contains cholesterol derived initially from the host plasma membrane. The PVM is porous, permitting the passive transfer of small soluble

molecules of < 855 Da [126]. The pores are maintained during parasite cytokinesis until complete cellularisation. Host factors related to lipid transport impact parasite replication during liver stages. Decrease or loss of scavenger receptor class B type 1 (SR-BI) resulted in reduction in parasite invasion and growth in hepatocytes [189]. SR-BI expression resulted in increase cell cholesterol levels and increased expression of liver fatty acid binding protein on the hepatocyte. One of the genes critical for liver stage development, UIS3, is likely to enable lipid absorption from the host. UIS3 binds the liver fatty acid binding-protein (L-FABP) of the host [190]. This interaction is critical for liver stage, demonstrating that lipid import is essential for development.

The FAS-II pathway generates octanoyl-ACP; a 6-carbon chain attached to the acyl-carrier protein. The synthesis of lipoic acid requires generation of this precursor for the post-translational modification of certain enzymatic complexes. Lipoic acid is an essential cofactor for a number of oxidative decarboxylases, including pyruvate dehydrogenase (PDH), the glycine cleavage system, alpha-ketoglutarate dehydrogenase (KGDH) and branched-chain oxo-acid dehydrogenase (BCDH). It has been suggested that the critical function of apicoplast FASII might lie in lipoic acid production, as for the FASII pathway of plant mitochondria [187]. In *Plasmodium falciparum,* disruption of LipB, a critical ligase for lipoic acid synthesis, parasites remained viable [191]. Inhibition of lipoic acid scavenge pathway in *Plasmodium falciparum* is lethal to parasite replication . *Plasmodium* parasites must use a combination of the synthesis and scavenge pathways to optimize their replication in different host environments. This is the metabolic pathway we focused on in Chapter 2.

2. LIPOIC ACID SYNTHESIS DURING THE PLASMODIUM BERGHEI LIFE-CYCLE

ABSTRACT

Plasmodium parasites require the post-translational modification of several α-ketoacid dehydrogenases to regulate their activity through the attachment of lipoic acid, a short-chain fatty acid derivative. Lipoic acid can be synthesized *de novo* within the apicoplast of the parasite or it can be scavenged from the environment. Several of the important enzymes that are required for lipoic acid synthesis and attachment to target enzymes have been identified within the *Plasmodium* genome.

Here, we report the generation of a *P. berghei* line disrupted in its apicoplast-targeted protein ligase *LipB* gene. The impact of deleting this gene was then assessed throughout the parasite life-cycle. Although we observed no significant impact on mosquito stage development or asexual blood-stage growth, deletion of *PbLipB* resulted in a significantly decreased infectivity of sporozoites *in vivo* and *in vitro.* These parasites also demonstrated a very late liver-stage arrest. Disruption of *PbLipB* reduced the levels of lipoylated proteins in both asexual blood and liver stages, but the apicoplast morphology was only significantly impacted during the liver stage. *ΔpbLipB* parasites also have slower replication rates than wild-type controls during blood-stage growth after treatment with a lipoic acid analogue, 8-bromo-octanoate (8-BOA). These results demonstrate the importance of the LipB gene in the liver-stage of *Plasmodium berghei*

development and an exchange between the synthesis and the scavenge pathways of lipoic acid, never before demonstrated in *Plasmodium* parasites.

INTRODUCTION

One of the metabolic products generated by fatty acid biosynthesis is lipoic acid (6,8-thioctic acid). This eight-carbon chain compound [192] is an essential cofactor for a number of multi-enzyme complexes found in all eukaryotic cells that generate metabolic products including heme and fatty acids [193]. In addition to being synthesized *de novo*, lipoic acid is also absorbed from dietary sources. Cells maintain active systems to import non-protein bound lipoic acid from their environment [192]. Lipoic acid rapidly traverses cell monolayers in a pH-dependent manner [194]. Lipoic acid is likely imported by transport system since its uptake can be inhibited by benzoic acid and medium-chain fatty acids. Studies have suggested a monocarboxylate transporter or Na+-dependent multivitamin transporter may be responsible for lipoic acid uptake into cells [195].

Lipoic acid biosynthesis in all cell types begins with the generation of an octanoic acid precursor. Two carbons of octanoic acid are removed for their replacement with two sulfur atoms to generate a dithiolane ring (C6 and C8) (Figure 2.1). Lipoic acid has one chiral center and naturally exists in both R and S-enantiomeric forms. However, only the R form is attached as a cofactor to proteins [196]. The dithiolane ring is critical for the function of lipoic acid, as it acts as an acceptor of reactive oxygen species.

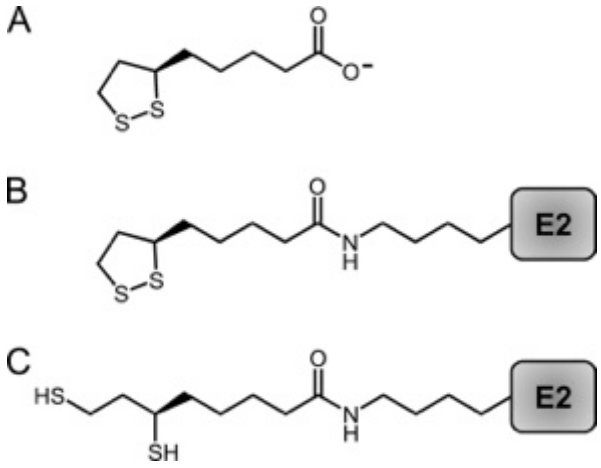


Figure 2.1. Different forms of lipoic acid intermediates
A) The R stereoisomer of lipoate. **B)** Lipoamide, the oxidized cofactor, bound to the E2 subunit. **C)** Dihydrolipoamide, the reduced form of lipoic acid, also bound to the E2 subunit of a lipoylated complex. Schematic reproduced from [197].

Oxidized (LA) and reduced (DHLA) forms create a potent redox couple. The dithiolane ring is the functional part of lipoic acid when it is attached to an enzymatic complex. The disulfur bond breaks and is attaches to the reaction intermediate to shuttle it between reaction subunits. After it transfers the product, the lipoate cofactor is regenerated through reduction of NAD+ by the E2 subunit [197]. Lipoic acid attachment always occurs on the N6 amino group of a lysine residue in a small protein domain (80-residues) [198] (Figure 2.2). The number of lipoyl acceptor domains on proteins can range based on the organism from one to three.

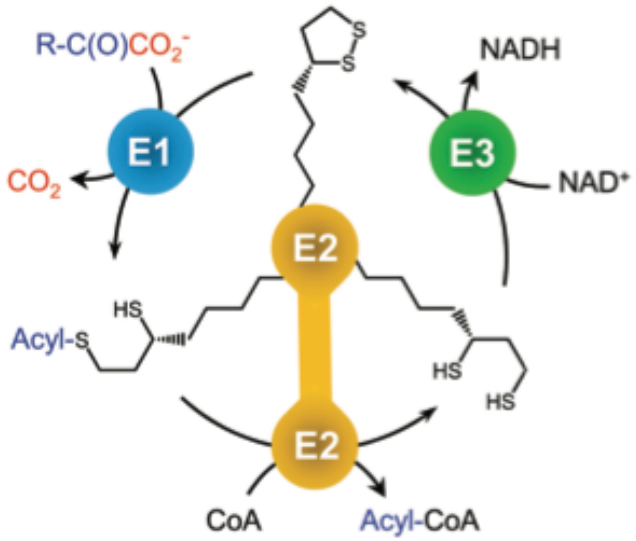


Figure 2.2. The PDH enzyme complex
Schematic shows how the lipoyl attachment to the E2 subunit of PDH is critical for function. The E1 subunit is responsible for the decarboxylation of the substrate that is then transferred to the lipoyl cofactor. The E2 subunit catalyzes the transfer of acyl to acyl-CoA The E3 subunit reduces NAD+ to regenerate the lipoyl domain. Schematic reproduced from [197].

In *Plasmodium,* covalent attachment of lipoic acid is required for the function of four large multi-subunit enzyme complexes: pyruvate dehydrogenase (PDH), alpha-ketoglutarate dehydrogenase (KGDH), branched-chain alpha-ketoacid dehydrogenase (BCDH) and the glycine cleavage system (the H-protein) [199]. PDH is located in the parasite apicoplast, a plastid-like organelle that performs a variety of metabolic functions including isoprenoid and fatty acid biosynthesis. In contrast to PDH, the other lipoylated complexes (KGDH, BCDH and the H-protein) have been localized to the mitochondria [199].

Lipoic acid also has a number of other biological functions. Lipoic acid has pharmacological properties as an anti-oxidant [193]. Lipoic acid, like other anti-oxidants, protects tissue from oxidant damage. In addition, attached lipoic acid can also serve as an anti-oxidant, and oxidative stress near the dehydrogenase complex can lead to oxidative destruction and can decrease its

function as a cofactor. Lipoic acid in humans is typically bound to proteins and free lipoic acid cannot be detected in the serum unless after the administration of a therapeutic dose [200]. Oxidative damage is a normal process of living systems. During oxidation, electrons are removed from these molecules and subsequently transferred in a chain of reactions to other molecules until they reach their final electron acceptor of oxygen. Oxidative damage occurs when the normal pathways of oxidation are not controlled and reactive oxygen species damage biomolecules such as DNA, proteins and lipids. Anti-oxidants function to control the oxidative processes by absorbing these "free radicals."

Another metabolic event of lipoic acid is the beta-oxidation of its pentanoic acid side chain. In bacterial species, such as *Pseudomonas putida,* lipoic acid is a principal metabolite of beta-oxidation [197], which produced carbon dioxide through the degradation of acetyl CoA in the citric acid cycle. In humans, the same beta-oxidation pathway degrades lipoic acid as for longer chain fatty-acids for energy production. However, seeing that the *Plasmodium* genome lacks the enzymes required for beta-oxidation of lipid species, lipoic acid is not used in these manner by the parasite.

In certain conditions, lipoic acid has been demonstrated to have a pronounced effect on the cell cycle. Alpha-lipoic acid induces hyperacetylation of histones *in vivo.* Lipoic acid was demonstrated to induce a reversible G1/G0 cell cycle arrest in cell lines by inhibiting histone deacetylase activity in tumor cell lines. Lipoic acid has also been shown to be a factor in the cell survival of rat hepatocytes by remediating loss of function of the Akt serine/threonine kinase, involved in stress response and cell cycle [192]. Lipoic acid supplementation of hepatocytes also

showed that two phosphatases that antagonize Akt, PTEN and PP2A, all components of the cell cycle, were inhibited by lipoic acid, demonstrating its ability to aid in the survival of liver cells.

Plasmodium parasites synthesize lipoic acid within the apicoplast, where the Fatty Acid Synthesis-II (FAS-II) pathway generates an octanoic acid precursor attached to the acyl-carrier protein (ACP) [187] (Figure 2.4) The derivation of lipoic acid from octanoyl-ACP requires two enzymes; Lipoate synthase (LipA) and Lipoyl octanoyl-transferase enzyme (LipB). LipA (MAL13P1.220) is responsible for catalyzing the introduction of two sulphurs at positions 6 and 8, forming a lipoyl appendage. The lipoyl appendage is an 8-carbon fatty acid chain, which when covalently bound in an amide linkage to multienzyme complexes results in a "swinging arm" that shuttles intermediates among active sites [201]. LipB (MAL8P1.37) transfers the octanoyl group from ACP to the conserved lysine residue on the PDH-E2 subunit in the apicoplast of *Plasmodium* parasites. LipA can work either before or after LipB has catalyzed this transfer. Both ligases have been identified in *P. falciparum* and are essential for the synthesis and attachment of lipoic acid to the E2 subunit of PDH in the apicoplast [202]. In *P. falciparum*, LipB was shown to be responsible for maintaining normal levels of lipoylated PDH but was itself nonessential to blood stage replication [202]. Δ*Pflipb* parasites were reported to have an increased growth rate during the asexual blood stages and a reduced level of lipoylation of PDH in the apicoplast. A second transferase enzyme, LplA2, was hypothesized to compensate for the loss of LipB and to be responsible for the remaining amount of lipoylation observed with the PDH E2 subunit [202]. LplA2 was demonstrated to localize to both the apicoplast and the mitochondria in *P. falciparum*. LplA2 also successfully complemented LipB/LplA deficient *E. coli* lines [202].

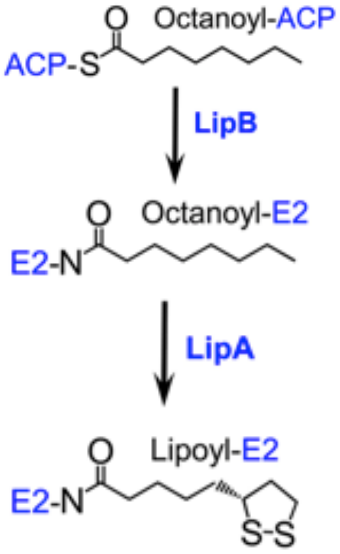


Figure 2.3. Lipoic acid synthesis pathway
Lipoic acid synthesis begins with the octanoyl-ACP precursor that is attached to the E2 subunit by LipB enzyme. LipA modifies octanoyl by introducing a dithiolane ring. Schematic reproduced from [197].

The pyruvate dehydrogenase complex (PDH) catalyzes the oxidative decarboxylation of pyruvate to form acetyl coenzyme A (Acetyl-CoA), which is a carbon intermediate that feeds back into the FAS-II pathway. Since there is only one PDH enzyme annotated in the *Plasmodium* genome (unlike plant species) and it is located in the apicoplast, it is assumed its major role in acetyl-CoA generation is for the fatty acid biosynthesis pathway [155].

In addition to the lipoic acid synthesis pathway, *P. falciparum* has an active scavenge pathway that is essential for both blood and liver stages and has been shown to lead to lipoylation of the mitochondrial enzyme complexes KGDH and BCDH. Scavenged radiolabeled lipoic acid is sequestered solely in the mitochondria where it is attached to these complexes [191]. The lipoic acid analogue, 8-bromo-octanoate (8-BOA), prevents lipoic acid scavenging by irreversibly binding to the lipoic acid protein ligase (LplA1), located in the mitochondria of *Plasmodium* parasites.

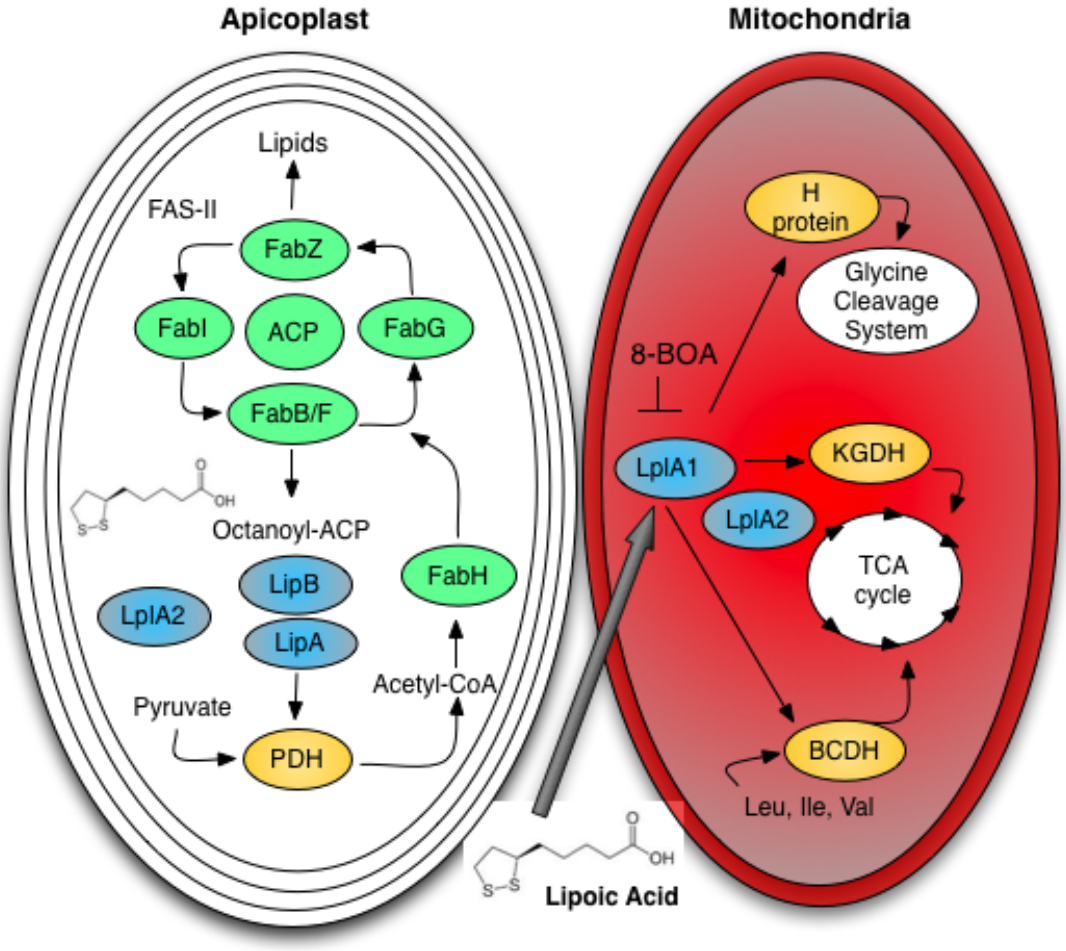


Figure 2.4. Lipoic acid synthesis and scavenge pathways in *Plasmodium* parasites
Schematic representation of lipoic acid synthesis and scavenging in the *Plasmodium* apicoplast and mitochondrion respectively. Enzymes responsible for the synthesis or attachment of lipoic acid to its target proteins are represented in light blue. LipB and LipA synthesize lipoic acid from the octanoyl-acyl carrier protein (ACP) precursor generated through the fatty acid biosynthesis type II (FAS-II) pathway. LipB is responsible for the attachment of octanoyl-ACP to the E-2 subunit of the pyruvate dehydrogenase (PDH) complex within the apicoplast. LipA is responsible for creating the thiosulfur bonds. PDH converts pyruvate into acetyl-CoA, which primes the FAS-II pathway. In the mitochondria, LplA1 attaches scavenged lipoic acid to α-ketoglutarate dehydrogenase (KGDH) and branched-chain alpha-ketoacid dehydrogenase (BCDH), which both feed into the tricarboxylic acid (TCA) cycle, as well as the H-protein of the glycine cleavage system. Attachment of scavenged lipoic acid can be inhibited by the analog 8-BOA that targets the ligase LplA1. A second lipoate ligase, LplA2, has been localized to both the apicoplast and the mitochondria.

8 BOA was transferred by *P. falciparum* LplA1 proteins *in vitro* to the H-protein acceptor domain [191]. It acts as a competitive inhibitor to free lipoic acid since it binds irreversibly to acceptor proteins. Addition of 8-BOA to *in vitro* cultures of *P. falciparum* inhibited blood-stage growth in a dose-dependent manner [191]. Recently, the essentiality of lipoic acid scavenging has been also been demonstrated for *Plasmodium* liver stages. 8-BOA treatment of parasites developing *in vitro* in hepatic cell lines prevented completion of liver stage development [203]. Investigations into the lipoic acid metabolism pathway of *Plasmodium* currently evoke a complete separation in the pathways of synthesis and scavenge with no exchange of lipoic acid occurring between the mitochondria and the apicoplast [191, 204]. The BCDH complex is involved in the degradation of branched-chain amino acids for the generation of acyl-CoA, which are intermediates of the tricarboxylic acid (TCA) cycle. The TCA cycle is commonly known in many cell types as the central process that allows them to metabolize carbon from glycolysis into a fully oxidized state to generate energy by oxidative phosphorylation and releasing carbon dioxide. The branched amino acids leucine, isoleucine and valine are deaminated to α-ketoacids that are decarboxylated and conjugated to CoA. In addition to contributing to the TCA cycle, these short chain primers can be used to generate longer branched fatty acid that can be important in modifying membrane fluidity. In certain organisms, BCDH activity is regulated by expression of the enzyme in response to accumulation of branched-chain ketoacids (prokaryotes) or phosphorylation of the enzyme complex (mammals). BCDH is expressed in both blood and liver-stages of *Plasmodium* parasites.

The KGDH complex converts α-ketoglutarate to succinyl-CoA, which also feeds into the TCA cycle. The TCA cycle enzyme succinyl-CoA synthetase consumes succinyl-CoA as a carbon

intermediate. It is regulated by the accumulation of metabolic intermediates like a high AMP/ATP ratio. While lipoic acid scavenging is sufficient to meet the metabolic needs of parasites during blood-stage growth, it has not been established if the synthesis pathway is necessary to meet the rigorous demands of liver-stage development. The observation that LipA expression is upregulated during early liver-stage development of *P. yoelii* nevertheless suggests an important role [104]. Furthermore, disruption of *PDH* in *P. yoelli* produced a phenotype in liver-stage forms, but did not affect the blood stage propagation in mice [205].

We have disrupted the *LipB* gene in *P. berghei* parasites for the purpose of investigating its role throughout the parasite life cycle. Our studies reveal that parasites lacking LipB progress normally through the asexual and sexual blood stages and develop normally in the mosquito, but fail to develop properly during the liver stage, indicating that lipoylation plays a critical role shortly after sporozoites invade a new host.

EXPERIMENTAL PROCEDURES

PROPAGATION OF *P. BERGHEI* PARASITES

P. berghei ANKA parasites (provided by MR4) were passaged in female Swiss-Webster mice (6–8 weeks of age purchased from Taconic Farms), via intraperitoneal injection. To determine pre-patent periods for parental ANKA vs. *Δpblipb* lines we used female C57/BL6 mice (Taconic Farms) as these are highly permissive and represent a sensitive model to detect low-level infections arising from the liver stage [105]. All animal experimentation followed protocols approved by the Institutional Animal Care and Use Committee of Columbia University.

PLASMID CONSTRUCTS AND PARASITE TRANSFECTIONS

Deletion of the *lipB* gene in *P. berghei* ANKA parasites was achieved by double crossover replacement of the targeted coding sequence with the *Toxoplasma gondii dhfr-ts* selectable marker, as published previously [206]. This marker mediates resistance to pyrimethamine that was added to the mouse drinking water at a concentration of 0.07 mg/ml in animal drinking water [206]. The *P. berghei* ANKA strain was transfected with plasmid pL0001-Δ*pblipb*, containing 5' and 3' untranslated regions (UTRs) of *PbLipB* as sites of homology. The cross-over event replaced the entire coding sequence with the *Tgdhfr-ts* selectable marker. The pL0001 vector was obtained from the MR4 organization (deposited by A. P. Waters, MRA-770). The pL0001-Δ*pblipb* plasmid contains 680bp of the 5' UTR, PCR amplified from the endogenous gene from *P. berghei* ANKA parasite genomic DNA. This fragment was sub-cloned between the EcoRV and the XbaI sites. 850bp of the *LipB* 3'UTR was also amplified from genomic DNA and sub-cloned between the Acc65I and HindIII sites. Plasmids were linearized by the XbaI, ScaI and Acc65I enzymes and verified by agarose gel electrophoresis prior to transfection. Transfection and with pyrimethamine selection were performed as previously described [206]. Correct integration of constructs and absence of the endogenous *LipB* locus was confirmed by PCR. Clonal parasite populations were obtained by limited dilution [206].

The plasmid designed to target GFP to the carboxyl-terminus of BCDH was generated from the GFP-containing pL0031 vector obtained from MR4 (MRA-800 pL0031) [207]. 875bp of the 3' end of the BCDH-E2 gene (PB001062.02.0) was amplified by PCR from *P. berghei* ANKA genomic DNA. Primers used were P2872 and P2873 (Table 2.1). The product was ligated into a Topo-easy vector (Promega) where site-directed mutagenesis primers were used to generate a BsmBI

site within the cloned product for linearization prior to transfection. The product was then sub-cloned between the restriction sites SacII and NcoI in the pL0031 vector, removing the endogenous stop codon and maintaining an open reading frame with the GFP coding sequence. This construct was linearized with BsmBI and transfected into *P. berghei* ANKA parasites to generate the tagged endogenous protein through a single cross-over recombination event. *P. berghei* ANKA parasites were transfected and selected with pyrimethamine as described above [206]. Correct integration into the BCDH-E2 endogenous gene was confirmed by PCR of the GFP-positive parasite population using primers P2957 and P2873 (Table 2.1). Single digit names correspond to locations on (Figure 2.5).

