The role and mechanism of hemoglobin variation in *Plasmodium falciparum* sexual differentiation

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The role and mechanism of hemoglobin variation in *Plasmodium falciparum* sexual differentiation

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The debilitating effects of human infection by the malaria-causing pathogen *Plasmodium falciparum* is believed to be the driving force behind the emergence of several different mutations of the hemoglobin (Hb) protein. Today, this group of disorders is the most common monogenic disorder within the human population, and unsurprisingly the most affected populations are those that have, and continue to be, most affected by malaria disease. Together, these two diseases incur a significant public and global health burden. While several variants of Hb provide protection against malaria, this protection is not sterile, and malaria prevalence has been shown to vary based on the prevalence of Hb variant- carriage within certain populations. The relationship between Hb variation and malaria is further complicated by recent epidemiological findings which have found Hb variation may promote the transmission of malaria from human host to mosquito vector. The host to vector transmission of *P. falciparum* is reliant on the formation of gametocytes, which arise from asexually replicating parasites during the intraerythrocytic stage of human infection. Defining specific factors that promote the formation of transmissible gametocytes from the disease-causing asexual stages has been an area of increased research, as targeting these stages may prevent transmission entirely. Here, we analyze the relationship between the most common Hb variant, Hb S, and the rate of gametocyte conversion in *P. falciparum*. We find gametocyte conversion rates (GCRs) are significantly increased among erythrocytes expressing Hb S compared to those expressing Hb A. We then find parasitic enzyme-mediated digestion of Hb S

occurs more rapidly than Hb A, suggesting an increased release of heme groups carried by each Hb subunit. Then, upon manipulating both intracellular and extracellular hemin concentrations, we found gametocyte conversion to significantly increase. Therefore, we conclude that heme is a causative factor for gametocyte conversion. As levels of both intracellular and extracellular heme are increased in individuals with Hb variants, we propose heme to be a contributing factor for increased gametocyte conversion observed among these populations. These results support further investigation into how heme concentrations may be directly manipulated to prevent commitment to gametocyte formation and ultimately disease transmission.

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Preface

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1.0 Introduction

Malaria is a dangerous infectious disease caused by apicomplexan parasites of the *Plasmodium* species. With evidence of early human civilizations suffering from malaria in as early as 1500 BCE, the true burden of disease is difficult to comprehensively quantify (1). Remarkably, it has been suggested that malaria may have been responsible for half of total deaths to have occurred through the 21st century (2). Though this is likely an overestimation, this evaluation raises important questions of the non-fatal consequences of *Plasmodium* infection, because where malaria does not kill, it destabilizes.

1.1 History and global impact of *Plasmodium*

Initially named in the Italian language for its believed origin, “mal aria” or “bad air”, the actual causative agent of malaria was only first discovered in 1880 by Charles Louis Alphonse Laveran, who observed *Plasmodium*-infected erythrocytes from malaria patients (3). Prior to this discovery, the disease was characterized based on fever paroxysms, a clinical symptom of malaria infection, with the terms “febris tertian” and “febris quartana” commonly used to distinguish the infections (4). Though originally Laveran hypothesized a connection between mosquitos and malaria, it was only formally confirmed that mosquitoes serve as the parasites’ vector in 1898 by Sir Ronald Ross (5, 6).

Plasmodium spp. have developed intricate methods to maintain their transmission and survival amongst diverse populations, civilizations, and climates. The parasite, which requires a

human and mosquito to sustain its existence, uniquely balances different facets of its lifecycle to optimally transfer between the two hosts. Interestingly, though historically malaria epidemics have produced astoundingly high fatality rates, most species exhibit limited pathogenicity, with less than 1% of total cases today becoming fatal (1, 7, 8). In not eliminating the host, the parasite is able to establish a cycle of almost continuous infection. Though resistance and immunity to infection can develop, more often than not the response is not sterilizing, and therefore may not entirely prevent reinfection. With continuous reinfection comes continuous transmission and over time, infected individuals develop devastating conditions such as chronic anemia and organ dysfunction. On a whole community scale, this results in the weakening of total populations. When entire communities are weakened, productivity suffers, ultimately leading to economic consequences which perpetuate the cycle of poverty (9-11).

1.1.1 *Plasmodium* species of human importance

All *Plasmodial spp* have an impressive history of overcoming environmental changes, whether it be the environment of the human host, mosquito vector, or external atmosphere. Currently, there are six recognized species of *Plasmodium* that can cause malaria disease amongst humans: *P. falciparum, P. knowlesi, P. malariae, P. ovale curtisi, P. ovale wallikeri,* and *P. vivax*. Each species varies in lifecycle, geographic distribution, Anopheline vector species, and disease severity (1, 4, 12, 13).

1.1.1.1 Plasmodium falciparum

Though *Plasmodium falciparum* likely emerged much later than all other currently recognized species of human concern, today it is sometimes regarded as the only species capable

of causing severe malaria disease (14, 15). Traditionally described as malignant tertian malaria due to the severity and paroxysm of fever, *P. falciparum* is the most prominent cause of death due to malaria. Due to this high mortality, *P. falciparum* would have been unable to sustain infection and transmission among early traveling populations. Thus, the pathogen likely emerged along with sedentary communities in Africa. Unlike species which are discussed below, *P. falciparum* parasites do not produce hypnozoites, negating its ability to "hibernate" during winter months. Moreover, *P. falciparum* also require higher average temperatures to complete the transmission cycle within the mosquito vectors, further restricting its territory to tropic and sub-tropic regions (16).

1.1.1.2 Plasmodium knowlesi

Plasmodium knowlesi, traditionally regarded as a primate species, was only shown to naturally infect humans in 1965, but it was likely first described in 1927 from a blood sample obtained from long-tailed macaques (17). Interestingly, *P. knowlesi* was known to be able to infect humans in 1932 