Primer Name	Sequence	Description
9 (P23020	ATACCCATATATCGTATCAACAGAGG	LipB gene F primer
10 (P2303)	ATTAATTTCAGTGATTAATGGATAATC	LipB gene R primer
7 (P2345)	TGTGCTTCACTCCCAGCAGC	Tgdhr gene F primer
8 (P2346)	GTCTCCACTGGAAGCCGTAGCC	Tgdhr gene R primer
2 (P2344)	CATAAAATGGCTAGTATGAATAGCC	LipB WT 5'utr R primer
3 (P2343)	GCATTATATGAGTTCATTTTACACAATCC	LipB WT 3'utr F primer
1 (P2448)	CATAACAATGAATTTACACCTAACCC	Recombinant locus 5' F primer
5 (P2320)	TGCATGCACATGCATGTAAATAGC	Recombinant locus 5' R primer
6 (P2317)	TGTGATTAATTCATACACAAACATAC	Recombinant locus 3' F primer
4 (P2453)	GACACAAATCAAACTATATTAATATGCG	Recombinant locus 3' R primer
P2304	AAGCATTAAATAAAATACATCCTTAC	18s rRNA subunit F primer
P2305	GGAGATTGGTTTTGACGTTTATGTG	18s rRNA subunit R primer
P3081	AGATCAAATTGCAAGTGGTCAATCCG	MSP1 gene F primer
P3082	TGGTACTTAAATCTTTGAATAGATCC	MSP1 gene R primer
P2957	TATGGAATTTATGTCCTACAGG	BCDH-GFP F primer
P2873	TGACCATGGATTTTCAAATCTTGAAGTGTCA T	BCDH-GFP R primer

Table 2.1. Primers used in Chapter 2

IMMUNOBLOT ANALYSIS OF LIPOIC ACID-BOUND PROTEINS

Cryopreserved *P. berghei* infected red blood cells were thawed on ice and then lysed in 1X PBS containing 0.2% saponin (1 mL for every 100 µl of packed iRBCs) for 3 min on ice. Samples were immediately diluted with 10 mL ice-cold 1X PBS and centrifuged in a swing bucket rotor at 3,500 x g for 10 min at 4˚C. The isolated parasites were washed twice with ice-cold 1X PBS and centrifuged as above. Parasites were resuspended in ice-cold RIPA lysis buffer (Boston BioProducts) supplemented with 10 µg/mL pepstatin A, 2 mM orthophenantroline, 2 mM EDTA pH 8.0, and 2X Complete EDTA-free Protease Inhibitor cocktail (Roche Applied Science). Lysates were incubated at -80˚C for 10 min and thawed at room temperature followed by vortexing for 1 min. This freeze/thaw/vortex cycle was repeated four times. Next, lysates were diluted with an equal volume of 1 x PBS lysis buffer. Total protein content was determined using a Bio-Rad Protein Assay. 5X SDS loading buffer (250 mM Tris-HCl, pH 6.8, 50 mM EDTA, 5% SDS (w/v), 25% glycerol (v/v), 0.02% bromophenol blue (w/v)) was added to each sample and boiled at 95˚C for 5 min. Sixteen micrograms of total protein were subjected to SDS-PAGE on a NuPAGE 4-12% Bis-Tris gel (Invitrogen) and then transferred to a nitrocellulose membrane. Membranes were incubated in a 1:1000 dilution of rabbit polyclonal anti-lipoic acid IgG (Calbiochem) overnight at 4˚ C and a 1:2500 dilution of secondary anti-rabbit IgG conjugated to HRP (GE Healthcare) for 45 min at room temperature (RT). This experiment was repeated three independent times.

IMMUNOFLUORESCENCE ANALYSIS OF BLOOD STAGE PARASITES

P. berghei wild-type *ΔpbLipB,* or *pbbcdh-gfp* asexual blood stage parasites were removed from infected mice, washed in complete schizont development media [206] and treated with 5nM

MitotrackerR CMXRos (Invitrogen) for 20 minutes at 37ºC. Asexual blood-stage parasites were fixed with 4% formaldehyde and 0.0075% glutaraldehyde. Cells were incubated for 30 minutes at RT and washed with 1 x PBS. Cells were permeabilized with 0.1% Triton X-100 for 10 minutes and washed with PBS. Cells were blocked in 3% BSA for 30 minutes. Anti-ACP antiserum was added at a 1:2000 dilution in 3% BSA for 1-2 hours (hr) at RT. Cells were washed 3 times with 1 x PBS. Anti-rabbit 594 secondary antibodies were then added in 1:1000 dilution for 1 hr at RT. Cells were washed twice in PBS and resuspended in complete medium containing Hoechst 33342.

P. BERGHEI INFECTIONS OF MOSQUITOES

Anopheles stephensi mosquitoes, obtained from the NYU Department of Parasitology insectary, were fed on Swiss Webster mice infected with *PbΔLipB* or *P. berghei* ANKA parasites. Mosquitoes were maintained in incubators at 19ºC with 70-80% humidity until the time of dissection.

IN VIVO LIVER-STAGE DEVELOPMENT ASSAYS

To determine the pre-patent period of parasite lines, C57/BL6 mice were infected with sporozoites by intravenous injection. Mosquitoes infected with *PbΔLipB* or ANKA parasites were dissected on day 21-post blood meal and used for injection into naive mice. Mice were infected with either 1,000 or 10,000 sporozoites. Pre-patent periods were determined by blood smears and are defined as the number of days it took for blood-stage parasites to appear following sporozoite injection.

IN VITRO LIVER-STAGE DEVELOPMENT ASSAYS

HepG2 cells (ATCC Company) were seeded at 2 x 10^6 per well in 8 well Lab-Tek culture dishes containing glass cover slips the day prior to sporozoite infection. Sporozoites were isolated from the salivary glands of infected female *Anopheles stephensi* mosquitoes and counted using a hemocytometer. HepG2 cultures were inoculated with 2 x 10^4 or 3 x 10^4 sporozoites per well. To quantify the size of the developing liver-stage parasites, developing exo-erythrocytic forms of GFP+ *P. berghei* (MRA-868) were visualized by fluorescence microscopy. Parasite size was completed using the Image-J program. *In vitro* cultures were also used for the generation of parasite cDNA after 48 hr of liver-stage development. RNA was harvested from infected HepG2 cells in Trizol and purified with an RNeasy kit (Invitrogen). We used a SuperScript III kit (Invitrogen) to generate cDNA. To determine the absolute number of developing liver stage parasites, cover slips were fixed 24 and 48 hours post-invasion (hpi) and were stained using either an anti – RFP antibody (wild-type controls used expressed Pb mCherry) or anti –Hsp70 *P. berghei* serum (*Δpblipb*). Parasites were counted using a Leitz-DM RB fluorescence microscope (Leica). At 65 hpi parasites that have successfully finished liver-stage development detach from the coverslips and float in the culture supernatant. These "detached cells" were stained with Hoechst 33342 and were counted using an Axiovert 200 fluorescence microscope (Zeiss). Residual parasites on the cover slips were fixed, stained and counted as described above at 65 hpi and 84 hpi.

IMMUNOFLUORESCENCE ANALYSIS OF LIVER-STAGE PARASITES

Liver-stage infections were prepared as described above. Infected cells were incubated for 15 min at 37°C in 5% CO_2 in the presence of 250 nM MitoTrackerRed CMXRos (Invitrogen) and

washed twice with medium before fixation. For fixation, cells were washed three times with 1 x PBS before adding 4 % paraformaldehyde /PBS for 20 min at RT. Cells were washed three times with 1 x PBS and stored in methanol at –20°C until staining. For staining, cells were washed three times with 1 x PBS and incubated for 1hr at RT in 10% FCS/PBS. Primary antibodies (chicken α-Exp1 1:1000, rabbit α-lipoic acid (LA) 1:1000, rabbit α-ACP 1:1000) were applied for 2hr at RT in 10%FCS/PBS. Cells were washed three times with PBS and secondary antibodies (donkey α-chicken Cy2 1:250, goat α-rabbit Fluor647 1:1000) and DAPI (1 µg/ml) were applied in 10%FCS/PBS for 1hr at RT. Cover slips were washed three times with PBS, dipped once into ddH20 and mounted on glass slides. Microscopic analysis was done on a FluoView TM FV1000 confocal microscope. For visualizing co-localization of the mitotracker signal with the α-LA signal, false colors were assigned to the signals (mitotracker: red, α-LA: green, α-Exp1: cyan, DAPI: blue). To determine the apicoplast morphology during late liver stage development, infected cells were fixed and stored as described above at the indicated time points. The same conditions were applied for the primary and secondary antibody staining as above. Microscopic analysis was done on a FluoView TM FV1000 confocal microscope. False colors were assigned to the signals: ACP: green; CSP, Exp1, MSP1: red.

SEQUENTIAL STAINING OF LIVER-STAGE PARASITES WITH *A*-LA AND *A*-ACP ANTIBODIES

Infected cells were fixed and permeabilized at the indicated time points with the same conditions as listed above. Cells were incubated with rabbit α-LA (1:2000 in 10% FCS/PBS) for 2hr at RT, washed three times with PBS and incubated with goat α-rabbit Hilyte647 (1:1000 in 10% FCS/PBS) for 1h at RT. Cells were washed three times with PBS. Following α-LA staining, cells were incubated with goat α-rabbit HRP (1:20 in 10% FCS/PBS) for 1hr at RT, washed three

times with 1 x PBS and incubated with 10% rabbit serum / 1x PBS for 1hr at RT. Cells were washed three times with PBS. Cells were incubated with rabbit α-ACP (1:500) and chicken α-Exp1 (1:2000) in 10% FCS/ 1 x PBS overnight at 4°C. Cells were washed three times with PBS and incubated with goat α-rabbit Alexa594 (1:5000), donkey α-chicken Cy2 (1:250) and DAPI (final concentration 1 μg/ml) in 10% FCS/PBS for 1hr at RT. Cover slips were washed three times with PBS, dipped in H_20 and mounted on glass slides using Daco Fluorescent Mounting Medium.

8-BOA TREATMENT OF BLOOD-STAGE PARASITES

In 3 independent experiments, blood-stage *Plasmodium berghei* parasites (ANKA or Δ*pblipb*) were drawn from an infected mouse, washed in complete schizont development media [206] and incubated on overnight incubation at 37°C with gentle agitation in complete media with dilutions of 8-BOA or DMSO control. 100 μM, 200 μM and 400 μM dilutions of 8-BOA in DMSO were placed in the cultures and incubated for 12-16 hr. Parasite cultures were washed and inoculated at 10,000 infected red blood cells into naïve mice by intravenous-injection. Parasitemias were monitored for 8-days by thin-smear microscopy.

RESULTS

DISRUPTION OF THE *LIPB* GENE IN *P. BERGHEI*

To disrupt *lipB* in *P. berghei* we replaced the entire coding sequence with the selectable marker, *Toxoplasma gondii dihydrofolate reductase (TgDHFR)* by double cross-over recombination [206]. We transfected *P. berghei* ANKA parasites with a linearized plasmid, pL0001-Δpblipb using the AMAXA electroporation system. The pL0001 vector (MRA-770) was obtained from MR4, as

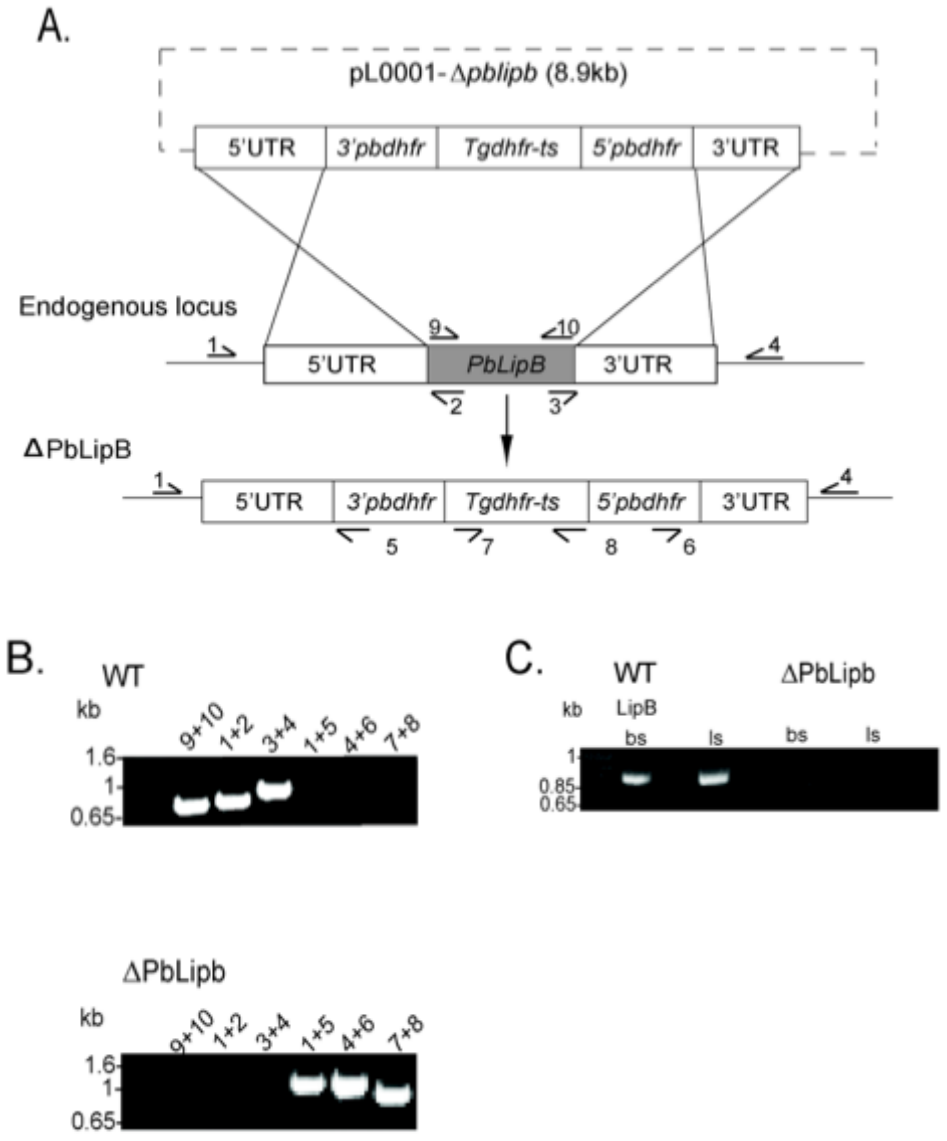


Figure 2.5. Generation of *LipB* knockout parasites in *Plasmodium berghei*
A) Schematic representation of the targeted disruption of the wild-type genomic locus to generate Δ*pbLipB* parasites. The location and orientation of the different primer sets (1 through 10) used for screening the transfectants are indicated. **B)** PCR confirmation of the modified locus of *ΔpbLipB* parasites. Primers used for screening are as listed in panel A. The top panel shows wild-type parasites have the *LipB* coding sequence. The bottom panel shows integration of the selectable marker into the *LipB* locus. **C)** Confirmation of lack of LipB expression in KO cells in blood and liver-stages. *pbLipB* is not expressed from *ΔpbLipB* cDNA from blood (bs) and liver stages (ls). *pbLipB* is expressed in wild-type *P.berghei* cDNA. The *lipB* gene is expressed during asexual blood and liver stages

deposited by AP Waters. The targeting vector contained 680 bp of homology to the 5' untranslated region (UTR) of *LipB* and 850bp of homology to the 3' untranslated region. Transfected parasites were injected back into naïve mice and selected using pyrimethamine. Resistant parasites were cloned and integration was confirmed by PCR (Figure 2.5).

In order to confirm the disruption of *LipB*, we performed an RT-PCR with primers specific to the coding sequence of the gene (Figure 2.5). Wild-type and cloned Δ*pbLipB* parasites were harvested at either blood or the liver stages (48 hours post-infection (hpi) of HepG2 cultures) and used to generate cDNA. HepG2 cells, a human liver carcinoma cells, were inoculated with wild-type or Δ*pbLipB* sporozoites to generate a liver stage infection with *in vitro* conditions. We confirmed lack of expression of *lipB* during both blood and liver stages in our knock-out parasites.

ASSESSMENT OF THE GROWTH RATE OF *P. BERGHEI LIPB* KNOCKOUT PARASITES DURING BLOOD STAGE

To determine whether there was any difference in blood stage growth in Δ*pblipb* parasites, we inoculated either 10,000 or 1000 asexual blood-stage parasites of either wild-type *P. berghei* ANKA or Δ*pblipb* strains by intravenous injection into Swiss/Webster mice. The parasitemia of asexual replication was determined by blood smear in three independent experiments (Figure 2.6A & B). Although [202], found a faster rate of asexual stage growth in *P. falciparum* parasites lacking *lipB,* our results indicate that in *P. berghei* disruption of the *LipB* gene does not impact blood-stage development.

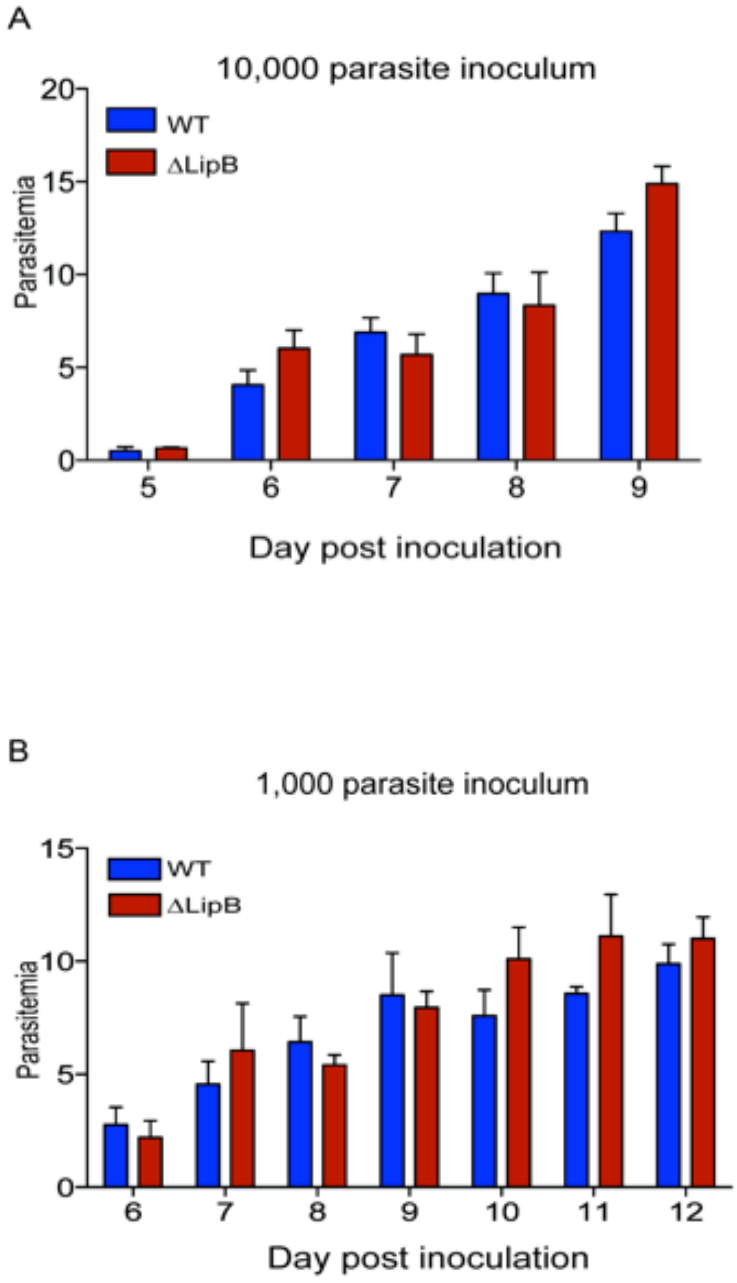


Figure 2.6. Δ*pbLipB* parasites have no change in blood-stage growth kinetics
(A & B) The blood-stage growth rate of Δ*pblipb* parasites is shown in comparison to the growth rate of *P. berghei* wild-type parasites. In three independent experiments, either 10^3 or 10^2 asexual blood-stage parasites were injected by intravenous injection into 6 week old female Swiss-Webster mice (n=5). Parasitemia was monitored by thin smear for 12 days or until death, shown as averages ± SEM.

ASSESSMENT OF LIPOYLATED PROTEINS IN BLOOD STAGE *P. BERGHEI* PARASITES

To determine the effect of *lipB* deletion on the composition of lipoylated proteins in the parasite, we compared the lipoylation pattern in wild-type and Δ*pblipb P. berghei* protein extracts by Western blot. Blood-stage parasite lysates probed with an anti-lipoate antibody revealed significantly lower levels of lipoylated PDH in the Δ*pblipb* parasite extract compared to control parasite extracts (Figure 2.7). This result is consistent with a current working model that proposes that LipB is responsible for lipoylation of PDH within the apicoplast [202]. LipB contains a predicted apicoplast targeting motif and PDH is the only protein within the apicoplast that is known to require lipoylation. Other lipoylated proteins identified in *Plasmodium* include BCDH, KDH and the H-protein, all demonstrated to be localized within the mitochondria [199]. These proteins are not predicted to be lipoylated by LipB, but by the mitochondrial ligases LplA1 or LplA2 [208]. Surprisingly, we found a reduction in lipoylation of the BCDH protein in the Δ*pblipb* parasites compared to wild-type, suggesting that LipB might play a role in BCDH lipoylation in *P. berghei.*

BCDH IS LOCALIZED TO THE MITOCHONDRIA IN *P. BERGHEI*

Given the reduced level of lipoylation of BCDH in Δ*pbLipB* parasites, we sought to determine the localization of this protein. A BCDH-GFP fusion plasmid was transfected into parasites and the fusion protein was localized by fluorescence microscopy.

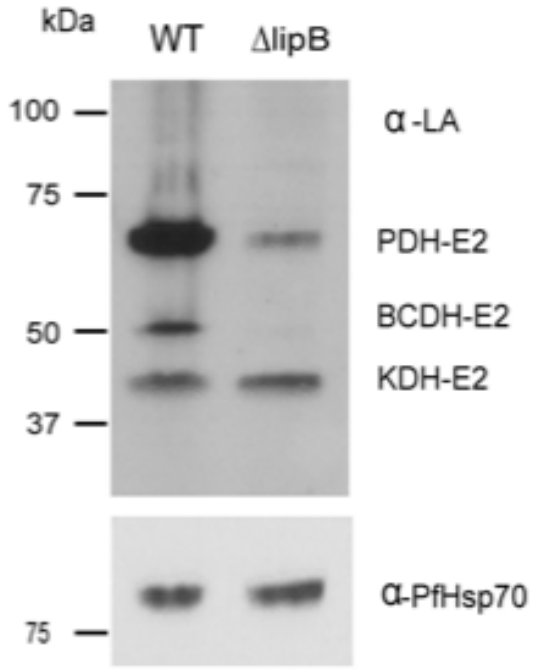


Figure 2.7. Δ*pbLipB* parasites have less lipoylated BCDH and PDH proteins
In three-independent experiments, antibodies specific for lipoylated proteins detected a different between wild-type and Δ*pbLipB* parasites. The three lipoylated proteins identified from top to bottom as pyruvate dehydrogenase E2 subunit (PDH-E2), branched-chain α-ketoacid dehydrogenase E2 subunit (BCDH-E2), and α-ketoglutarate dehydrogenase E2 subunit (KDH-E2). In the Δ*pbLipB* parasites, the level of PDH lipoylation is reduced to 16% of the level detected for the wild-type parasites. There is only 2% of lipoylated protein for the BCDH in the Δ*pbLipB* parasites versus the wild-type. The membrane was re-probed for *P. falciparum* Hsp70 as a loading control. BCDH-E2 is detected in the *ΔpbLipB* parasites at longer exposures (data not shown).

Co-staining of live parasites with the mitochondrial marker. Mitotracker red localized BCDH to this organelle (Figure 2.8A). In contrast, BCDH-GFP did not co-localize with the apicoplast marker, acyl-carrier protein (ACP), which was localized using anti-ACP antibodies (Figure 2.8B). Approximately 50 - 80 BCDH-GFP cells were imaged with either mitotracker staining or with the anti-ACP antibodies on three separate occasions. We did not try to localize the BCDH protein by immunofluorescence because of a lack of protein specific antibody. Anti-lipoic acid antibodies would not be useful for immunofluorescence either. They would detect all lipoylated

proteins within the parasite and we wanted to find the organelle-specific location for BCDH. Given the results we obtained with the BCDH-GFP fusion line and the western-blot data of parasite extracts, it appears that LipB is responsible for the lipoylation of a mitochondrially-targeted protein. We did not localize LipB itself because of its strong apicoplast targeting motif as predicted by PlasmoAP [169]. These results suggest an exchange between the lipoic acid synthesis and scavenge pathways.

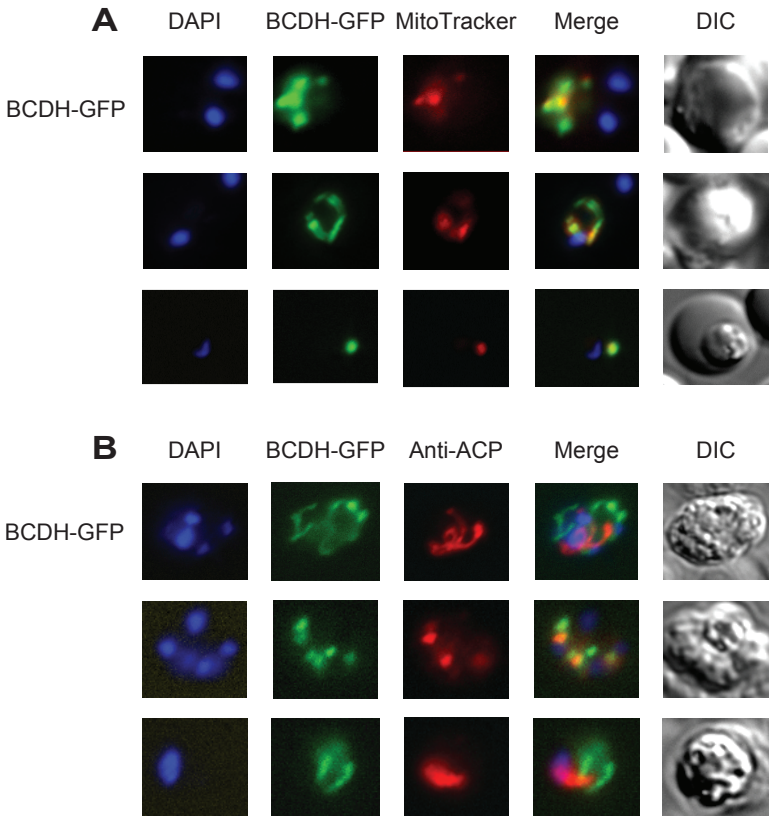


Figure 2.8. The BCDH protein localizes to the mitochondria in blood stage
A) Parasites expressing BCDH-GFP fusion proteins were stained with Mito-Tracker Red, revealing co-localization with the mitochondrial dye. **B)** The BCDH protein is not localized to the apicoplast. BCDH-GFP parasites were fixed and stained with anti-ACP antibodies to identify the location of the apicoplast. There was no co-localization of BCDH protein and the apicoplast during blood stages.

Δ*PBLIPB* PARASITES DO NOT SHOW ANY DEFECTS IN APICOPLAST MORPHOLOGY

To determine if apicoplast morphology was perturbed in the *lipB* knock-out parasites, we performed immunofluorescence microscopy with anti-ACP antibodies on fixed wild-type and Δ*pbLipB* parasites (Figure 2.9). The apicoplast undergoes a striking morphological change over the course of blood-stage development, beginning as a compact dot in the ring stage and elaborating into a highly branched structure during schizogony in anticipation of subsequent cell division, ensuring inheritance to each daughter merozoite [170]. Δ*pbLipB* parasites showed comparable amounts of dotted versus branched apicoplasts in comparison to wild-type parasites (Δ*pbLipB* parasites: 61% branched, wild-type: 52% branched). In our *P. berghei* knock-out parasites, apicoplast development proceeded normally, suggesting that disruption of the lipoic acid synthesis pathway is not impacting the health of the apicoplast during blood stages.

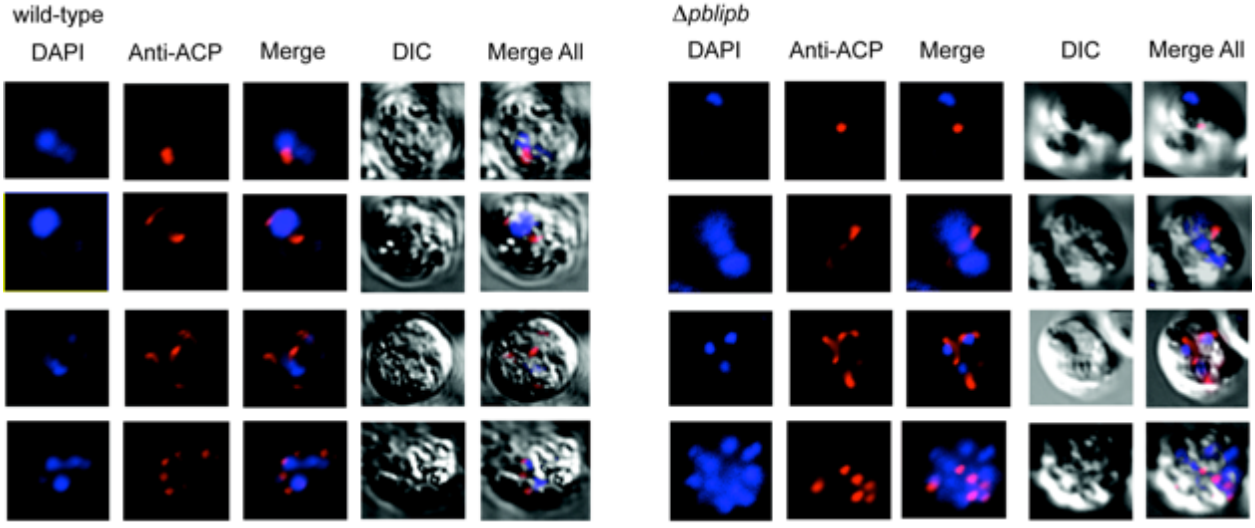


Figure 2.9. Δ*pbLipB* parasites show no defect in apicoplast morphology in blood-stages
Blood stage *ΔpbLipB* and wild-type *P. berghei* parasites were fixed and stained with an anti-ACP antiserum to identify the apicoplast. No visible difference in apicoplast morphology between *ΔpbLipB* and wild-type parasite lines was observed.

Δ*PBLIPB* PARASITES DO NOT DEMONSTRATE A DISCERNIBLE PHENOTYPE DURING MOSQUITO STAGES

We did not see a significant deficiency in the mosquito stages of development based on a comparison of the number of sporozoites isolated from mosquitoes infected with knock-out or wild-type parasites. In three independent experiments, infected mosquitoes were dissected 18-21 days after feeding on infected mice with similar parasitemias dissected salivary glands and viewed midguts for the presence of oocysts on day 21 post blood-meal by phase-constrast microscopy. This experiment was performed three times and sporozoite numbers were calculated from groups of 5–30 mosquitoes per parasite strain (Table 2.2). We observed mean ± SD sporozoite loads of 15,313 ± 8,055 and 13,611 ± 6,286 for the WT and KO respectively. These assays indicate that the loss of LipB did not noticeably impair parasite development in the *Anopheles* mosquito vector, and provide evidence that normal levels of lipoylated protein are not required for *P. berghei* development within the mosquito host.

Experiment	Parasite line	# of Mosquitos Dissected	# of Sporozoites/ Mosquito
I	WT	10	6667
	Δ*pbLipB*	10	9375
II	WT	10	22606
	Δ*pbLipB*	32	20833
III	WT	20	16667
	Δ*pbLipB*	20	10625

Table 2.2. PbΔ*LipB* sporozoites have normal sporozoite numbers

Δ*PBLIPB* PARASITES DO NOT COMPLETE LIVER-STAGE DEVELOPMENT *IN VIVO*

To test whether disruption of *LipB* impacted the liver stage of *Plasmodium* development, mice were inoculated with sporozoites and the pre-patent period was determined. The pre-patent period, defined as the number of days until detection of parasites in the blood-stream,

demonstrates the infectivity of sporozoites to establish infection and complete liver-stage development in the mammalian host.

Experiment	Parasite line	Sporozoites	Number of Infected mice	Average Prepatent Period
I	WT	1,000	5 of 5	5.0 days
	Δ*pbLipB*	1,000	1 of 5	9.0 days
II	WT	1,000	4 of 4	5.0 days
	Δ*pbLipB*	1,000	1 of 4	9.0 days
III	WT	10,000	5 of 5	3.0 days
	Δ*pbLipB*	10,000	5 of 5	7.6 days

Table 2.3. *In vivo* infectivity of *ΔpbLipB* in comparison to wild-type sporozoites

Sporozoites were dissected from salivary glands of infected mosquitoes and injected intravenously into C57/BL6 mice, Using dosages of either 10,000 or 1,000 sporozoites, we consistently saw a delay in the pre-patent period with the Δ*pbLipB* sporozoites, which only generated a blood-stage infection in 1 of 5 and 1 of 4 mice at the 1,000 sporozoite inoculum (Table 2.3). In contrast, all of the mice inoculated with wild-type sporozoites developed a blood-stage infection after a 5-day pre-patent period. At an inoculum of 10,000 sporozoites, all mice infected with Δ*pbLipB* sporozoites developed a blood-stage infection, but with a significantly increased pre-patent period, suggesting that *lipB* is important for normal liver-stage development in *P. berghei*. 1,000 or 10,000 infectious sporozoites were intravenously injected into mice and the pre-patent period was detected by thin-blood smears. Disruption of the *LipB* gene greatly reduced the infectivity as shown by the delay in prepatent period.

Δ*PBLIPB* PARASITES HAVE MAJOR DEFECTS DURING LATE LIVER-STAGES AND DO NOT PRODUCE DETACHED CELLS DURING *IN VITRO* CULTURE

In order to investigate when the defect during liver stage development is occurring, equal numbers of *P. berghei* ANKA and Δ*pbLipB* sporozoites were plated onto HepG2 cells. From three independent experiments there was no significant difference between the absolute number of developing parasites 24 and 48 hpi (Figure 2.10B). In addition, the size of the exo-erythrocytic forms at 48 hpi was examined to determine whether the arrest seen during *in vivo* experiments is detectable early during *in vitro* development (Figure 2.10A). The area of the developing parasites, as delineated by Exp1 staining of the PVM, was measured and found to not differ significantly between the two parasite lines. Approximately 20-30% of wild-type ANKA parasites were capable of successfully finishing *in vitro* liver-stage development, resulting in the detachment of the hepatocytes from the culture surface. As the parasite develops within the host cell, the parasite blocks the apoptotic machinery and host cell does not demonstrate any signs of cell death [132]. As the PVM begins to break down, merozoites mix with the host cell organelles and parasite proteases degrade host cell organelles [128]. The parasite induction of host cell apoptosis is visualized during *in vitro* culture as detachment of infected hepatocyte cells from its neighboring cells. This typically occurs at 62-70 hpi. We counted the number of infected hepatocyte host cells floating in the supernatant of the culture at 65 hpi. No detached cells were found for the Δ*pbLipB* parasites at 65 hpi, nor at the later time point of 84 hpi (Figure 2.10). Furthermore, we decided to detect expression of merozoite-surface protein-1. Msp-1 is a marker for the parasite plasma membrane (PPM), which at late time points invaginates around the individual daughter merozoites [132]. Msp-1 is usually expressed at the later-stages of parasite development; i.e. 48hpi or later. We identified expression of Msp-1 at 48hpi in hepatocyte

cultures infected with wild-type parasites, but could not detect transcripts from Δ*pbLipB* infected cultures. *P. berghei* 18s ribosomal RNA was used as a control for proper isolation of RNA and cDNA synthesis from infected cultures. This result suggests that Δ*pbLipB* expression of msp-1 is abnormal; expressing later than wild-type. Therefore, although infectivity of knock-out parasites appears normal at 48 hpi, Δ*pbLipB* parasites have defects in other aspects of liver-stage development.

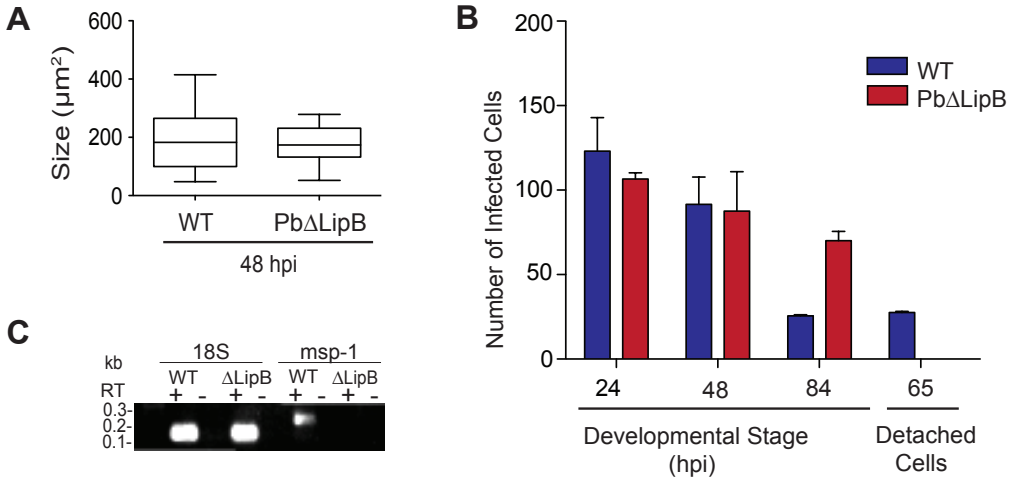


Figure 2.10. Δ*pbLipB* parasites fail to form detached cells *in vitro*
A) Δ*pbLipB* parasites showed no defect in size at 48 hpi in comparison to wild-type parasites. The average sizes of the developing exo-erythrocytic forms were similar at this time point. Data represents two independent experiments in duplicate. Data presented as a box and whiskers plot that illustrates the mean, interquartile range (25-75%), <95% confidence interval. **B)** Δ*pbLipB* parasites do not complete liver stage development *in vitro* to form detached cells. In two independent experiments, HepG2 cells were infected with equal numbers of sporozoites. Parasite numbers were determined by counting fixed/ stained parasites at time points 24, 48, 65 and 84 hpi and counting of live Hoechst stained detached cells at 65 hpi. No detached cells were ever detected. Error bars represent the standard error of the mean. **C)** Expression of 18s and msp-1 detected by RT-PCR of parasite cultures at 48 hpi.

To further probe the timing of the developmental block in the *lipB* knockout parasites, HepG2 liver cells were infected with either wild-type or Δ*pbLipB* parasites and assessed for parasite development by antibody staining for MSP1. Antibodies to MSP1 (PB000172.01.0) were used to recognize the morphology of the PPM at different time-points of development. Comparison of the staining of the wild-type and Δ*pbLipB* parasites revealed abnormal development of the knockout parasites at later time points (Figure 2.11). Wild-type ANKA parasites have well-defined individual nuclei by 54 hpi. The PPM evenly invaginates around each of the individual merozoites at this and later time points during development. However the *lipB* knockout parasites, in contrast, demonstrate heterogeneous MSP1 staining that appears to clump and that is not well invaginated around the individual daughter cells. The abnormal MSP1 staining in the Δ*pbLipB* parasites suggests that the plasma membrane is segregating improperly during the late time points of development. The images shown for Δ*pbLipB* parasites at the late schizont and cytomere stages of development show how the PPM staining is punctuated and does not surround the individual nuclei of the replicating parasite. Rarely were normal Δ*pbLipB* parasites found during *in vitro* cultures. These were only seen at very late time-points, around 84 hpi, when all normally developing parasites would have completed development and detached within their host cell membranes into the supernatant. The defect we are seeing in the MSP-1 staining clearly shows that the parasite plasma membrane is improperly segregating late in development in Δ*pbLipB* parasites.

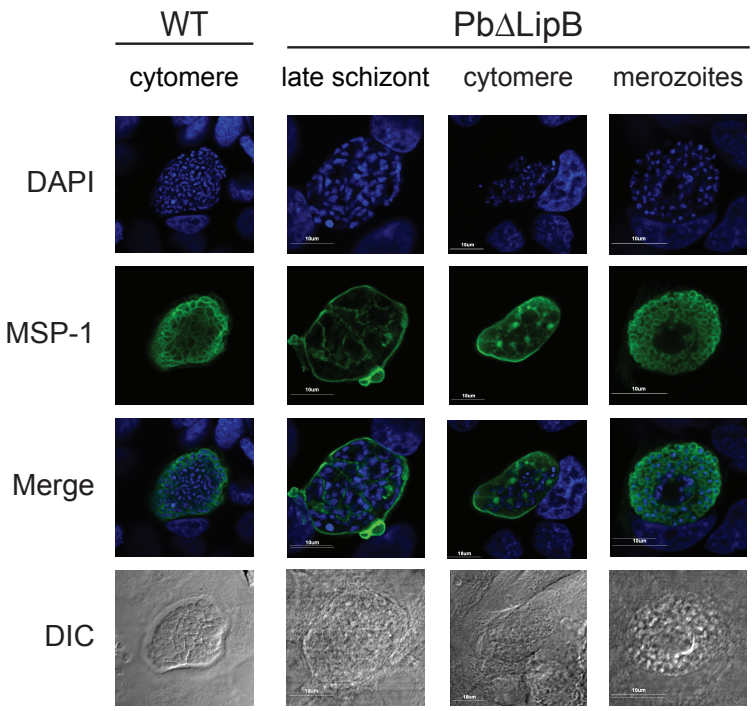


Figure 2.11. Δ*pbLipB* parasites are defective in late liver-stage development
Developing parasites were imaged over a liver stage time course by immunofluorescence for MSP1 (Green). The left panel shows wild-type (WT) cells at 54 hpi. The right three panels show *ΔpbLipB* parasites progressing through their liver stages of development within HepG2 cells. MSP1 staining of the parasite plasma membrane (PPM) appears to clump and is not evenly dispersed at late time points in the *ΔpbLipB* parasites.

Δ*PBLIPB* PARASITES HAVE REDUCED LEVELS OF LIPOYLATED PROTEINS IN THE APICOPLAST DURING LIVER-STAGE DEVELOPMENT

To determine whether the defect in late liver stages was correlated with a lack of lipoylated proteins, we performed sequential staining with antibodies to the apicoplast marker, ACP, and an antibody that detects lipoylated proteins. The results demonstrate that the control wild-type parasites had extensive staining with the anti-lipoic acid antibody throughout the apicoplasts, whereas the Δ*pbLipB* parasites had more non-lipoylated structures (Figure 2.12). This phenotype was witnessed in 10-30 parasites per strain per time point. This result supports our hypothesis that disruption of *lipB* reduces lipoylation in the apicoplast. Portions of the apicoplast remained

lipoylated in the knock-out parasite lines. This is possibly due to the presence of other lipoic synthase enzymes in the apicoplast [204].

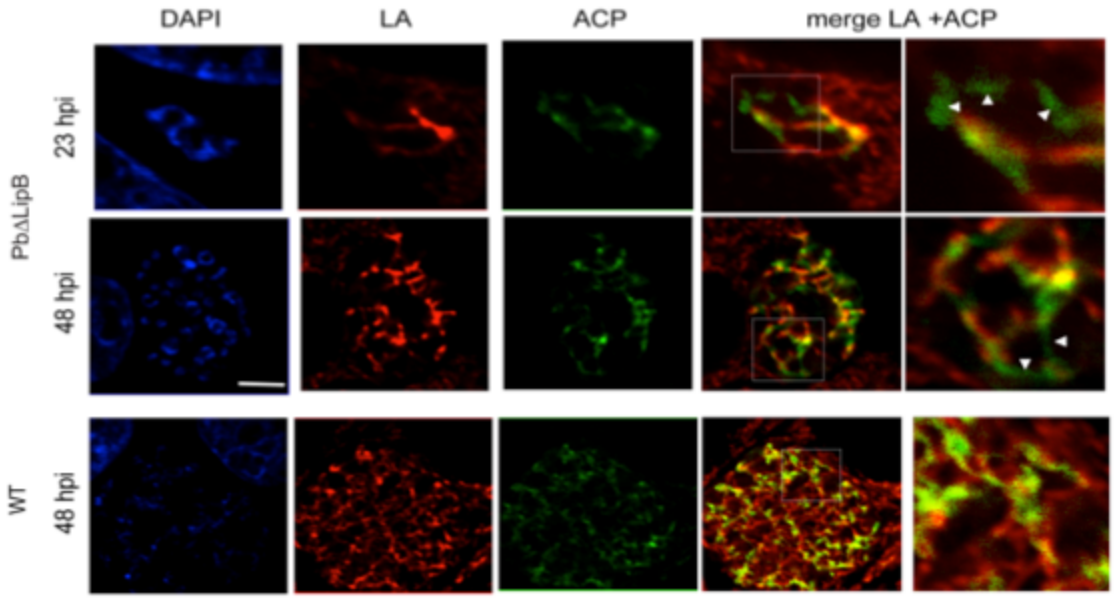


Figure 2.12. Δ*pbLipB* parasites have less lipoylated protein in the apicoplast parasites
ΔpbLipB sporozoites inoculated onto HepG2 cells were stained for lipoylated proteins (anti-LA) and apicoplast structure (anti-ACP) at 23 hpi and 48hpi. In comparison to wild-type parasites developing at 48 hpi of liver stage, *ΔpbLipB* parasites show less lipoylated structures in the apicoplast.

Δ*PBLIPB* PARASITES SHOW LESS BRANCHING OF THEIR APICOPLAST DURING LIVER-STAGES

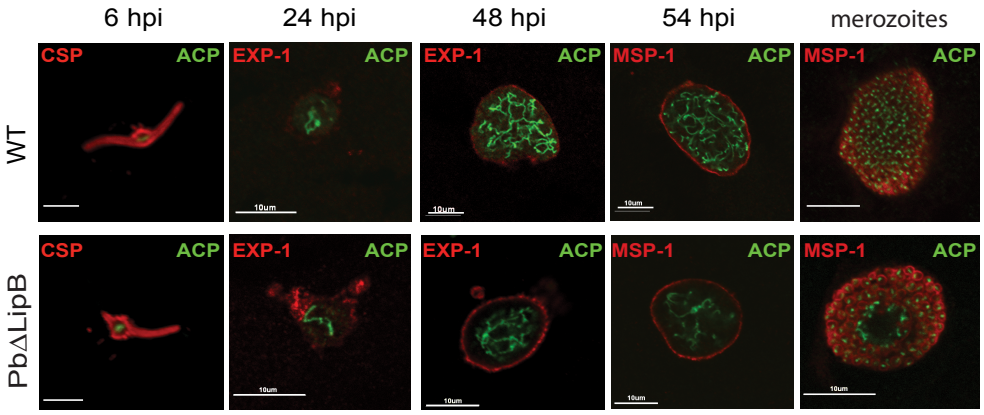
Given the reduced lipoylation in the apicoplast described above, we examine the overall morphology of apicoplast development during the liver stage. We observed appreciably fewer branched structures during later time points, i.e. 48 hpi and 54 hpi (Figure 2.13). Unlike our results for the morphology of the apicoplast during the blood stages (Figure 2.13), these results demonstrate that blocking lipoic acid synthesis impacted the morphology of the apicoplast during liver stage development. *ΔpbLipB* sporozoites inoculated onto HepG2 cells showed slightly less

branching of the apicoplast over time points of development, as detected with anti-ACP antibodies (green). Red channel is as follows: anti-CSP at 6 hpi, anti-Exp1 for 24 and 48 hpi, and anti-MSP1 at 54 hpi and merozoites (WT at 62 hpi, *ΔpbLipB* at 72 hpi).

To further examine this defect, we measured the size occupied by the ACP stained area by tracing the confines of the fluorescent images. This measurement is by the total parasite area to adjust for smaller growing parasites. This was measured by the total surface of the Exp1 staining. The data shows that in *ΔpbLipB* parasites, the apicoplast occupies a smaller total area of the parasite at the later time points, in comparison to the WT. However this analysis was not statistically significant.

Additionally, we counted the number of ACP-positive branch points reaching into the periphery of the developing parasite and contacting the MSP-1 labeled parasite plasma membrane (PPM) for 40 parasites per time point, divided equally between WT and KO cultures. WT parasites yielded 7.4 ± 5.8 and 13.1 ± 4.3 (mean ± SD) “contact points” between branches of the apicoplast and the PPM, at 48 and 54 hpi, respectively. In comparison, KO parasites had an average of 4.4 ± 3.6 and 3.9 ± 2.1 contact points at 48 and 54 hpi (the latter time point was significantly different between the WT and KO lines; $P<0.0001$ based on Mann-Whitney U test). These data suggesting impaired apicoplast development in the KO line as it progresses to the late liver stage.

A



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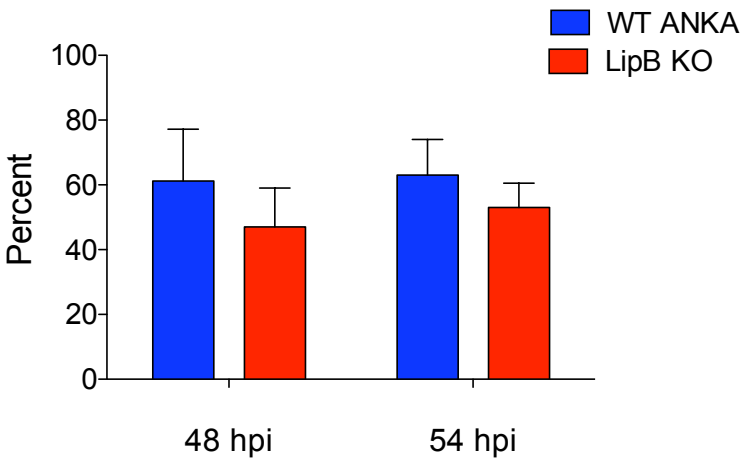


Figure 2.13. Δ*pbLipB* parasites have a less branched apicoplast in liver-stage
A) Morphology of the apicoplast during different time points of development by staining with anti-ACP antibodies. The top panel shows wild-type parasites with the corresponding markers to identify parasite structure at the stages of development. The bottom panel shows the knock-out parasites at the same time-points. **B)** Quantification of the branching of the apicoplast between the wild-type and knock-out parasites at 48 and 54 hours post-infection. Data is represented as a percentage of the area occupied by the anti-ACP fluorescence of the total parasite area (marked by the Exp-1 area of fluorescence).

8-BOA TREATMENT INHIBITS SCAVENGING OF EXOGENOUS LIPOIC ACID IN Δ*PBLIPB* PARASITES

In order to determine whether the scavenge pathway is active in our knockout cell lines, liver stage Δ*pbLipB* parasites were treated with 8-BOA (Figure 2.14). The developing parasites at 48 hpi were then stained with Mitotracker red was used to distinguish the mitochondria and the anti-Lipoic acid antibody, to find the lipoylated proteins.

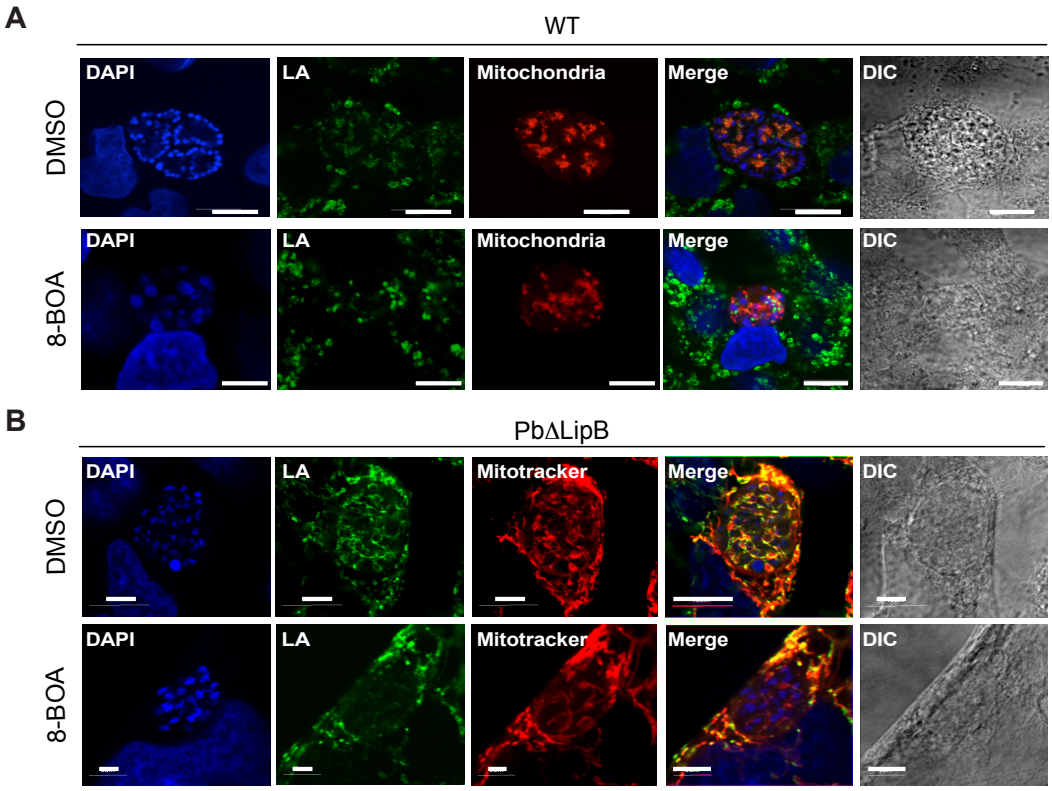


Figure 2.14. 8-BOA inhibits scavenging of lipoic acid species into the mitochondria
A) HepG2 cells infected with wild-type parasites at 48 hpi were stained with anti-LA to identify the lipoylated structures, in the presence of DMSO treatment as a control. **B)** *ΔpbLipB* parasites after 48 hours grown in the presence of 200 µM 8-BOA showed reduced levels of lipoylated protein in the developing parasites.

In comparison to wild-type ANKA parasites Δ*pbLipB* parasites treated with 8-BOA show less lipoylated protein in the mitochondria of developing parasites. These results confirm that the

scavenge pathway brings exogenous lipoic acid into the mitochondria of *P. berghei* liver stages for attachment to the dehydrogenases. Δ*pbLipB* parasites showed very few lipoylated structures outside of the mitochondria. Therefore after treatment with 8-BOA, the developing parasites showed almost no residual lipoylation during liver stage development.

In a similar set of experiments, we inhibited scavenging of lipoic acid during the asexual blood stage cycle with the 8-BOA inhibitor. In these experiments, *P. berghei* parasites were incubated overnight at different concentrations of 8-BOA and then counted for the number of nuclei per infected red blood cell (Figure 2.15). We wanted to determine whether 8-BOA would reduce the replication of *Plasmodium* parasites during an overnight incubation period. This experiment was repeated three times, counting between 100 - 200 infected red blood cells per parasite line.

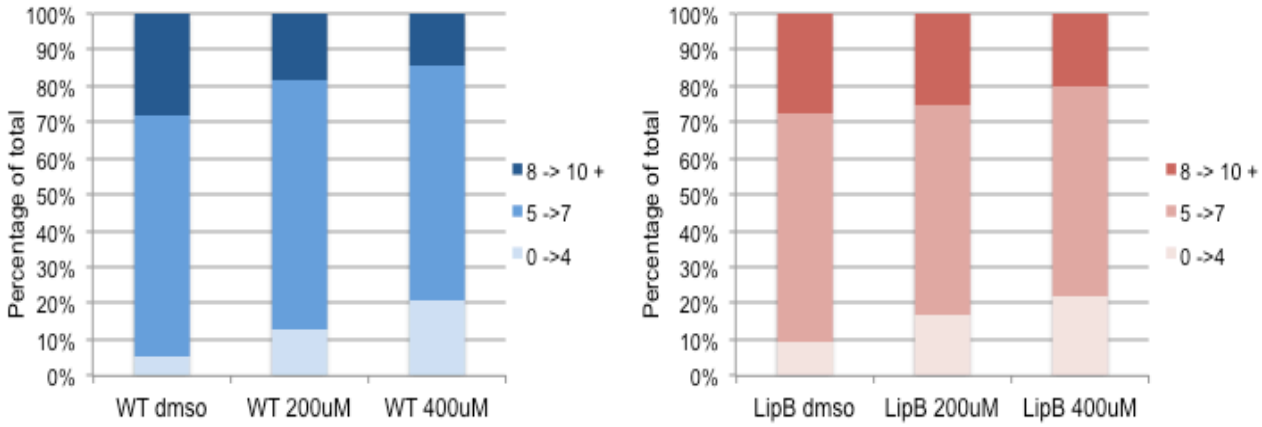


Figure 2.15. No significant different in schizont development between *ΔpbLipB* and wild-type blood-stage parasites treated with 8-BOA over-night
Parasites were counted for the number of nuclei per parasite, incubated with different concentrations of 8-BOA or DMSO control. In three experiments, equal numbers of infected red blood cells were incubated and counted by DAPI-staining for nuclei. Results demonstrate the percentage of total number of parasites counted (100-200 per experiment) as grouped by number of nuclei per RBC. Data represents the average of the percentages calculated in three experiments. There was a reduction in the amount of multi-nucleated, fully mature schizonts in culture, however no significant difference between the wild-type and the knock-out parasites.

We identified fewer fully formed schizonts after over-night development, in a dose dependent manner with 8-BOA treatment. This confirms that 8-BOA is a toxic inhibitor to parasite replication, as previously demonstrated in *P. falciparum* parasites [191]. However, there was no significant difference between the wild-type and the knock-out parasites.

In parallel, these 8-BOA treated parasites were injected back into naïve mice to determine the blood-stage growth kinetics. 8-BOA treatment increased the recovery time for both *P. berghei* ANKA and Δ*pbLipB* parasites in a dose-dependent manner. We observed a more significant impact of the 8-BOA treatment on the Δ*pbLipB* parasites than the wild-type ANKA cells (Figure 2.16). The Δ*pbLipB* parasites required a longer recovery time to reach the same levels of parasitemia as the untreated control. These results suggest that although we don't see a difference between the impact of 8-BOA between the wild-type and the knock-out after the overnight incubation period, there is a difference in their processes of lipoic acid attachment. By disrupting LipB gene, we have inhibited the way parasites are repopulating their lipoylated proteins. This implies that a degree of exchange between the scavenge and synthesis pathways within the asexual stages.

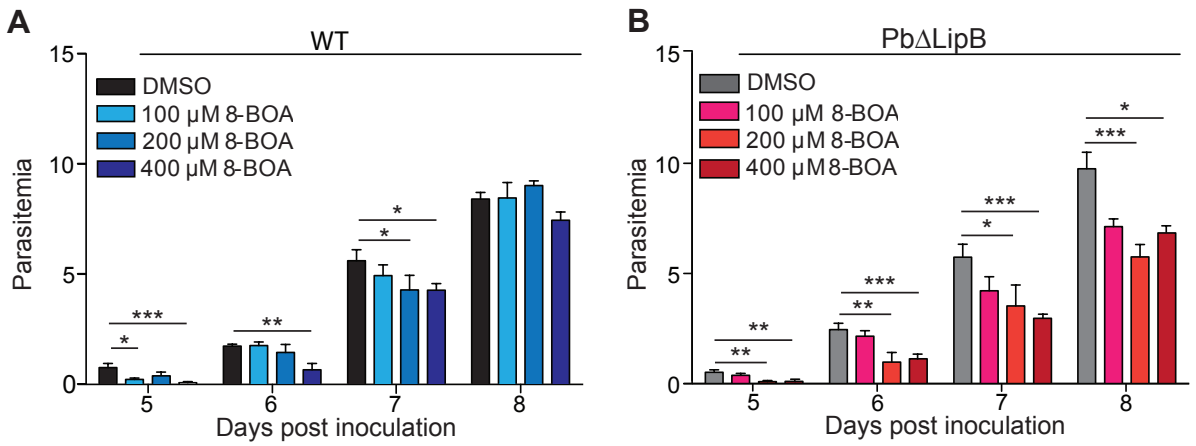


Figure 2.16. Blood-stage *ΔpbLipB* parasites are more sensitive to lipoic acid scavenge inhibitor 8-BOA
A) *P. berghei* wild-type or **B)** *ΔpbLipB* parasites were treated with concentrations of 100, 200 and 400 µM 8-BOA or DMSO control during overnight incubations. Resuspended cultures of 10,000 parasites were inoculated into naïve mice by intravenous injection. Parasitemia was tracked by thin smear microscopy. The 8-BOA treatments showed a dose-dependent effect and a stronger impact on the *ΔpbLipB* parasites than on wild-type control. Statistics were generated from a Mann-Whitney t-test for all 15 parasitemias from the 3 independent experiments.

DISCUSSION

Since lipoic acid is an essential cofactor used to covalently modify a number of enzymatic complexes, we decided to disrupt *pbLipB* to determine the essentiality of lipoic acid synthesis during the *Plasmodium* life-cycle. Our studies reveal that while lipoic acid synthesis is not required for mosquito stage development or blood-stage, it is critical for normal liver stage development.

Δ*pbLipB* parasites arrest during liver-stage development *in vitro* and have reduced infectivity *in vivo* as evidenced by a significantly extended pre-patent period. Additional experiments suggested that the developmental defect occurs late during liver stage growth. Δ*pbLipB* parasites in early liver-stage development have the same parasite size and absolute number as wild-type

controls. Δ*pbLipB* parasites had abnormal MSP-1 staining. This staining showed that Δ*pbLipB* parasites do not have normal development of the PPM. This membrane appeared to be unable to invaginate around each of the developing merozoites. This suggests that disruption of LipB had an impact on membrane biogenesis. Disruption of LipB leads to a reduced levels of lipoylation of PDH in the apicoplast and its main role in *Plasmodium* parasites is considered to be the generation of acyl-CoA for fatty acid biosynthesis. Perhaps Δ*pbLipB* parasites show abnormalities in the PPM because it is not synthesizing adequate amounts of fatty acids. Although we did not observe any detached cells, Δ*pbLipB* parasites were nonetheless capable of establishing a blood-stage infection *in vivo*, albeit at a reduced rate. These results indicate that synthesis of lipoic acid synthesis plays an important role during liver stages development although it is not absolutely required.

Liver stage Δ*pbLipB* parasites demonstrated reduced levels of lipoylated proteins in the apicoplast. In addition these parasites developed apicoplasts with an abnormal size and morphology. This phenotype is likely due to the lack of lipoylation of PDH within the apicoplast. A similar defect in the apicoplast morphology during liver-stage development was found when P. yoelii parasites were disrupted for the PDH-E1 and E3 subunits [205].

In an earlier study, *P. falciparum* Δ*pbLipB* lines demonstrated a residual amount of lipoylated PDH-E2 subunit and attributed it to the action of the LplA2 ligase that localizes to both the apicoplast and the mitochondria [202]. It is possible that the residual amount of lipoic acid on the apicoplast in our knock-outs is because of lipoylation by LplA2 in *P. berghei*. RT-PCR studies with *P. berghei* confirmed the expression of *pblplA2* during both blood and liver stages (data not

shown). The residual amount of lipoylation of PDH, due to LplA2, could also be the reason why we do not have a complete arrest during *in vivo* infections. Disruption of LplA2 would be an interesting follow-up experiment to determine whether it has an essential role during liver-stages.

While *pbLipB* was not essential for development during the blood-stages, we did find that it is required for normal levels of lipoylation of the PDH and BCDH proteins. There was no significant change in the growth rate of parasites and we also saw no impact on development of the apicoplast organelle. Our results are contrary to the phenotype found upon disruption of *lipB* in *P. falciparum*, in which knock-out parasites replicated faster than the control lines [202]. Lipoic acid can act as a mediator of histone hyperacetylation and cell cycle arrest. Changes in the levels of lipoylation of proteins within the mitochondria could impact the histone acetylation and *Plasmodium* has a unique TCA cycle in which acetyl-CoA generated in the mitochondria is exclusively used for histone modification [209].Lipoic acid can also inhibit histone deacetylase in other cell types [210].These other functions of lipoic acid, beyond its role as a post-translational modification, could explain the changes in growth kinetics of Δ*PfLipB* lines. However, we did not see any change in growth kinetics *in vivo* in Δ*pbLipB* parasites. Explanation for this difference could be the different types of environments used to measure blood-stage growth. Or else, it could mean that the lipoic acid synthesis pathway has a different function in *P. falciparum* parasites versus *P. berghei*. Authors of the study did not propose an explanation as to why their knock-out parasites replicated faster in *P. falciparum* than control lines. However, in both studies, disruption of LipB did not hinder parasite replication in blood-stage. Several phenotypes presented in this chapter were not studied in the Δ*PfLipB* study; i.e. morphology of the apicoplast during blood-stages and sensitivity to 8-BOA treatment.

Our results demonstrate that while *lipb, pdh* and *bcdh* are expressed during blood-stages in *Plasmodium berghei*, they are not critical for development and can be inactivated *in vivo* without an impact on pathogenesis. However, under conditions in which parasites were treated with the lipoic acid analog, 8-BOA that competitively inhibits lipoic acid scavenging, Δ*pbLipB* parasites required a longer time to reach the same parasitemias as wild-type. This suggests that lipoic acid synthesis might be needed for pathogenesis under lipoic acid limited conditions.

The central components of the FAS-II pathway (FabI, FabB/G, FabZ) and PDH are unessential for blood-stage but critical for liver stage development [105, 106, 205]. Δ*pbFabI* parasites, like Δ*pbLipB,* also failed to form detached cells during *in vitro* culture, however most of the late liver-stage parasites were not MSP1 positive [105]. Δ*pyPDH* parasites also lacked MSP-1 staining in the mature late liver-stages and had abnormal apicoplast morphology [205]. A unique feature of the phenotype of Δ*pbLipB* parasites was that it arrested later than other knock-outs related to lipid metabolism in liver stages. This is likely due to the residual amount of lipoylation of PDH, which could enable parasites to develop until very late time points with a limited amount of functional PDH proteins. Although we created a *P. berghei* line stably deleted for the lipB, it is not a complete knock-out in terms of PDH and BCDH protein function. Our western-blot data showed 16% of signal intensity for the PDH protein and 2% for BCDH protein in comparison to the wild-type parasite extracts. As mentioned above, BCDH protein becomes more visible at longer exposures. We have not completely abolished the enzymatic function of these complexes, but instead decreased the amount of functional protein per cell. This is one explanation for why Δ*pbLipB* parasites develop to later time points than other knock-outs in the same pathway.

Other explanations for the phenotype of Δ*pbLipB* parasites could be related to its functions outside of fatty acid generation. Lipoic acid is unique to other carbon chain fatty acid since it is known for its potent antioxidant properties. It is unknown whether the parasite would require more antioxidation during liver-stage of development, but it definitely an interesting avenue to consider.

A third explanation is whether the reduction of BCDH lipoylation is responsible for the arrest we see during liver stages. BCDH degrades branched chain amino acids, valine, leucine, isoleucine, to generate acetyl-CoA or succinyl-CoA, intermediates for the TCA cycle [199]. TCA-cycle intermediates have been detected in the blood stage of *Plasmodium* [209]. Since we have greatly reduced the amount of lipoylated BCDH, we can conclude that it is non-essential for blood stages. BCDH is overexpressed during liver-stage in comparison to blood-stage [104], however its role and a general understanding of the importance of the TCA-cycle during liver-stage remains to be clarified. *Plasmodium* liver-stage parasites replicate at an enormously abundant rate, which might require the energy generated from the TCA-cycle. However, we are not certain that BCDH is unlipoylated in Δ*pbLipB* liver-stage parasites since our western-blot data used parasite extracts from blood-stage parasites. In addition, the apicoplast and the mitochondria are not in close proximity during liver-stages of development like during blood-stage [133]. It is possible that LipB is only responsible for the lipoylation of BCDH during blood-stage replication.

Our data suggest that some amount of lipoic acid exchange is occurring between the synthesis and the salvage pathways located in the apicoplast and the mitochondria, respectively. Recently,

it has been shown that scavenging of lipoic acid is essential to complete liver-stage development in rodent *Plasmodium* species [191]. Treatment with 8-BOA did not completely arrest liver-stage development *in vitro*, since some parasites were capable of forming full merozoites [203]. Studies in *P. falciparum* blood stages showed no effect of 8-BOA treatment on lipoylation of apicoplast proteins [191]. However, it is possible that exchange is occurring in a small and unidirectional manner; such that lipoic acid generated in the apicoplast is exported into the mitochondria, explaining our result of reduced lipolyation of BCDH and increased sensitivity to 8-BOA. A close physical association between the mitochondria and the apicoplast has been demonstrated during the blood stages and these organelles are known to share some metabolic pathways such as heme biosynthesis [211]. However, the two organelles do not share such close proximity throughout the liver stages [133], suggesting less exchange of metabolites during this stage. The uptake of lipoic acid into human cells is an energy-dependent reaction, mediated by a sodium-dependent vitamin transporter [195] and 8-BOA is likely actively transported into *Plasmodium* [191]. However, the mechanisms of lipoic acid uptake into the parasite or the processes that could regulate its movement between the organelles remain to be determined.

The results from this study advance our understanding of the importance of lipoic acid metabolism throughout the parasite life cycle. As mentioned, Δ*pbLipB* parasites arrested even later than other related knock-outs [205, 105, 106]. This is an important finding since parasites that arrest late during liver stage development confer the strongest amount of protection to subsequent challenge [107]. *LipB* is a very viable candidate for the development of a whole attenuated sporozoite vaccine. Testing these resulting in *P. falciparum* knock-out parasites would be the next step in translating this research into real applications to treat malaria

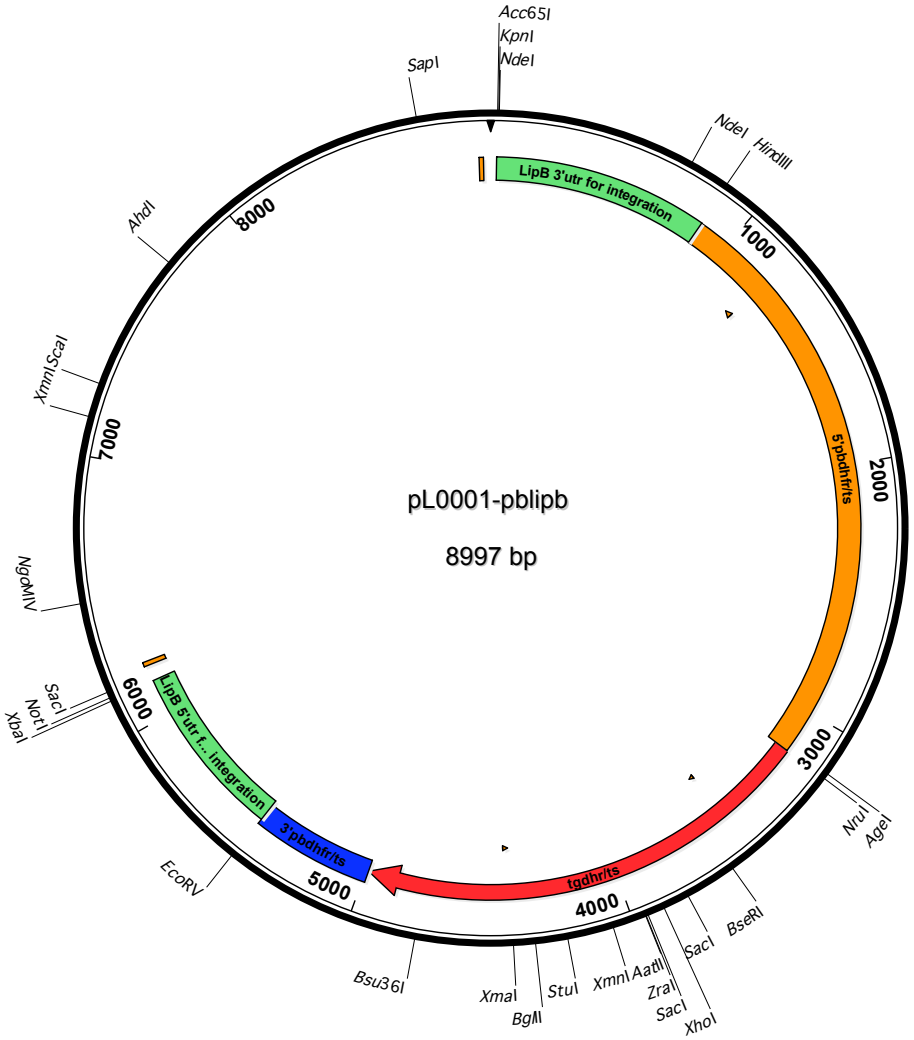


Figure 2.17. Map of pL001-plipb

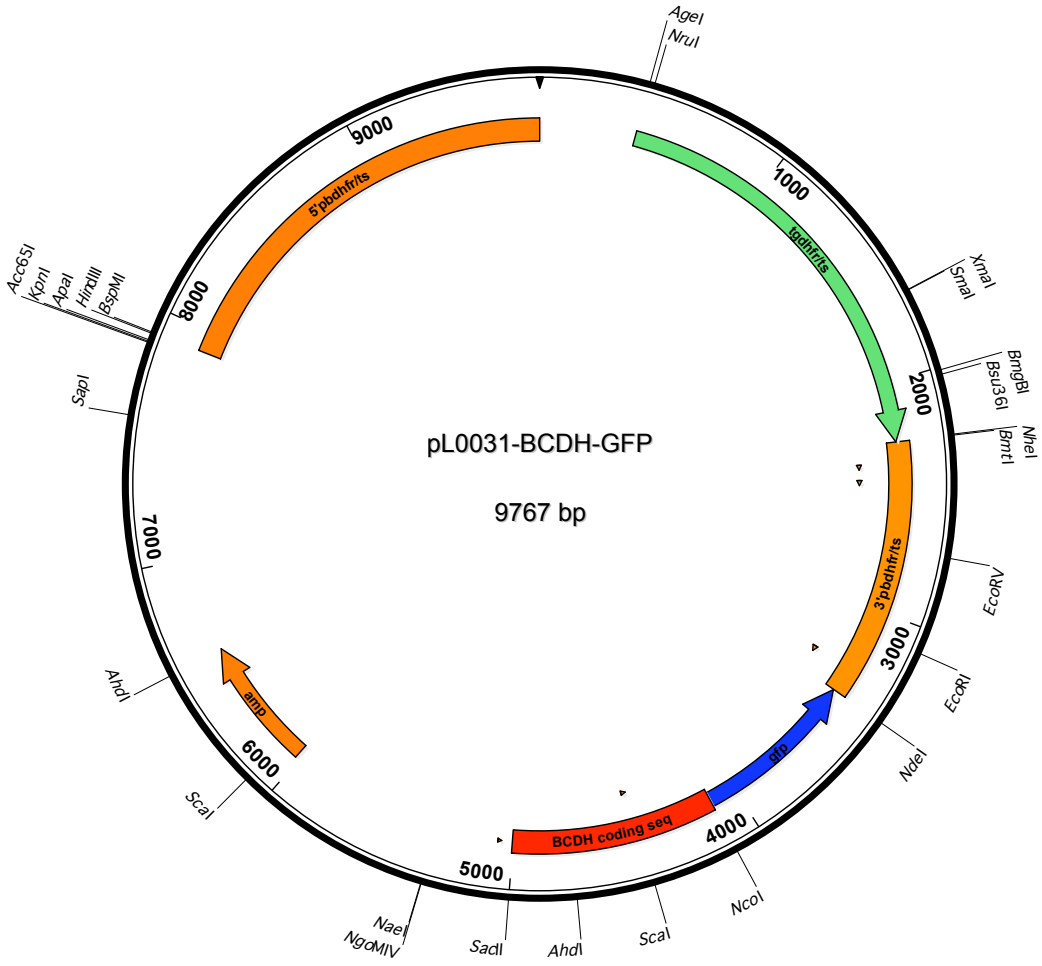


Figure 2.18. Map of pL0031-BCDH

3. THE ROLE OF HOST LIPIDS DURING PLASMODIUM PATHOGENESIS

ABSTRACT

Plasmodium parasites require the import of exogenous fatty acids for replication during the blood and liver-stages. The parasite uses these fatty acids for a number of functions, including membrane formation and metabolic co-factors. We wanted to assess how modifications to the fatty acid content the host would impact the replication of *Plasmodium* parasites. To understanding how *Plasmodium* parasites scavenge nutrients from their environment, we decided to examine their replication in mice that have decreased levels of circulating fatty acids in the blood-stream and fatty acid content in the liver. Using two methods to reduce the levels of circulating fatty acids in mice, we have monitored the replication rates of parasites *in vivo*. In the first, we have administered a fatty acid-lowering agent, clofibrate, which chemically stimulates beta-oxidation of fatty acid. In a second method, we used a genetic approach to block lipid synthesis in the host. ΔXBP1 mice are disrupted for a transcription factor that plays a key role in fatty acid synthesis in the liver. Both of these animal models demonstrated significant decreases in blood-stage growth kinetics of *P. berghei* ANKA parasites. We found an even greater impact on the blood-stage growth kinetics of *ΔpbLipB* parasites versus the wild-type control parasites. We examined the impact of reduced fatty acid content in the liver through the inoculation of *P. berghei* ANKA sporozoites and determination of the pre-patent period. There were modest increases in the pre-patent period in the fatty acid depleted conditions.

INTRODUCTION

Plasmodium requires a critical amount of fatty acid scavenging from the host to complete its life-cycle. During the blood stage, *Plasmodium falciparum* metabolizes a broad range of serum-derived fatty acids into the major lipid species of the parasite membrane and lipid bodies [177]. It has been demonstrated through *in vitro* experiments that the minimal serum components required for *Plasmodium falciparum* blood-stage growth are palmitic and oleic or steric acids. These serum components are metabolized into the major constituents of membranes and lipid bodies of the parasite; phosphatidylcholine, phosphatidylethanolamine, diacylglycerol and triacylglycerol [181]. The reliance on host lipids for parasite metabolism during intracellular growth is further supported by studies on parasites disrupted in genes for fatty acid synthesis. FabI, of the FAS-II pathway of fatty acid synthesis, is not-essential during blood-stages of *P. falciparum* [105]. The same result was found by disrupting *fab B/F* in *P. yoelii* [212]. These studies demonstrate that during blood stage replication *Plasmodium* derives all of its fatty acid needs, likely through scavenging from the host serum.

Conversely, in the liver stage, the *Plasmodium* parasite might meet the majority of its demands for fatty acids via *de novo* synthesis by the FAS-II pathway. Deletion of FabI decreases infectivity of the liver stages of *P. falciparum* and *P. berghei* [105]. The requirement for *Plasmodium* parasites to generate their own lipids during liver stage development is likely due to a greater need for fatty acids for membrane biogenesis in this life-cycle stage. The liver is also the key organ for fatty acids generated in the human host. In addition to its functions in detoxification of the blood-stream, protein synthesis, and production of chemicals necessary for digestion, the bulk of lipoproteins for the body are produced in the liver. Therefore, any

modifications in liver function could lead to abnormalities in the level of lipid species in the bloodstream [213].

Scavenging of lipid-species by *Plasmodium* parasites from the host during liver-stage development has not been well characterized. Only cholesterol and lipoic acid scavenging have been demonstrated during liver-stage infection. Cholesterol is scavenged from the host to enrich in the parasite PVM for rigidity and fluidity [138, 226]. Scavenged lipoic acid is essential in liver-stage for its attachment to multi-enzymatic complexes within the parasite mitochondria [203]. It has been hypothesized that parasites maximize their potential to scavenge host nutrient through association with hepatocyte organelles, such as the host endoplasmic reticulum (ER) [126]. A close proximity between the PVM and the host mitochondria has also been described, which might enable the uptake of other nutrient needs.

New insights into the metabolic processes of *Plasmodium* parasites were found by a transcriptional profiling study of *P. falciparum* parasites isolated from human patients [186]. Contrary to the transcriptome data set for *P. falciparum* replication during *in vitro* culture, samples from patients demonstrated three unique transcriptional states, suggesting that the parasite has the capacity to transcriptionally respond to varying host metabolic conditions in which it propagates. The first state, which represents active growth based on glycolytic metabolism, is similar to the transcriptional profile observed with *in vitro* cultures. The second and third states resemble a starvation response, in which the parasites metabolize alternative carbon sources to support growth. These latter stages are often indicative of an environmental stress response, which involves the induction of gene sets associated with glycolysis, amino-acid

metabolism and nitrogen metabolism and general growth processes such as nuclear transcription and cytoplasmic translation. The up-regulation of metabolic pathways during *P. falciparum* propagation *in vivo* implies that lipid metabolism of parasites is enhanced as required during low lipid conditions. Similarly, our findings from Chapter 2 demonstrated an increased sensitivity to the lipoic acid scavenge inhibitor, 8-BOA, in the Δ*pbLipB* parasites we generated. In order to follow-up this finding in a broader context, we set out to determine whether parasites would require lipoic acid or fatty acid synthesis during *in vivo* replication in a fatty acid depleted environment.

In our study, we have used two independent approaches to determine the role of host fatty acids during *Plasmodium* blood and liver stage development *in vivo.* In the first approach, we decreased host fatty-acid levels by stimulating beta-oxidation of lipid species with the drug, clofibrate. Fibrates have been demonstrated to lower fatty acids and triglycerides [214]. Fibrates function by stimulating the peroxisomal beta-oxidation pathway in the liver. Clofibrate acts by binding to Peroxisome Proliferator Activated Receptor-alpha (PPAR-α), which is predominantly expressed in the liver, kidney, heart and adipose tissue. Activation of the PPAR-alpha receptor, in hepatocytes leads to the proliferation of peroxisome organelles that are the site of beta-oxidation in the liver. The resulting increased beta oxidation causes decreases in the levels of circulating triglycerides and fatty acids and liver total lipid content [214]. PPAR-α is normally activated under nutrient-deficient conditions as an adaptive response to prolonged fasting. Beta-oxidation is the process of breaking down fatty acids to generate acetyl-CoA to enter the citric acid cycle. The three steps of the beta-oxidation pathway include, activation of fatty acids in the cytosol, transport of activated fatty acids into the mitochondria or peroxisomes and beta-

oxidation of the fatty acid. In peroxisome beta-oxidation, fatty acids oxidation ceases at octanoyl-CoA. Unlike beta-oxidation in the mitochondria, in the peroxisomes it is not coupled to ATP synthesis. It also requires other enzymes for beta-oxidation than the mitochondrial pathway; the carnitine acyltransferase enzyme (for the transport of the activated acyl group into the peroxisome), the acyl-CoA oxidase (the first step of oxidation) and the peroxisomal beta-ketothiolase (different substrate specificity than the mitochondrial beta-ketothiolase). Clofibrate administration does not decrease fatty acid synthesis, it reduces fatty acid levels in the serum and in the liver content. A previous study demonstrated that clofibrate-treated mice showed a reduced level of *P. berghei* blood-stage growth [215].

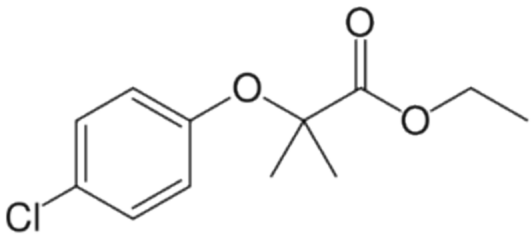


Figure 3.1. Chemical structure of clofibrate

In our second approach, we used the XBP1 liver-specific conditional knock-out mouse in order to decrease *de novo* fatty acid synthesis in the liver [216]. This transcription factor is ubiquitously found in adult tissues, with its highest expression in the fetal exocrine glands, osteoblasts and liver tissue. XBP1 is essential for hepatocyte differentiation as XBP1-deficient embryos die in utero[217]. In the liver, XBP1 expression is induced by glucose and it directly regulates the expression of critical genes involved in fatty acid synthesis [216]. XBP1 is activated by inositol requiring enzyme 1 (IRE1), a proximal sensor of ER stress, which splices the XBP1 mRNA into a functional transcript [218]. The splicing by IRE-1 removes 26 bp from XBP1 mRNA and the spliced 267 amino acid XBP1 protein becomes a transcriptional activator once it translocates into the nucleus. XBP1 is a key factor in the unfolded protein response (UPR) in the endoplasmic

reticulum (ER) [219]. XBP1 also regulates genes involved in ER-associated degradation (ERAD), redox metabolism, autophagy, vesicular trafficking, glycosylation and protein entry into the ER. The UPR ensures correct folding, processing, export and degradation of proteins that emerge from the ER during both stressed and normal conditions.

Fatty acid biogenesis occurs in the ER within mammalian cells. XBP1's role in controlling fatty acid biosynthesis is relates back to the cell's need to regulate activity within this organelle and avoiding ER stress. Similarly, XBP1 is also known to be important for the development of B cells [219]. XBP1 expression is critical during B lineage development from pro-B to plasma cells, where its high transcript level is found in plasma cells. XBP1 plays a critical role as a regulator of the ER stress response, particularly in B cells during high levels of protein synthesis that are required for plasma cells to secrete anti-bodies

Deficiency of XBP1 in the liver, using a conditional knock-out approach, leads to a significant decrease in serum triglycerides, cholesterol and free fatty acids while retaining normal liver lipid content in the liver [216]. XBP1 exon 2 is floxed on either side and an inducible Mx1-CRE is introduced on a separate chromosome. Poly-IC injections stimulate CRE expression and create a stable knock-out in homozygous-floxed mice. We used this conditional knock-out mouse to test the growth of *Plasmodium* parasites.

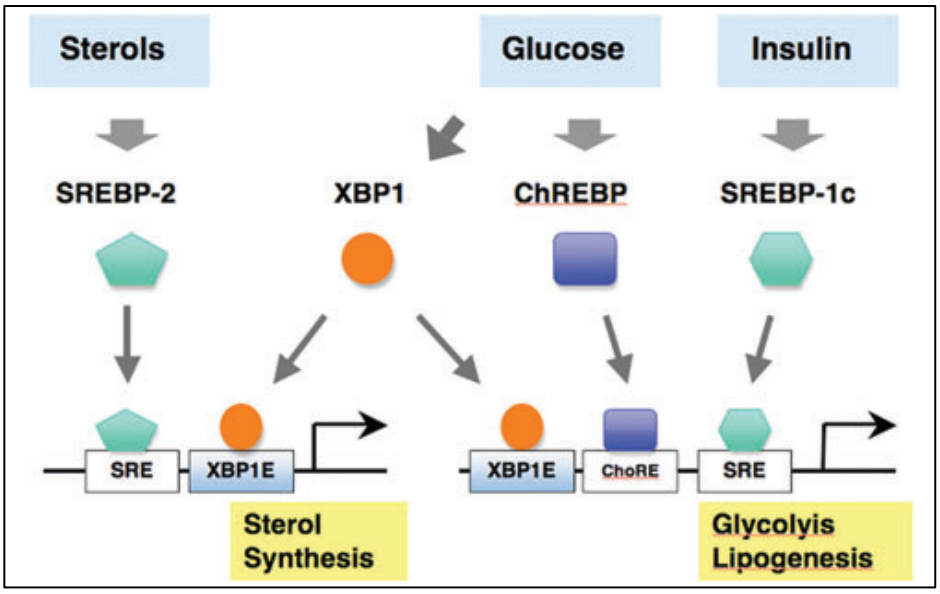


Figure 3.2. The role of XBP1 during hepatic lipogenesis

The XBP1 transcription factor has a unique role for fatty acid metabolism by promoting the expression of both sterol and glycolysis lipogenesis in the liver. Glucose stimulates the processing of the XBP1 mRNA. Schematic is reproduced from [219].

In this study, we have used these two independent models to study the impact of a reduction in lipids on the pathogenesis of malaria. We have found that blood stage parasites when inoculated into clofibrate-treated mice or XBP1 KO mice had decreased growth kinetics. Both models demonstrated modest changes during liver stage infection.

METHODS

ANIMAL USE AND CARE

XBP1Δ mice were generated as previously described [216]. The mice were generated on the C57/BL6 background at Harvard University, Cambridge Massachusetts. Mice were imported into Columbia University facilities and cared for according to University standards under a protocol approved from the Institute for Animal Care and Use Committee. Mice over 5 weeks

old were injected with 250 µg of poly(I:C) (Amhersham) every two days, for a total of three times, to induce CRE recombinase-mediated deletion of exon 2. This deletion results in a frameshift of the open reading frame, producing a truncated and non-functional product. Primers specific to exon 2 were used for PCR confirmation of excision of exon 2 post-poly I:C injections (Figure 3.8).

CLOFIBRATE DOSAGES IN MICE

Female Swiss-Webster mice of 6 weeks of age were purchased from Taconic and housed in Columbia University mouse facilities. Groups of five mice for each independent experiment were injected with dosages of 0.5 mg/kg or 5.0 mg/kg of clofibrate resuspended in DMSO or a control volume of DMSO by intraperitoneal injection for 9 days. Volume injected was < 0.1ml per mouse of <10% DMSO to avoid toxicity. We confirmed the reduction of levels of circulating lipids five and ten days after the completion of treatment (also represented as day 0 and day 5 of parasite inoculation). WAKO enzymatic kits were used to determine the levels of non-esterified fatty acids (NEFA) and triglycerides (TG) in treated and untreated mice. The lipid levels are represented as the average percent reduction of lipid levels for treated versus untreated control mice, for three independent experiments.

PARASITE PROPAGATION

Plasmodium berghei ANKA, *ΔpbLipB* and *ΔpbFabI* parasites were inoculated into mice from cryopreserved stocks. Parasites were diluted to inoculums of 10,000 or 1,000 parasites per 0.1ml of media. Clofibrate treated mice with infected with parasites 5 days after the completion of drug treatment. Blood-stage parasites were inoculated into mice by intravenous (IV) tail vein

injection. Sporozoites were obtained from mosquitoes (reared at NYU Medical Center) dissected on day 18 - 20 post-feeding and injected by IV into mice at inoculums of 10,000 or 1,000. Parasite growth in mice was determined by thin-smear microscopy of mouse blood.

ALBUMIN DETECTION IN MOUSE PLASMA

Groups of five mice were tested for their circulating levels of albumin in the plasma. Approximately 30-50υl of blood was drawn with an anti-coagulant every other day from the tail-vein during a 9-day clofibrate treatment. Blood was diluted 1:30,000, according to the protocol of the AbCAM Mouse Albumin ELISA kit. The clorometric read-out values generated from the ELISA were converted into gram per deciliter (g/dL) with the slope generated by the standard curve processed on the same day. Data is represented as the average value of five mice and error bars represent the standard deviation of all values.

Name	Nucleotide Sequence	Description
P2656	ACTTGCACCAACACTTGCCATTTC	F flox primer
P2657	CAAGGTGGTTCACTGCCTGTAATG	R flox primer
P2658	GCGGTCTGGCAGTAAAAACTATC	F cre primer
P2659	GTGAAACAGCATTGCTGTCACTT	R cre primer
P2660	CTAGGCCACAGAATTGAAAGATCT	F IL-2 control primer
P2661	GTAGGTGGAAATTCTAGCATCATCC	R IL-2 control primer
deltaXBP-F	AGAAAGCGCTGCGGAGAAC	F primer to verify excision of exon 2 in XBP1 KO mice
deltaXBP-R	GGAACCTCGTCAGGATCCAG	R primer to verify excision of exon 2 in XBP1 KO mice
XBP1WT205-F	CCTGAGCCCGGAGGAGAA	F primer that only detects the WT non-excised XBP1 locus
XBP1WT272-R	CTCGAGCAGTCTGCGCTG	R primer that only detects the WT non-excised XBP1 locus

Table 3.1. Primers used in Chapter 3

STATISTICAL ANALYSIS

All stats analyses were performed using the Prism 4 software. Analysis of the significant difference between the clofibrate treated-mice used the Mann-Whitney t-test program to generate significance.

RESULTS

BLOOD-STAGE PARASITE REPLICATION RATES ARE REDUCED IN CLOFIBRATE-TREATED MICE

A previous study showed that clofibrate treatment decreased *Plasmodium* blood stage growth. In this study, we inoculated parasites into the dosed mice after completion of the treatments and did not continue dosing during the stages of infection. The impact of clofibrate treatment on host lipids mice was confirmed through the use of WAKO enzymatic kit for the values of NEFA and TG in the serum. Values from both days 0 and 5 post-parasite inoculation of treated mice show a reduction in the serum lipid levels (Figure 3.3). The absolute values of the nonesterified fatty acid and triglycerides for the 3 independent clofibrate experiments are also shown in Table 3.2. In three independent experiments, mice were treated with clofibrate over the course of 9 days. In all three experiments, the NEFA values were detected by WAKO enzymatic kit and represented as the average mEq/L of the 5 mice per treatment group. In two experiments, the TG values were detected, also by WAKO kit and shown above as the average mEq/L. Values were determined on 5 and 10 days after the completion of clofibrate treatment.

Experiment Group	Lipid Species	Day Post Treatment	Average Value (mEq/L)	SEM	Percent Reduction
I	Non-esterified Fatty Acids				
Untreated		5	0.7024	0.198	
0.5mg/kg		5	0.505	0.074	28%
Untreated		10	0.996	0.142	
0.5mg/kg		10	0.427	0.329	57%
II	Non-esterified Fatty Acids				
Untreated		5	0.164	0.085	
0.5 mg/kg		5	0.130	0.039	21%
5.0 mg/kg		5	0.102	0.039	22%
Untreated		10	0.338	0.158	
0.5mg/kg		10	0.220	0.037	35%
5.0 mg/kg		10	0.112	0.086	66%
II	Triglycerides				
Untreated		5	0.267	0.145	
0.5 mg/kg		5	0.206	0.062	23%
5.0 mg/kg		5	0.204	0.062	24%
Untreated		10	0.234	0.068	
0.5mg/kg		10	0.205	0.086	12.5%
5.0 mg/kg		10	0.204	0.081	13%
III	Non-esterified Fatty Acids				
Untreated		5	0.148	0.060	
0.5 mg/kg		5	0.125	0.007	15.5%
5.0 mg/kg		5	0.121	0.002	18.3%
Untreated		10	0.156	0.060	
0.5mg/kg		10	0.105	0.108	33%
5.0 mg/kg		10	0.072	0.011	54%
III	Triglycerides				
Untreated		5	0.132	0.019	
0.5 mg/kg		5	0.110	0.017	16.5%
5.0 mg/kg		5	0.090	0.008	31%
Untreated		10	0.491	0.088	
0.5mg/kg		10	0.449	0.086	9%
5.0 mg/kg		10	0.436	0.076	12%

Table 3.2. Values of Non-esterified Fatty Acids and Triglycerides in clofibrate treated mice

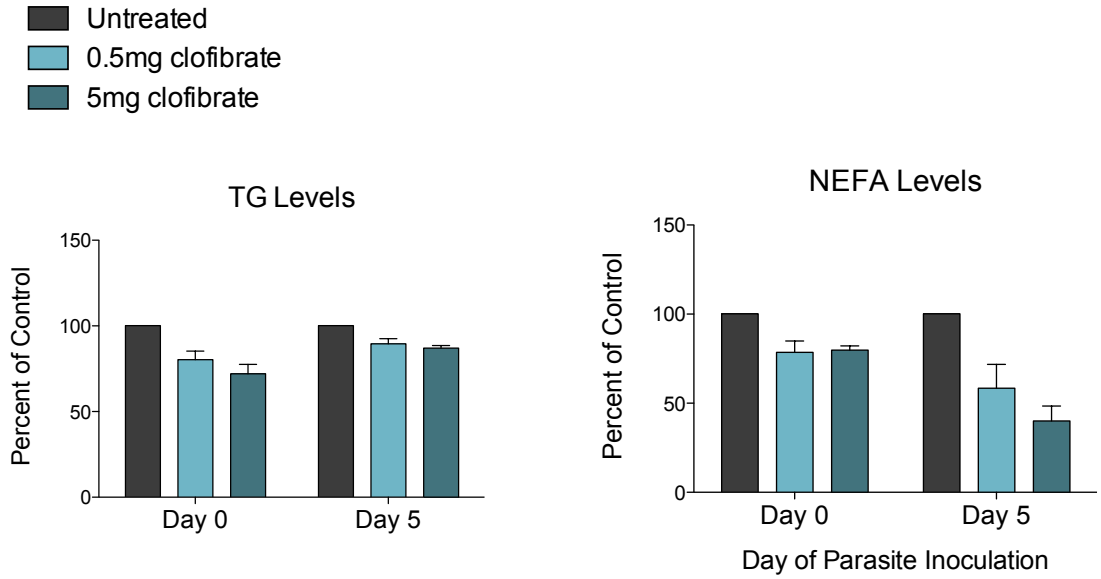


Figure 3.3. Triglyceride (TG) and non-esterified fatty acid (NEFA) in the serum in clofibrate-treated mice as a percentage of control
The data represent the average of the percentage of three experiments when TG and NEFA levels were measured on days 0 and 5 post infection with parasites. For each experiment, 5 mice from each group were measured. The percent reduction represents the average of the treated groups divided by the average of the untreated groups, subtracted from 100. Error bars represent standard error of the mean.

To investigate the impact of reducing the lipid levels on malaria blood-stage growth, *P. berghei* wild-type, *Δpblipb* and *ΔpbFabI*, parasites were inoculated into clofibrate-treated mice 5 days after the completion of the clofibrate dosing (i.e. Day 0 as annotated in Figures 3.4, 3.5 and 3.6). All inoculations were performed at 10,000 parasites per mouse and comparison of the blood-stage growth kinetics of the different parasite strains was completed in parallel. Parasitemias were tracked over the course of 10 days or until parasites reached lethal levels of parasitemia; i.e. mice were sacrificed as they demonstrated severe illness due to the parasite infections. Infections are always lethal, therefore in our experiments no mice ever recovered from infection after

viewing detectable levels of parasitemia. We found that clofibrate treatment dramatically reduced the rate of blood-stage growth kinetics for all parasite lines (Figures 3.4 – 3.6).

Additionally, we also observed that a decrease in the rate of blood-stage development correlated with the decrease in lipid levels. Mice treated with a ten-fold higher dosage of clofibrate treatment showed greater impact on blood-stage replication for all parasite lines. In addition to reducing the rate of blood-stage replication, clofibrate treatment was found to have an even greater impact on the *Δpblipb* parasite rate of replication in comparison to wild-type or the *ΔpbFabI* parasites (Figure 3.5). These results show that changing the lipid levels of the host, by as little as 20%, can have a significant impact on the pathogenesis of *Plasmodium* parasites.

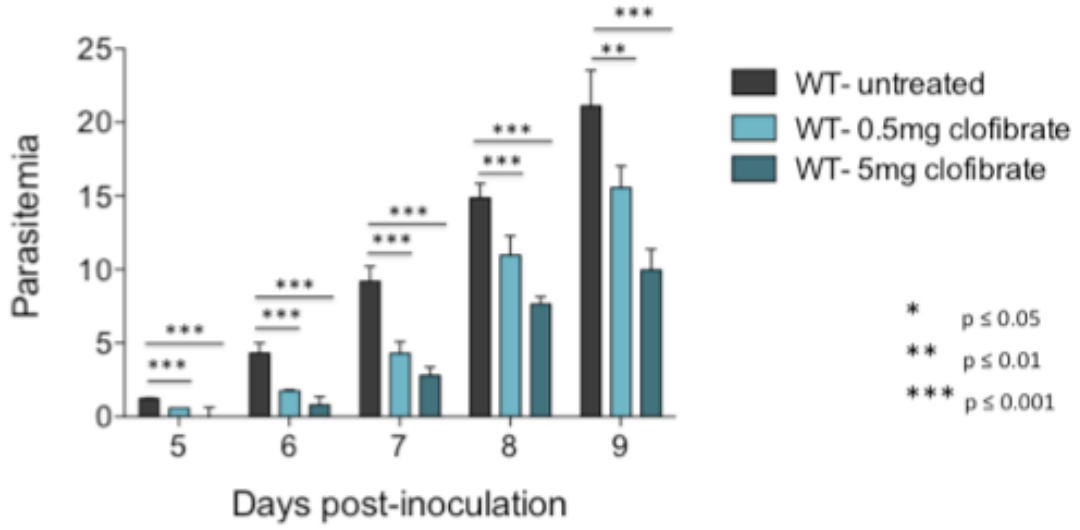


Figure 3.4. Kinetics of blood-stage growth of wild-type parasites in clofibrate-treated mice Data represent the average parasitemias tracked for three independent experiments: 3 experiments with the dosages of 0.5 mg/kg and 2 experiments with the dosages of 5.0 mg/kg of clofibrate. Each independent experiment contained five mice per group. Error bars represent standard error of the mean. Significance was determined using a Mann-Whitney t-test.

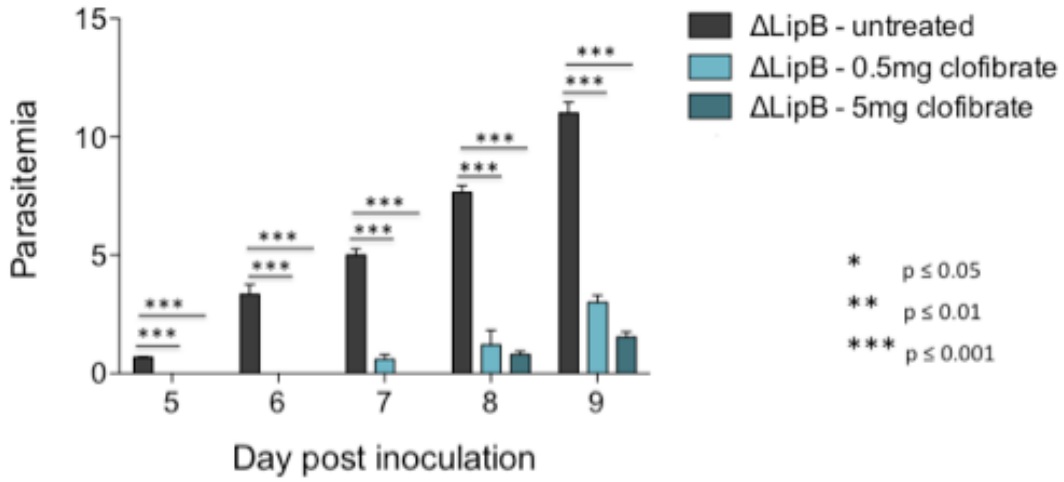


Figure 3.5. Kinetics of blood-stage growth for *Δpblipb* parasites in clofibrate-treated mice
Data represent the average parasitemias tracked for three independent experiments: 3 with 0.5 mg/kg dosage and 2 with 5.0 mg/kg dosage of clofibrate. Each independent experiment contained five mice per group. Significance was determined using a Mann-Whitney t-test.

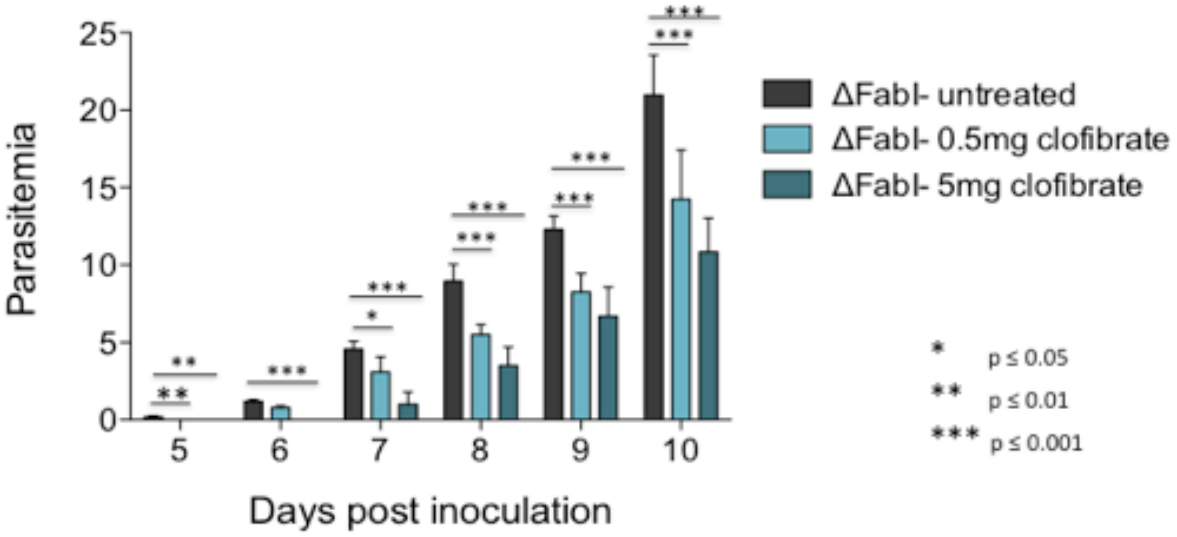


Figure 3.6. Kinetics of blood-stage growth for *ΔpbFabI* parasites in clofibrate-treated mice
Data represents the average parasitemias tracked for two independent experiments; both with the dosages of 0.5 mg/kg and 5.0 mg/kg. Each independent experiment contained five mice per group. Error bars represent standard error of the mean. Significance was determined using a Mann-Whitney t-test for the 15 mice, data gathered from 3 separate experiments.

SPOROZOITE INFECTIONS OF CLOFIBRATE-TREATED MICE SHOW AN INCREASED PRE-PATENT PERIOD

To determine whether host liver lipid levels impact liver-stage development of *P. berghei,* mice that were dosed with 0.5 mg/kg or 5.0 mg/kg clofibrate were infected with sporozoites. In two independent experiments, the pre-patent period was determined by thin-smear microscopy and is defined as the number of days it takes after sporozoite inoculation to a detectable blood-stage infection. This experiment revealed an increase in the average pre-patent period for the clofibrate-treated mice in comparison to the control, ranging from 0.5 days to 2.0 days (Table 3.3).

Experiment	Clofibrate Dose	Sporozoites Inoculated	Percent of Infected mice	Average Prepatent Period
I	Control	1,000	100%	4.0 days
	0.5 mg/ kg	1,000	66%	4.0 days
	5.0 mg/ kg	1,000	100%	4.5 days
II	Control	1,000	100%	4.0 days
	0.5 mg/ kg	1,000	100%	4.0 days
	5.0 mg/ kg	1,000	100%	4.5 days
III	Control	10,000	100%	4.0 days
	0.5 mg/ kg	10,000	100%	4.0 days
	5.0 mg/ kg	10,000	100%	4.5 days
IV	Control	10,000	100%	4.0 days
	0.5 mg/ kg	10,000	100%	4.0 days
	5.0 mg/ kg	10,000	100%	4.5 days

Table 3.3. Pre-patent period of *P. berghei* ANKA sporozoites in clofibrate-treated mice
Mice dosed with clofibrate were inoculated with 1,000 or 10,000 *P. berghei* ANKA wild-type sporozoites, administered intravenously. Detection of blood-stage parasites and determination of the pre-patent period was conducted by daily thin-smear microscopy. The lower lipid of detecting microscopy is 0.05%.

A greater difference in the pre-patent period was seen in mice treated with higher doses of clofibrate-treatment. While the phenotype we are seeing is subtle, the pre-patent period is longer at the clofibrate dose of 5.0 mg/kg, implying a dose-response.

CLOFIBRATE TREATMENTS REDUCE THE AMOUNT OF CIRCULATING ALBUMIN IN MICE

Although there is no published information relating clofibrate treatment to a physical reduction in circulating albumin levels, we were interested in determining whether albumin levels changed over the course of clofibrate treatments. We found that clofibrate treated mice had reproducibly lower levels of albumin in comparison to the untreated controls. Although there was a high degree in variability between the groups of the untreated control mice and the treated mice, we found reproducibly lower levels versus the untreated control mice.

Treatment Group	Lipid Species	Days of Treatment	Average Value (g/dL)	SEM	Percent Reduction
	Albumin				
Untreated		2	5.50	2.40	
5.0 mg/kg		2	3.20	0.78	26%
Untreated		4	5.30	2.50	
5.0 mg/kg		4	2.59	1.57	47%
Untreated		6	4.98	23.6	
5.0 mg/kg		6	2.50	1.20	50%
Untreated		8	4.80	3.30	
5.0 mg/kg		8	3.50	2.80	27%

Table 3.4. Values of albumin content in mouse plasma during clofibrate treatment

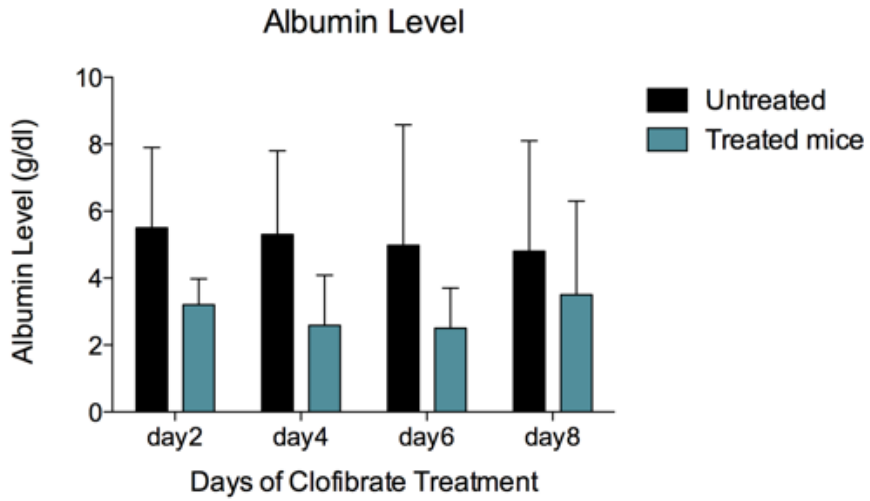


Figure 3.7. Albumin levels in mouse plasma during clofibrate treatment
Albumin was tested by ELISA-Abcam enzymatic kit. Data represents the average of 5 mice tested over the course of one clofibrate treatment regimen.

WILD-TYPE *P. BERGHEI* PARASITES HAVE A SLOWER RATE OF BLOOD-STAGE REPLICATION IN XBP1 KNOCK-OUT MICE

XBP1 KO mice have decreased levels of circulating lipids. We assessed a decrease in the levels of circulating lipids in the XBP1 KO mice. Similarly to the clofibrate-treated mice, we confirmed a reduction in the non-esterified fatty acid (NEFA) and triglyceride (TG) levels for the XBP1-KO mice after poly-IC mediated excision of exon 2 (Figures 3.8 and 3.9). The data is presented in the same manner as Figure 3.3; as a percent reduction of lipid levels measured as compared values to the control mice. The data is the average from two experiments with 4-6 mice per group. We saw between a 10-50% reduction in the levels of NEFA and between a 20-80% reduction in the levels of TG for XBP1 KO mice. These values are similar to the levels of lipid reduction in the serum published for the KO mice [216].

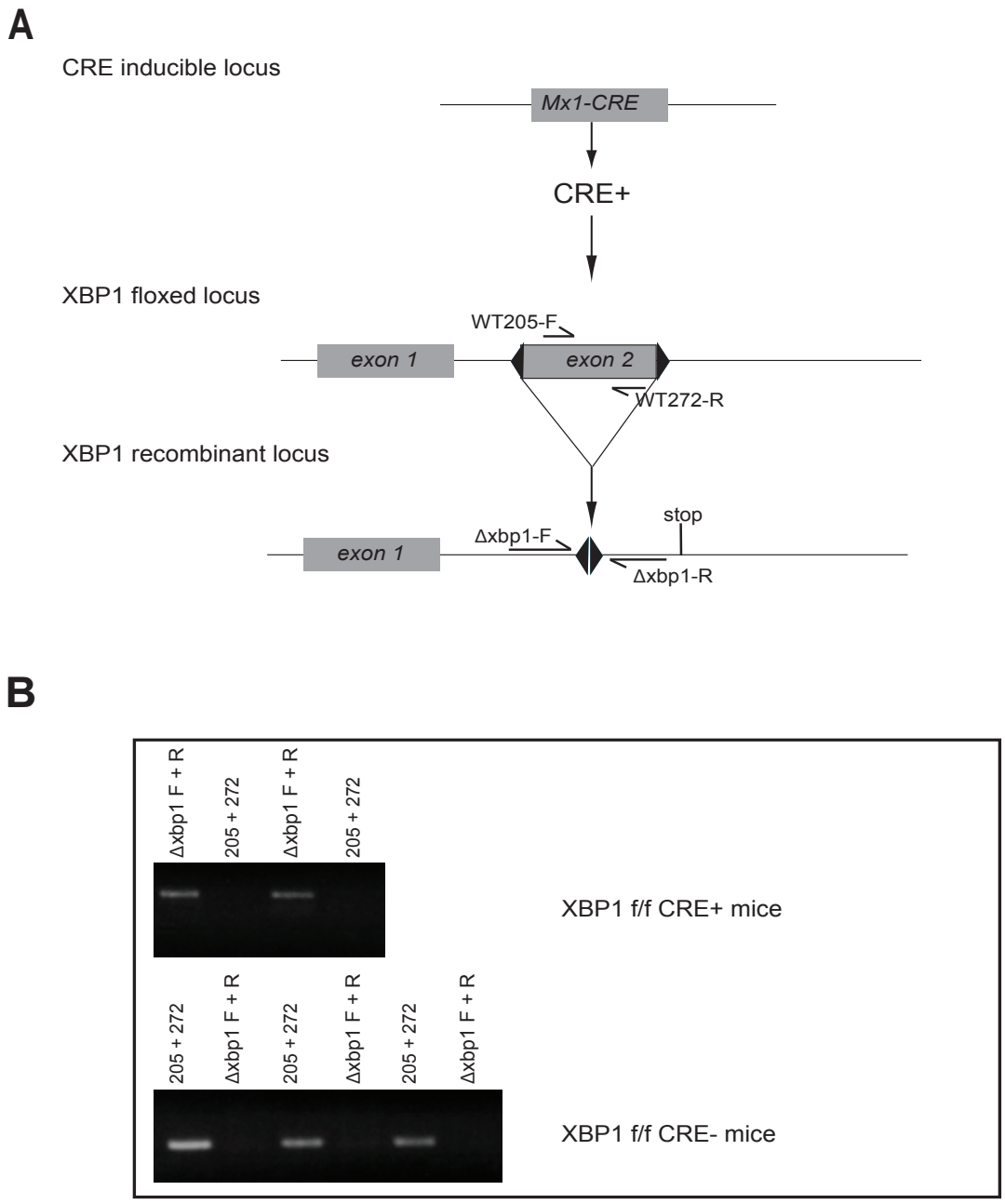
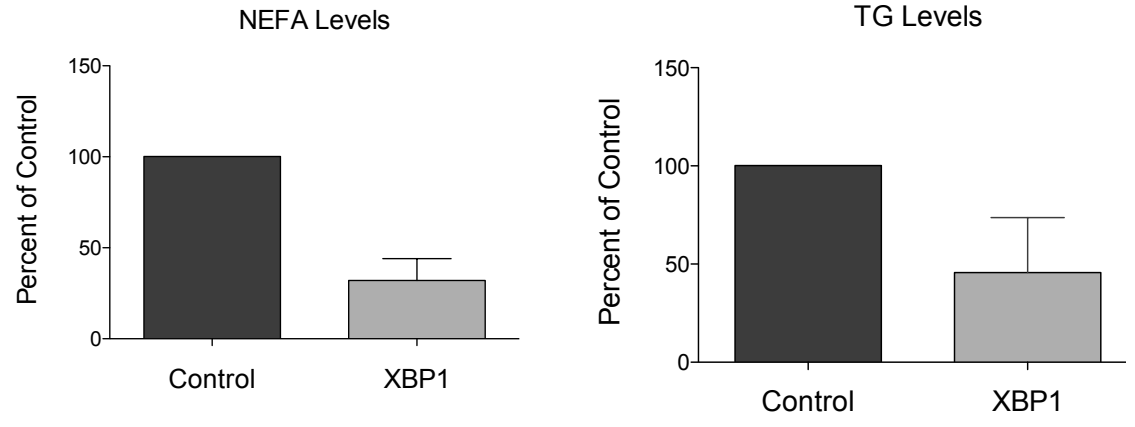


Figure 3.8. Confirmation of XBP1 exon 2 excision by RT-PCR
Excision of XBP1 was determined by locus specific primers. Data represents 5 mice from experiment I in which cDNA was generated from homogenized liver tissue after the experiment was completed. The top panel shows 2 mice with expression of the longer delta-XBP1 product (deltaXBP-F + deltaXBP-R, product 120 bp) and is absent for the shorter WT product. The bottom panel shows 3 XBP1 CRE- control mice were and show only detectable levels of expression of the WT-control gene (primers XBP1WT205 F + XBP1WT272, product 70bp) and absence of the delta-XBP1 product. These results confirm proper excision of the XBP1 locus after induction by poly-IC injection.

Experiment Group	Lipid Species	Average Value (mEq/L)	SEM	Percent Reduction
I	Non-esterified Fatty Acids			
Control (Cre-)		0.462	0.029	
ΔXBP1 (Cre+)		0.641	0.042	28%
I	Triglycerides			
Control (Cre-)		0.135	0.014	
ΔXBP1 (Cre+)		0.092	0.011	32%
II	Non-esterified Fatty Acids			
Control (Cre-)		0.188	0.050	
ΔXBP1 (Cre+)		0.094	0.042	50%
II	Triglycerides			
Control (Cre-)		0.280	0.019	
ΔXBP1 (Cre+)		0.071	0.034	75%

Table 3.5. XBP1 KO mice have lowered lipid levels as compared to littermate controls
The absolute values of the non-esterified fatty acids (NEFA) and triglycerides (TG) as determined in the serum for CRE positive and CRE negative control mice. Lipids were determined 2-3 weeks after poly-IC injections to induce excision. Serum lipids levels were determined for all mice per group for each experiment.



. Representation of the NEFA and TG values of XBP1 KO mice
Graphs represent the average value of the two experiments, as a percentage of the control mice. Error bars represent the standard error of the mean.

In order to assess *Plasmodium* blood stage growth kinetics in XBP1 KO mice, we inoculated these mice wild-type blood-stage parasites 2 to 3 weeks after the final poly-IC injection. We found a greater than two-fold reduction in the blood-stage growth kinetics in the KO mice as compared to wild type control (Figure 3.10). These findings confirm our results with the clofibrate treated mice, that blood-stage *P. berghei* parasites are sensitive to changes in the fatty acid content of the mouse serum and will replicate with slower blood-stage growth kinetics..

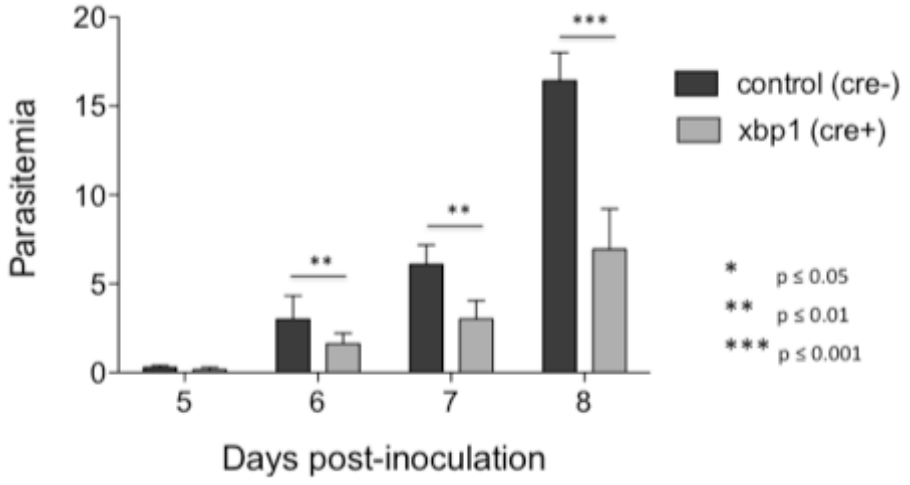


Figure 3.10. Blood-stage growth kinetics of *P. berghei ANKA in XBP1 KO mice*
In two experiments, 6-8 mice per group were inoculated with 10,000 blood-stage parasites by I.V. injection into XBP1 flox/flox CRE positive mice and control CRE negative mice. Blood-stage parasitemia was tracked by thin-smear microscopy over the course of 8 days. Statistics were generated by Mann-Whitney t-test of all parasitemic values generated in the two experiments.

SPOROZOITE INFECTIONS IN XBP1 KNOCK-OUT MICE DO NOT HAVE SIGNIFICANTLY INCREASED PRE-PATENT PERIODS

In two independent experiments, XBP1 KO mice were inoculated with 1,000 sporozoites and the pre-patent period was determined by thin-smear microscopy. Mice used were litter-mates (age-matched) and were also the same sex. In comparison to control mice there was a modest increase (0.25 days) in the number of days for the parasites to complete liver-stage development in XBP1 KO mice (Table 3.6). Our results demonstrate that XBP1 KO has no significant impact on liver stage development, illustrating that in these mice the quantities of lipids for parasite proliferation is not limiting.

Experiment	Clofibrate	Sporozoites Inoculated	Percent of Infected mice	Average Prepatent Period
I	Control (Cre-)	1,000	100%	5.2 days
	ΔXBP1 (Cre+)	1,000	100%	4.8 days
II	Control (Cre-)	1,000	100%	5.0 days
	ΔXBP1 (Cre+)	1,000	100%	5.2 days

Table 3.6. Pre-patent period of *P. berghei* ANKA sporozoites in XBP1 KO mice

DISCUSSION

Lipid scavenging from the host is critical for pathogenesis of *Plasmodium falciparum.* In our studies, we utilized two independent animal models enabled us to study how changes in serum and liver lipids impact malaria progression. In one model we treated mice with clofibrate, which reduces the fatty acid and triglyceride levels in the serum by inducing beta-oxidation of fatty acids in the liver. In our second model we utilized XBP1 KO mice, which have a reduced level of fatty acid synthesis in the liver. With both of these models we demonstrated that changes in host lipids impact the kinetics of blood stage replication.

Using these animal models, we tested the impact decreasing lipid levels has on the development of liver stages. We demonstrated an impact on liver-stage development for both the clofibrate-treated and the XBP1 KO mice. For the XBP1-KO mice, there was a slight increase (0.25 to 0.5 days) in the pre-patent period in comparison to control mice. XBP1 KO mice are not reported to have changes in the lipid content within the liver [216]. Conversely, there was a more substantial change in the pre-patent period for the clofibrate-treated mice. The increased pre-patent period varied from 0.5 to 2.0 days in the clofibrate treated mice in comparison to control treated mice. At both inoculums we are seeing an increase in the average pre-patent period in the treated mice versus the mock-treated controls. Clofibrate treatment impacts the lipid content of the liver [220] by a reduction in triglycerides by 30%, and phospholipid content by 30% when administered within the food as 0.5% of total food content [221]. Results from the clofibrate-treated mice demonstrate that decreasing the lipid content of the liver slows the pathogenesis of malaria parasites. A corresponding experiment with obese mice showed an accelerated rate of sporozoite development in fatty livers[188]. The studies we performed were with 5 mice per dosage of clofibrate in two experiments. To definitively prove such a small change in the pre-patent period, we would need to at least double the size of the experiment and use at least 20 mice per group. A relevant future experiment to conduct is to inoculate clofibrate treated mice with *ΔpbLipB* sporozoites and monitor the pre-patent period. We hypothesize that these parasites would be blocked in treated mice. This hypothesis assumes that the parasite is unable to scavenge enough lipoic acid from its host environment under the treated conditions to compensate for the disruption in the synthesis pathway in knock-out parasites. These studies demonstrate initial

findings of how liver lipid content could be an important factor in determining malaria pathogenesis, but require several follow-up experiments.

The statistically significant impact that clofibrate treatment has on *Δpblipb* blood-stages is intriguing. These findings imply that certain genes of the fatty acid synthesis or lipid metabolism pathway of the parasite are necessary for normal blood-stage development in lowered lipid conditions. This is an interesting finding, since we previously did not see any phenotype for the *ΔpbLipB* parasites during blood-stage growth in normal mice (Figure 2.6). This is also intriguing because we do not see the same impact on blood-stage growth kinetics for *P. berghei* wild-type or *ΔpbFabI* parasites in the clofibrate treated mice, especially since LipB and FabI are both involved in the lipoic acid synthesis pathway in the apicoplast. This finding could be explained by how LipB has a role in the lipoylation of mitochondrial proteins, as demonstrated in Chapter 2. FabI is currently known to only function within the apicoplast and impact fatty acid biosynthesis. The reduction in BCDH E2-lipoylation within the mitochondria , a protein that is assumed to be lipoylated through the scavenge pathway, would have increased sensitivity to changes in the levels of circulating lipoic acid for attachment. The currently unclear role LipB has on BCDH-E2 lipoylation is likely the reason we are seeing a more significant impact in blood-stage growth kinetics of *ΔpbLipB* parasites versus in the *ΔpbFabI* parasites in clofibrate treated mice.

While there is no published data whether clofibrate modulates the levels of lipoic acid in the serum of treated mice, it has been demonstrated that clofibrate binds to albumin, displacing thyroxine, a thyroid hormone[222]. Lipoic acid similarly circulates in the serum of mammals

attached to albumin[223]. Clofibrate treatment of mice is thus likely displacing lipoic acid from albumin in a similar manner to thryoxine, reducing the likelihood of infected red blood cells scavenging albumin-bound lipoic acid attached albumin into the parasite cytosol. Our data also showed an overall reduction in the total content of albumin in the circulation during the course of clofibrate treatment. Lipoic acid can also be reduced by beta-oxidation in the same pathway as longer chain fatty acids, suggesting that free circulating lipoic acid could be degraded in peroxisomes as well. These are three possible ways clofibrate is acting to decrease either the total lipoic acid content within the serum or its availability for uptake into the parasite by reducing its transportation in the circulation as attached to albumin.

These results raise an interesting point on whether genes involved in fatty acid or lipoic acid metabolism are up-regulated during low lipid conditions. As mentioned earlier, a study examining *P. falciparum* infections from patients demonstrated new transcriptional profiles, resembling adaptations to the human host [186]. Both the clofibrate and the XBP1 KO mice provide *in vivo* conditions to examine whether changes to the transcriptional profile of *P. berghei* parasites are occurring in low lipid environments.

For the future use of these models systems, we recommend the use of clofibrate treated mice over XBP1 KO mice. XBP1, as mentioned before, has a wide range of activities *in vivo*. XBP1 is also involved in the production of dendritic cells (DCs) [224]. Although lethal forms of *Plasmodium*, like *P. berghei ANKA,* are unable to generate DCs that prime CD8 T cells [225], it does raise a caveat to the data we have produced, and should be taken into future consideration. The levels of clofibrate used in this study were lower than other published reports in determining

the impact of clofibrate on fatty acid and triglyceride levels in mice. The results we have produced even at this low level dosage of clofibrate suggest that we would demonstrate a more significant arrest in *Plasmodium* growth in mice treated with higher levels of clofibrate. Other studies have published clofibrate levels as 500 mg/kg that were safe to be administered to Swiss/Webster mice [226]. The significant impact on blood-stage growth kinetics that we have demonstrated with the 0.5 mg/kg and the 5.0 mg/kg dosages demonstrate how sensitive *Plasmodium* parasites are to their host conditions. The administration of higher dosages of clofibrate on mice inoculated with sporozoites will likely have a significant impact on the pre-patent period. In order to reduce lipid content within the liver to demonstrate a significant impact on parasite replication, higher concentrations of clofibrate are required. Clofibrate treatment remains an easy and safe method to reliably decrease the fatty acid and triglyceride content in mice, therefore testing a wide range of dosages will open new avenues for determining the impact of host environment on *Plasmodium* liver-stages.

Other methods to reliably reduce the levels of cholesterol species in mice include the use of the group of the statins. Statins inhibit 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase in order to suppress cholesterol biosynthesis [214]. The family of statins shares similar chemical characteristics by containing the aromatic ring structure and an aliphatic fatty acid side chain.

Another possible approach would be to use diets that are low in fatty acids or other lipid species, which could mimic the effects of clofibrate. For example, synthetic low fat diets have been used for the study of atherosclerosis in mice [227]. Recently it has been shown the importance of omega-

3 fatty acids and their potential in reducing the levels of circulating non-esterified fatty acids and triglycerides in mice[228]. These specific diets also offer the potential to modify *in vivo* the levels of circulating lipids to monitor the progression of *Plasmodium* infections. It would be interesting to test their alternate ways to reduce fatty acid and triglyceride contents in the host to determine whether we find the same phenotype with *P. berghei* parasites. Further exploration of these model systems would advance the our understanding of parasite's need for fatty acid and triglyceride species from its host environment within the different stages of its life-cycle.

4. CONCLUSIONS AND FUTURE DIRECTIONS

THE MALARIAL LIFE-CYCLE IS COMPLEX AND ENCOMPASSES DEVELOPMENT IN DIFFERENT ENVIRONMENTAL CONDITIONS.

Plasmodium parasites replicate within a number of different environments to complete its life-cycle and transmit between different hosts. These environments vary widely, therefore the parasite has evolved mechanisms to thrive in each of these conditions, to remain pathogenic and evade host immunity. A key question in malaria biology is how parasites alter their metabolic processes to adapt to constantly changing environmental conditions. Also, do parasites modify their metabolism within the same stage of the life-cycle to respond to changes within that stage? Like most pathogenic infections, *Plasmodium* must generate a balance between synthesis and scavenge from their host. Identifying which metabolites the parasite requires is important for understanding the disease. It could also potentially lead to the development of new therapeutics that block either the synthesis or salvage processes. Finally, knowing the impact the host environments has on parasite replication can lead to a better understanding of disease severity and clinical outcome [186, 229].

In this study, we have addressed these two questions independently. In Chapter 2, we have outlined the role of synthesis of the metabolite lipoic acid in *Plasmodium berghei* parasites. In our second aim, in Chapter 3, we have examined the role of host lipids on the pathogenesis of *Plasmodium* parasites.

KEY QUESTIONS CONCERNING MALARIA PATHOGENESIS

CHAPTER 2: WHEN IS LIPOIC ACID SYNTHESIS REQUIRED FOR *PLASMODIUM* PATHOGENESIS?

Lipoic acid is an important metabolite for eukaryotic cells, including many pathogenic species [197, 230]. Lipoic acid, when attached to enzymatic complexes, serves as their "swinging arm" to shuttle intermediates among active sites. Its attachment to these complexes is critical for the function of these enzymes. Lipoic acid is introduced into the cells through either the synthesis or the scavenge pathways and *Plasmodium* parasites generate lipoic acid through both.

In this study, we have specifically targeted *LipB* for disruption in *Plasmodium berghei* parasites and demonstrated that lipoic acid synthesis is critical for normal liver-stage development. We demonstrated both *in vivo* infections and *in vitro* development assays, the importance of *PbLipB* during liver stages. We observed that disruption of lipoic acid synthesis in the *P. berghei* parasites did not confer any change in virulence during the blood or mosquito stages of development. However, it had an increased dependence on scavenging of lipoic acid from the environment. Furthermore, *PbΔLipB* parasites demonstrated reduced virulence in blood-stages under fatty acid limiting conditions. This work demonstrates that the lipoic acid synthesis pathway is of critical importance during liver stage development of *Plasmodium* parasite

This life-cycle specific need for the lipoic acid synthesis pathway is most likely due to a greater metabolic need during this stage of development than during the mosquito or blood stages. In the work we have presented, we confirmed that disruption of lipoic acid synthesis by the parasite reduces the levels of lipoylated proteins, the pyruvate dehydrogenase and the branched-chain dehydrogenase (PDH and BCDH). PDH is responsible for transforming pyruvate into acetyl-

CoA by pyruvate decarboxylation. Its role in *Plasmodium* metabolism has also been determined to be within the liver stages of development [205]. BCDH is responsible for the degradation of branched-chain amino acids. Its role during the *Plasmodium* life-cycle has not yet been determined. It's likely that is also critical for liver stage development as well, due to its expression pattern [104].

CHAPTER 3: DO CHANGES IN FATTY ACID AND TRIGLYCERIDE LEVELS OF THE HOST IMPACT *PLASMODIUM* PATHOGENESIS?

As discussed in Chapter 1, *Plasmodium* parasites require the uptake of exogenous lipids from the host for blood-stage replication and different host conditions can change the disease severity and clinical outcome of *Plasmodium* infections. How the content of host lipids impacts the pathogenesis of *Plasmodium* parasites remains to be fully characterized.

In this study, we have used two approaches to study the pathogenesis of *Plasmodium berghei* in mice with different fatty acid levels. In the first approach, we used a lipid-lowering agent, clofibrate. Clofibrate, by stimulating beta-oxidation of fatty acids in the liver, reduced the lipid levels of the serum and liver. We measured a reproducible reduction in the rate of blood-stage growth for *Plasmodium berghei* parasites. The impact on blood-stage growth was more severe for the development of *ΔpbLipB* parasites, in comparison to the wild-type and *ΔpbFabI* parasites. Clofibrate treatment of mice also impacted the development of sporozoites in the liver. *P. berghei* parasites required a slightly longer time for liver stage development in the treated versus the untreated control mice.

In our second approach, we used a genetic approach to modify the levels of fatty acids in mice. We employed mice that were deleted for XBP1, a transcription factor that is involved in *de novo* fatty acid synthesis in the liver. XBP1 knock-out mice are conditionally deleted for the XBP1 gene through CRE-mediated excision. As a result, XBP1 mice were significantly reduced in the serum fatty acid content. We demonstrated a similar reduction in the blood-stage growth kinetics for the XBP1 knock-out mice versus the wild-type controls. *Plasmodium berghei* parasites grew significantly slower in these mice, supporting our findings from the clofibrate treated mice and demonstrating that serum fatty acid content is a major contributing factor in the rate of *Plasmodium* blood-stage replication.

THE FUTURE DIRECTIONS FOR THIS PROJECT

The work presented in this thesis demonstrates the importance of the host environment on malaria pathogenesis as well as demonstrated stage specific needs for the metabolite, lipoic acid. Remaining questions on these research topics could be addressed by the following investigations and experiments.

The work presented in Chapter 2 focused on the role of lipoic acid synthesis during the *Plasmodium* life-cycle by disrupting the lipoic acid protein ligase LipB. We identified a reduction in the levels of lipoylated proteins, however it would be interesting and important to determine whether we see the same phenotype by mutating the acceptor domains of these proteins. For instance, the PDH and BCDH proteins have annotated domains within the E2 subunits that receive the lipoic acid attachment. It would be a good complement to our study to see the phenotype we saw in liver-stage development would be replicated by specifically

mutating these domains in *Plasmodium berghei* so that they could not accept lipoic acid and the multi-subunit complexes would be non-functional. Since we identified a reduction in both levels of lipoylated BCDH and PDH proteins, it would be beneficial for future experiments to understand the impact of each enzyme has separately on the life-cycle.

Furthermore, investigation into the role of BCDH during *Plasmodium* development is important. Disruption of BCDH in *Plasmodium berghei* would enable us to investigate when it plays a role during the life-cycle. It is highly expressed during liver-stage, leading us to hypothesize that it has an important function during this stage. However, there currently is no published information about the needs for this protein in *Plasmodium* biology. BCDH-E2 is one of the proteins that are lipoylated in the mitochondria by scavenged lipoic acid. Since lipoic acid scavenging is essential to blood-stage replication of *Plasmodium falciparum* parasites[191] yet neither the *P. falciparum* or the *P. berghei ΔpbLipB* parasites demonstrated loss of blood-stage replication with reduced levels of lipoylated BCDH protein [202]. Therefore, investigation into the role of BCDH could reveal a need for this protein during liver stages of *Plasmodium* parasites.

Finally, the findings from this study presented new questions into the potential exchange of lipoic acid species between the synthesis and the salvage pathways. It is important to further study the impact of the lipoic acid analogue, 8-BOA. The data presented in this work showed that *ΔpbLipB* parasites had reduced rates of blood-stage growth after treatment with 8-BOA. This result implies that wild-type parasites import lipoic acid from the apicoplast to the mitochondria to meet their metabolic needs, whereas in *ΔpbLipB* parasites this import is absent. It would be interesting to see which proteins are lacking lipoylation after treatment with 8-BOA. This type of

experiment could be done though western blot analysis of parasite extracts after over-night incubation with 8-BOA. We predict that there would be a reduction in the levels of lipoylated proteins within the mitochondria, the KGDH and the BCDH, but not the PDH. We also hypothesize that *ΔpbLipB* parasites when treated in the same conditions would have an even greater reduction in the levels of lipoylated mitochondrial proteins in comparison to the wild-type parasites.

In Chapter 3, we presented work in which fatty acid species were reduced in mice through the use of clofibrate treatment. Future studies using these methods include identification of the lipoic acid levels in the serum of mice treated with lipoic acid. As stated within that chapter, we think that *ΔpbLipB* parasites are more attenuated in blood-stage replication than wild-type parasites because lipoic acid is typically attached to albumin as it migrates through the serum. We think they are more attenuated during blood-stage replication than the *ΔpbFabI* parasites because of LipB's role in the lipoylation of mitochondrial proteins. In addition to the possibility of clofibrate binding to albumin and inhibiting lipoic acid transport in the serum, we demonstrated that clofibrate treatment reduces the levels of circulating albumin. This is another reason why we are seeing a dramatic reduction in blood-stage growth kinetics in *ΔpbLipB* parasites. It is generally difficult to measure the levels of lipoic acid in the circulation of humans or mice because they are so low. In order to measure the level of lipoic acid in the circulation, we would need to give additional lipoic acid to the serum of mice and measure whether or not there is a substantial change between the clofibrate treated and untreated mice. Lipoic acid can be measured by methods such as high performance liquid-chromatography (HPLC) [231].

Follow-up studies with the XBP1 knock-out mice would help to confirm the unique phenotype of the *ΔpbLipB* parasites in clofibrate treated mice. XBP1 knock-out mice have similar levels of reduced non-esterified fatty acids and triglycerides as the clofibrate-treated mice, even though the two mice models decrease lipids through different pathways. It is important to confirm that the phenotype of blood-stage growth kinetics is replicated in the XBP1 knock-out mice. Future experiments would consist of inoculating these mice with *ΔpbLipB* and *ΔpbFabI* parasites lines to determine their blood-stage growth rates. We expect a similar result to the clofibrate-treated mice. These results would provide further support that the lipid levels of the host serum make a significant impact on parasite replication, independent of how the lipid levels are reduced *in vivo.*

The data presented in this work demonstrated a subtle impact on liver-stage development in clofibrate-treated mice and XBP1 knock-out mice as determined by the pre-patent period. An important addition to this data-set would be to inoculate clofibrate-treated and XBP1 knock-out mice with *ΔpbLipB* and *ΔpbFabI* sporozoites. We predict that these parasites completely arrest in liver-stage development, since both parasite strains are significantly attenuated in untreated and wild-type mice. In addition, a more sensitive way to detect changes in liver-stage development would be to determine the transcript levels of 18s by quantitative reverse transcription (qRT-PCR). 18s was used in Chapter 2 to control for detection of liver-stage infection by RT-PCR. Detection of 18s by qRT-PCR is regularly used to determine the liver-stage burden during *in vivo* infections[232]. The transcript for the 18 small ribosomal subunit is commonly used to detect parasite transcript from the total RNA of the sample. This method is very sensitive to differences in parasite burden in the host and might elucidate a more significant phenotype during liver-stage development in the two mouse models.

As mentioned in Chapter 3, clofibrate treatment of mice has been demonstrated to vary in the concentrations administered, with the highest reported dosage of 500 mg/kg [226]. The dosages used in this study are on the low-end of the range used within clofibrate-literature. Therefore, the phenotypes we are seeing in both blood and liver stage demonstrate how sensitive *Plasmodium* parasites are to their environments. To further probe into the fatty acid needs of *Plasmodium* during liver-stage development in particular, future experiments include increasing the dosage of clofibrate administered to mice before their inoculation with *Plasmodium* sporozoites. Since dosages up to 500 mg/kg are safe for administration into mice, we recommend using this dosage to try to significantly arrest parasites during liver-stage development *in vivo.* In addition, the combination of higher clofibrate dosages with attenuated parasite strains, such as *ΔpbLipB* and *ΔpbFabI* sporozoites, will likely lead to full attenuation in mice. Comparing how the *ΔpbLipB* and *ΔpbFabI* sporozoites respond in the lipid-reduced host environments would provide us greater insight into how these proteins function during liver-stage development.

Since clofibrate induces beta-oxidation of fatty acids in hepatocyte cells and results in a decreased lipid-content within the liver, we also propose elaborating this area of research to replicate our results *in vitro.* Possible experiments would include pre-treatment of cultured hepatocytes (such as HepG2 cells used in Chapter 2) with clofibrate and subsequent inoculation with sporozoites. Read-outs to determine whether the clofibrate treatment has a significant impact on parasite liver-stage replication would include quantifying the size of the developing parasite, like the use of fluorescent parasites as illustrated in Chapter 2. Other quantitative determinations could include the total number of developing parasites and the number of detached cells at different time-points of liver-stage development. Adjusting the use of clofibrate

to *in vitro* hepatocyte conditions would allow for better determination of the reduction in fatty acid content upon treatment. Certain methods, such as Folch extraction, could be employed. This is also a model system to test for the *in vitro* activity of antimalarial compounds that inhibit liver-stages. As briefly discussed in the introduction, the lipid content of the host serum impacts the efficacy of blood-stage antimalarias. Liver-stage specific drugs might also demonstrate significant differences in their efficacies when changes the lipid content of the host. Clofibrate administration of cultured hepatocyte cells is an attractive method to test this hypothesis.

Finally, we identified a reduced rate of parasite replication for both blood and liver-stage development and a valuable future experiment would be to determine whether gene expression patterns are altered in these conditions. As mentioned in the introduction, one group [186] described unique transcriptional profiles in *Plasmodium* parasites taken directly from human patients. These profiles had never been seen before from *in vitro* parasite cultures, suggesting that *Plasmodium* parasites respond to their environmental conditions by up-regulating certain metabolic pathways. A pilot study would be to determine the expression levels of known fatty acid metabolism genes in parasites removed from clofibrate or XBP1 knock-out mice with parasites in untreated or wild-type mice as a control. If there are differences in this initial gene set, a complete understanding the genomic transcriptional patterns could be identified by a microarray. Support for the hypothesis that *Plasmodium berghei* parasites are transcriptionally responding to changes in fatty acid levels is provided by data presented in Chapter 3 in which the *ΔpbLipB* parasites are more significantly impacted in their blood-stage replication versus wild-type. These findings suggest that lipoic acid synthesis, although not essential with normal serum fatty acid levels, is necessary under limiting conditions. It is likely there are other metabolic

pathways that are similarly needed in the fatty-acid limited conditions we have created.

Although the research we presented in this work did not touch upon the role of lipoic acid as a potent antioxidant, it is another future direction for this project. Lipoic acid has the ability to form a redox couple that can quench reactive oxygen species [233, Packer, 2011 #528]. Other infections that replicate in the liver, in particular Hepatitis C virus, induce oxidative stress in the liver of patients and play a key role in pathogenesis of the virus [234]. Additionally, *Plasmodium*-infected hepatocytes up regulate their redox pathways [235]. Redox-perturbing agents would be useful for future studies in investigation of the redox pathways of the host. In particular, dithiothreitol decreases the oxidative state within the endoplasmic reticulum of the cell. It has also been demonstrated to result in increased splicing of XBP1 transcripts, leading to an up regulation of genes controlled by this transcription factor [236]. It would be very interesting to use this redox agent on mice and see whether there are any changes to the liver stage development of wild-type and *ΔpbLipB* sporozoites. It would also be a useful control to verify our results generated with the ΔXBP1 mice.

RESULTS FROM THIS RESEARCH RELATE TO GENERAL *PLASMODIUM* BIOLOGY

The work stated in this thesis examine the fatty acid and lipoic acid needs of the malaria parasite *Plasmodium berghei*, which causes malaria in mouse species. We used this species of malaria as a model system to probe unanswered questions in *Plasmodium* biology. There are several aspects of this project that we can relate to its greater field of *Plasmodium* research.

The main impetus for generating a *P. berghei* strain disrupted for LipB was because it was shown to be non-essential for blood-stage development in *P. falciparum* [202]. The *P. falciparum* LipB knock-out strain showed that although lipoic acid synthesis was not required during *in vitro* blood-stage replication, the modification generated a replication rate faster than the wild-type control. We did not find the same results for *in vivo* blood-stage growth with *P. berghei LipB* knock-out strains. Our results showed similar blood-stage growth rates when mice were inoculated with either 10,000 or 1,000 parasites. Currently, we do not have an explanation for this difference between the LipB knock-out strains in different species. Both sets of experiments were done with clonal parasite populations. Perhaps the difference is because *P. falciparum* replication rates are determined with *in vitro* conditions and *P. berghei* blood-stage replication occurs *in vivo*. Removing *P. falciparum* from its natural environmental conditions when examining blood-stage growth kinetics might amplify small changes that are not seen when parasites are replicating *in vivo* [202]. There were no other significant differences between the knock-out parasites in different species. Similar to our results identifying the lipoylated proteins by Western blot, the *P. falciparum* LipB knock-out parasites showed reduced levels of PDH and BCDH proteins and no change in the levels of KGDH protein.

P. falciparum LipB knock-out parasites were treated with Triclosan, an antibiotic known to target the FAS-II pathway. Although it was later determined that *PfFabI* is not the target of Triclosan [105], an earlier publication showed Triclosan as a potent antimalarial [237]. However, *P. falciparum* LipB knock-out parasites did not have any significant change in their sensitivity to Triclosan treatment. We did not replicate this study with the *ΔpbLipB* parasites since the target of Triclosan in *Plasmodium* is currently unknown.

The study also localized the protein Pf LplA2 in *P. falciparum* by both fusion to GFP and immunofluorescence. LplA2 is another lipoate protein ligase that was found to target to both the apicoplast and the mitochondria in *P. falciparum* parasites [204]. The authors hypothesized that LplA2 is capable of compensating for the loss LipB, leading to a residual amount of PDH lipoylation. Our study did not venture into whether or not LplA2 is compensating for the loss of LipB in *P. berghei* although we had similar levels of residual PDH lipoylation. This study instead localized PbBCDH since its not predicted to be lipoylated by LipB [204].We found that PbBCDH localized to the mitochondria, as predicted. A more detailed understanding of how LipB is contributing to the lipoylation of this protein is needed, for both parasite species. Neither study localized LipB since it has a strong apicoplast targeting motif and anti-LipB antibodies are not available for immunofluorescence.

P. falciparum LipB knock-out parasites were not treated with 8-BOA, unlike this study. 8-BOA inhibits lipoic acid scavenging by acting as a competitive inhibitor. This understanding is based on work in *P. falciparum* and we have assumed the same mechanism of action in *P. berghei* parasites [191]. It would be interesting to see if there is also increased sensitivity to 8-BOA treatment during *in vitro* conditions with *P. falciparum* LipB knock-out parasites.

A large portion of the work presented in this document focused on lipoic acid as a component of the larger fatty acid metabolism pathway in *Plasmodium*. However, lipoic acid is unique in comparison to the other longer fatty acids that are synthesized by the FAS-II pathway or scavenged from the environment. As mentioned previously, *Plasmodium* utilizes the fatty acids

palmitic and oleic acids scavenged from its host environment [176]. These fatty acids are metabolized into more complex lipid species; including phosphatidylcholine, phosphatidylethanolamine, diacylglycerol and triacylglycerol. These complex lipid species are known constituents of the membranes and lipid bodies [177]. Whereas, lipoic acid is not known to be incorporated into complex lipid species or into the cell membrane [191]. Therefore, changes in the contents of the cellular membrane witnessed in *ΔpbLipB* parasites are a down-stream effect of the reduced capacity of enzymatic components that require lipoic acid modification; PDH-E2 activity would be limited within the apicoplast, leading to decreased generation of acetyl-CoA as FAS-II intermediates. Similarly, 8-BOA treatment to *Plasmodium* parasites inhibits lipoic acid scavenging and is toxic to parasites because the multi-subunit enzymes that require its attachment cannot function. Therefore, although lipoic acid is similarly an essential short-chain fatty acid that is scavenged from the environment, like the longer chain fatty acids, it plays a unique role in parasite metabolism. In most cells, lipoic acid, like long chain fatty acids, can also be reduced into shorter chain metabolites via beta-oxidation, however the genes required for beta-oxidation of fatty acids have not been found in the *Plasmodium* genome.

Lipoic acid is also known as a potent antioxidant. This is a unique function that distinguishes it from other fatty acid chains. LA is capable of scavenging reactive oxygen and reactive nitrogen species *in vitro.* The antioxidant needs of *Plasmodium* parasites remain uncharacterized. The essential role of lipoic acid in *Plasmodium* biology could be related to this capacity, but further research is required to clarify its role as an antioxidant during infections.

Here we've demonstrated that the synthesis of lipoic acid is only required during liver-stage but scavenging of lipoic acid is essential in liver and blood-stage. *Plasmodium* parasites balance their metabolic needs through either synthesis or salvage processes. Certain metabolic pathways in *Plasmodium* are completely dependent on scavenging, including amino acid and purine nucleotides[209]. Scavenging of fatty acids is essential for blood-cycle replication of *Plasmodium falciparum* parasites [176]. Fatty acid components from human serum, in particular serum albumin and purified bovine serum albumin, are essential for *P. falciparum* growth *in vitro* [176]. Since the parasite's fatty acid composition reflects that of the medium, it implies that the parasite is incredibly dependent on scavenging from its environment [177]. The parasite has demonstrated the capacity to modify scavenged fatty acids, specifically to elongate and desaturate radioactive fatty acids provided in the serum. Similarly, lipoic acid from the environment is attached to proteins to function as a cofactor in mitochondrial keto-dehydrogenase enzymes, BCDH and KGDH[191].

The mechanisms of scavenging and import of lipoic acid and other longer chain fatty acids have not been demonstrated on a molecular level. However, several lipid import genes have been annotated in the *Plasmodium* genome [187]. Due to the wide range of host environments *Plasmodium* infects, it is likely that it has developed different mechanisms of scavenging. The large family of acyl-CoA synthases and acyl-CoA binding proteins demonstrate unique patterns of expression that may function in a stage-specific manner[180]. This family of fatty acid scavenging proteins appears to be necessary for both blood and liver-stage [104]. Stage-specific expression of scavenging proteins may be one way *Plasmodium* regulates import based on its environment.

The unique nature of the Acyl-CoA synthease (ACS) family leads to the potential for the development of chemotherapeutics to block fatty acid import from the host. There are twelve ACS genes in the *P. falciparum* genome, the most of any known organism. The duplicity of the gene family suggests an essential need for ACS proteins [179]. Furthermore, they are located within epigenetically repressed centers on the genome commonly associated with genes involved in immune evasion (i.e. *var, rifin and steavor*) [180]. Data from our group found the expression all four *P. berghei* ACS genes in mixed blood-stages but only *PbACS-9* was expressed in the oocyst stage and *PbACS-10* during liver stages. We were also unable to disrupt *PbACS-10* and *PbACS-11*, suggesting essentiality of these proteins during blood-stages. These results demonstrate a stage-specificity for the *P. berghei* gene family and we hypothesize an even more complex pattern of the gene expression in *P. falciparum*. Finally, Triacsin is a known chemical inhibitor of ACS enzymes in other cell types and had an IC_{50} value within the nanomolar range for *P. falciparum* cultures [238]. Data from our group could not replicate such a low IC_{50} value however if the published values are true, it is an interesting new method to target the fatty acid scavenge pathway in *Plasmodium*.

The mechanisms for lipoic acid scavenging in *Plasmodium* are currently unknown. Lipoic acid is naturally occurring in red blood-cells and is attached non-covalently to albumin in human serum[239]. Lipoic acid may be imported directly into the parasite, as attached to albumin, into the parasite cytosol or may require uptake through a small molecule transporter, such as the H+-coupled transporter that transports pantothenate [240]. Further study is required to identify the molecular mechanisms for lipoic acid import.

Several critical synthesis pathways in *Plasmodium* have been localized to the apicoplast organelle [187]. Synthesis pathways such as the lipoic acid, fatty acid, isoprenoid and heme synthesis pathways have been identified in the *Plasmodium* genome. The lipoic acid synthesis pathway requires an active FAS-II pathway, to generate the octanoyl-ACP precursor. The fact that both the FAS-II and the lipoic acid synthesis pathway are both non-essential for blood-stages but are critical for liver-stage development, confirms the interdependence of the pathways. FAS-II intermediates are generated by the Pyruvate Dehydrogenase enzyme (PDH). FAS-II generates the ocantoyl-ACP precursor that is used by the lipoic acid synthesis pathway for attachment to the PDH complex. PDH degrades pyruvate, generating acetyl-CoA, which feeds back into the FAS-II synthesis pathway. These synthesis pathways occur in the apicoplast organelle. Fosmidomycin is an apicoplast specific inhibitor of *P. falciparum* blood-stage growth. Treatment of blood-stage cultures could be rescued with isoprenoid precursors [241]. Based on these findings, the isoprenoid pathway appears to be the only essential synthesis pathway in the apicoplast during blood-stage development. This complements our study if we propose that other metabolic pathways targeted to the apicoplast would serve their function during non-blood-stages of the life-cycle. It would be interesting to follow-up this hypothesis by determining the mosquito and liver-stage needs of isoprenoid and heme synthesis.

The majority of the work presented in Chapters 2 and 3 examined the phenotypes of parasites during the blood and liver-stage of development. As the data has demonstrated, these stages have unique metabolic needs, generate different clinical symptoms and provide opportunities for intervention.

ΔpbLipB parasites were significantly arrested during liver-stage development however showed no significant difference in blood-stage replication unless treated with the lipoic acid scavenge inhibitor 8-BOA or in mice with measurably lower circulating lipids. Blood-stage replication occurs after *Plasmodium* parasites have completed replication in the liver and invade red-blood cells of the host. In *Plasmodium falciparum*, parasites multiply within the confines of the red blood cell host, lysing the cell when the cycle of replication completes. *Plasmodium falciparum* parasites take 48 hours to complete their development within the red blood cell and upon release triggers the immune response that generates the classical waves of fevers and chills. These symptoms are the earliest indications that a patient is infected. Blood-stage is also critical for the transmission of the disease, since an infected person will generate a certain percentage of gametocytes in the blood. These parasite forms when taken up by the mosquito will mature and continue the life-cycle. Therefore the development of antimalarials for this life-cycle stage is ideal to generate drugs that block both blood stage asexuals and gametocytes, to prevent transmission. *Plasmodium* gametocytes can be tested for their sensitivity to particular chemotherapies [68].

In this study, we demonstrated a significantly reduced rate of blood-stage growth of *Plasmodium* parasites in mice with lower circulating fatty acids and triglycerides. However we did not identify whether these conditions lead to an increased amount of gametocytemia. From *in vitro* work with *P. falciparum*, its been identified that low fatty acid conditions promote gametocytemia. However more research would help to clarify whether this is a general stress response or a particular response to the lipid content of the environment. This would be an interesting direction for future work with the clofibrate and XBP1 knock-out mice.

We examined the replication of *Plasmodium berghei* parasites in mice as a model system for human disease. Liver-stage of development is clinically silent in patients. This stage begins when an infected mosquito releases sporozoites that migrate to the liver, invade and start to replicate. This period of development yields thousands of merozoites, which escape the infected hepatocyte and are released into the blood-stream to infect red blood cells. The amount of replication during liver-stage is much higher than during blood-stage. Liver-stage development is critical for the life-cycle of *Plasmodium* parasites; without this stage of development parasites are not capable of invading red blood cells. Because this stage is clinically silent, most patients do not yet know they are infected. Therefore, therapies that target liver-stage are taken as prophylaxis treatments. Liver-stage drugs would need to be taken on a consistent basis to prevent *Plasmodium* infections.

The work we've shown here has focused on genetically attenuated *Plasmodium berghei* strain that arrests during liver stage development for the generation of a genetically attenuated sporozoite vaccine. Literature presented in the introduction has shown that parasites that arrest during liver stage, in both mice and humans, can provide protective immunity to subsequent challenge. Liver-stage vaccines would provide protection to *Plasmodium* infections without experiencing any of the symptoms that occur during blood-stage development.

All of the experiments presented in Chapters 2 and 3 were done using the mouse species of malaria, *Plasmodium berghei* ANKA. We decided to use this parasite strain based on the similar pathology to *P. falciparum* since both are lethal strains when they infect mammals. *P. berghei*

has been commonly used as a model to represent cerebral malaria and severe malarial anemia [242]. We chose to use the mouse strains of C57/Bl6 and Swiss/Webster for the series of liver-stage or blood-stage experiments, respectively. Mouse strains of malaria are of particular importance in understanding the elements of liver stage development. However, like for all model systems, the data presented in this work require the replication of these findings in *P. falciparum* and the other human infective species. There have been many caveats in applying the findings from the mouse malaria strains to different aspects of the human disease. For instance, within the field of human cerebral malaria a point of contention has been the extent of parasite sequestration in the brain and whether the rodent species have the same amount of sequestration [95].

Even within the field of rodent parasites, there has typically been more arrest with the *P. yoelli* versus *P. berghei* strains when disrupted for the same genes. For example, the disruption of *fab b*/f in *Plasmodium berghei* resulted in incomplete attenuation, whereas in *P. yoelii* it completely arrests [243]. However several studies that have translated genetically attenuated rodent strains into *P. falciparum* strains have demonstrated similar outcomes. *P. falciparum* parasites disrupted for p52 gene, an ortholog of *P. berghei* p36p, when tested for development with primary human hepatocytes demonstrated similar attenuation to the rodent strain [244]. Also, when *P. falciparum* parasites were generated for a double-knock out of p52 and p36, it resulted in no observable parasites after 4 days of inoculation into hepatocyte *in vitro* cultures [243]. Disruption of either of these genes in rodent malaria strains causes complete arrest *in vivo,* similar to the phenotype witness for their corresponding rodent models. However, in clinical trial with *P. falciparum* disrupted of p52 and p36 caused a breakthrough blood-stage infection in one volunteer. These results indicate that we might have different phenotypic outcomes at each stage of vaccine

development and that all GAP rodent models require complete testing before jumping forward to stages of vaccine development. These steps would include thorough testing in both *P. yoelii* and *P. berghei* strains and in different strains of mice (i.e. BALB/c and C57/Bl6). And finally, a corresponding analysis of GAP *P. falciparum* development in hepatocyte cultures would be required.

Initial investment into research with rodent malaria strains has proven to be a worthwhile pursuit. In the field of vaccine development, mouse malaria models have successfully predicted vaccine failures, although not always effective vaccines [245]. They have a useful amount of predictive value for vaccines. Drug treatment studies of mouse malaria have demonstrated inconsistent results in comparison to their human malaria counterparts. One major reason is that mice might be treated before the onset of symptoms in certain cases, which is irrelevant to their counterpart human studies. This is just an indication that mouse studies are useful and powerful tools when experiments are conducted in a manner close to the natural pathogenesis of the disease as possible. The work presented here was conducted with this mind. All of the experiments were replicated on independent occasions with control studies run in parallel. Both blood and liver stage phenotypes that we present should however be replicated with *P. falciparum* parasites, if possible. The use of the humanized mouse models would offer the best of both worlds [117]. Future experiments with genetically attenuated *P. falciparum* parasites should take advantage of this model system. The arrest we demonstrated in the *ΔpbLipB* parasites was significant that we predict a similar outcome in *P. falciparum* parasites.

TRANSLATING THESE FINDINGS INTO TREATMENTS AND CURES FOR MALARIA

The results from this study demonstrated how lipoic acid synthesis is required in liver-stage and during blood-stages in lipid-limiting environments. This implies that lipoic acid is a critical factor for *Plasmodium* pathogenesis in both developmental stages. The levels of circulating lipoic acid are typically below the levels of detection in humans. Lipoic acid levels are reportedly between 33-145 ng/ml bound to serum albumin in human serum [246]. Free lipoic acid is present at millimolar levels and administration of 200 to 600mg of lipoic acid will only show a less than 50 μM accumulation in blood plasma [192]. Since its levels in circulation are naturally so low, it is not commonly a measurement of malnutrition.

Lipoic acid is a well-promoted dietary supplement. It is absorbed from dietary sources and transiently accumulates in many tissues. Its role as a powerful antioxidant is well studied and supplemental lipoic acid treatment has a protective effect on several diseases, including cardiovascular disease, liver disease, insulin resistance and diabetic polyneuropathy [247]. However, based on the data presented in this work, it would be recommended to not take lipoic acid supplements in malarial-endemic areas. Limiting the amount of free lipoic acid in the environment reduces the parasite burden in blood and liver-stages, demonstrated in both *P. falciparum* and *P. berghei* parasites. There is no recommended way to reduce the natural level of lipoic acid from humans. Since many malarial-endemic areas typically have high levels of malnutrition, it is likely that lipoic acid levels are below average.

Results from Chapter 3 raise questions into the relationship between *Plasmodium* pathogenesis and malnutrition. The data we have presented show how the levels of fatty acids and triglycerides have a significant impact on the virulence of *Plasmodium* infections. Based on this data, one could recommend reducing the intake of lipid species to prevent cases of severe malaria. However, it has been well documented that malnutrition leads to a lower functioning immune system [38]. There is likely a balance between the loss of immune function due to undernourishment and the possibility of reducing the malarial-burden with less pathogenic forms of *Plasmodium* in low fatty acid conditions. This balance would exist on a region-specific basis, depending on the species of *Plasmodium* present, the age of the patent and the normal diet of the region. However, there are severe consequences to malnourished young children, especially children under five. Not only does stunting pose an enormous problem for human development, malnutrition is also associated with cognitive impairment and decreased school performance [248]. Therefore, any benefits from maintaining a low lipid diet for the purpose of combating malaria is likely overweighed by their downsides.

Finally, it is very important to have a comprehensive understanding of the relationship between the effectiveness of antimalarials and the levels of serum lipids in the host. As mentioned in the introduction, arteminisin is one of the most commonly used antimalarials yet it has a short plasma half-life of 1-3 hours. This can lead to failure rates since it is metabolized so quickly. There have been a number of publications in addressing whether high fat diets lead to more bioavailability of antimalarials with high levels of lipid solubility [249, 250] [71]. It is clear that the administration of a single high-fat meal would not significantly impact the bioavailability of piperaquine, yet long-term effects of high fat versus low fat diets on antimalarial activities has

yet to be addressed. The mouse models we presented in Chapter 3 would be useful tools in discovering whether a long-term reduction in fatty acids impacts the absorption of antimalarials. It would also be useful to determine whether there is a correlation between antimalarial activity and cases of malnutrition. Further investigation into the fatty acid and other lipid levels impact has on antimalarial therapies will have significant consequences on antimalarial treatment regimens.

FUTURE DIRECTIONS FOR THE FIELD OF LIPID BIOLOGY IN *PLASMODIUM* PARASITES

The future of work in the field of fatty acid and lipid metabolism in *Plasmodium* parasites should take advantage of the stage specificity of the pathways. Data from this work and other publications from the field have indicated the necessity of scavenging fatty acids from the environment for the pathogenesis of *Plasmodium* parasites during blood stage replication. These findings imply an essential role for genes involved in the scavenge pathway during this stage of replication. Furthermore, many of these genes are expressed to a higher degree at blood-stage and are even specifically regulated for expression within the intraerythrocytic period [181].

We suggest using this knowledge of the importance of scavenging of host lipids during blood-stage for the development of chemotherapies that can treat *Plasmodium* infections after the demonstration of clinical symptoms. We suggest targeting the Acyl-CoA Synthase gene family as these genes failed to be disrupted in *Plasmodium berghei* (data from our lab, not shown). Other proteins involved in the fatty acid scavenge pathway include the Acyl-CoA Binding proteins. The considerable polymorphism and redundancy in these gene families make them

likely essential in parasite development and unique in structure which make the good drug targets.

Other *Plasmodium* blood-stage lipid synthesis pathways that are also candidates for chemotherapeutics include the synthesis of major phospholipids, phosphatidylcholine and phosphatidylethanolamine [172]. The parasite completes a major amount of modification to imported fatty acids, which may also be essential for parasite development. The *Plasmodium* phosophlipid pathway has already shown to be targeted during blood-stage with compounds that mimic the structure of membrane precursors, such as ethanolamine, serine or choline [251]. Compounds that target these pathways inhibit parasite proliferation in the micromolar range.

In addition, targeting the lipoic acid scavenge pathway offers new potential for the development of malarial chemotherapies. The potency of 8-BOA treatment for both *P. falciparum* and *P. berghei* parasites demonstrates that inhibition of lipoic acid salvage pathway would be lethal to parasites. Although our studies with 8-BOA showed remaining amount of lipoylated protein in the human hepatocyte cells (HepG2) after treatment, 8-BOA is still active against mammalian lipoylated proteins. Other parasite specific chemical inhibitors to the lipoic acid scavenge pathway should be developed.

Other *Plasmodium* lipid synthesis pathways we recommend targeting for disruption and the generation of the genetically attenuated parasite (GAP) vaccine. The critical difference between genes that have the potential for GAP development and pathways we recommend for targeting by chemotherapies, is whether they can be disrupted in blood-stage *Plasmodium* parasites. Genes

that can be disrupted through genetic modifications of *P. falciparum* or other human malarial strains, enable further testing for their arrest in liver-stage and ability to stimulate the immune response. If genes are unable to be disrupted in blood-stages, in *P. falciparum* or rodent malaria models, they are essential and offer potential for chemotherapeutic development.

As shown in this work, lipoic acid synthesis pathway is not essential for blood-stages, however it has a critical role in *Plasmodium* liver-stage development. Similar to components of the FAS-II pathway, we suggest targeting these pathways for the generation of GAP vaccines to combat malaria.

The lipid and fatty acid pathways of *Plasmodium* parasites offer potential for generating GAPs by design. Part of the attraction of using genes involved in the fatty acid and lipoic acid synthesis pathways for the development of GAPs is that they arrest later than other known genetically attenuated parasite strains. Parasites developing into the later stages of liver-stage development provide superior antimalarial immunity [107]. Parasites that are disrupted for FAS-II genes develop later than radiation attenuated sporozoites and also other knock-out parasites tested. FAS-II knock-outs also generate a larger memory CD8+ T cell population than the other parasites, which confers greater level of protection to challenge. However, as mentioned earlier, there can be discrepancies between the mouse models of GAPs and disrupting the orthologous gene in human malaria. Mouse models of genetically attenuated parasites offer great potential for their design and testing, but validation in human malarial species should always be a consistent branch of the experimental procedure. The new developments of the humanized mouse provide great potential for testing these GAP strains.

Finally, the future directions in the field of lipid biology of *Plasmodium* should include further investigation into how the fatty acid content of the host impacts the disease. Data we have shown in Chapter 3 demonstrated how changes in the fatty acid content of the host impact the rates of *Plasmodium* replication. However, the model systems presented in Chapter 3 to reproducibly lower fatty acids during *Plasmodium* replication in vivo should be further explored in the areas of transmission and drug solubility. The lipid content of the host can have a significant impact on how drugs are absorbed and their effectiveness in clearing infections. The nutritional status of the host also has a major impact on whether the immune system can successfully respond to infection. The interconnection that exists between nutrition, immunity, severity of disease and treatment outcome should be addressed in a multi-factorial manner. For instance, future work on malaria chemotherapeutic treatment should take into account the host's nutritional status, in particular the fatty acid and triglyceride content. Likewise, the effectiveness of GAP vaccines may vary widely depending upon the nutritional status of the host and their ability to generate a protective immune response. Understanding interdependence between the host and pathogen fatty acid and lipid profiles in a detailed, molecular level is fundamental to understanding the disease and providing the best recommendations for antimalarial therapies and preventative treatments. The lipid metabolism of *Plasmodium* parasites and their hosts is a critical component in the design of new drugs, the administration of existing treatments, and the development of effective vaccines for the eradication of this disease.

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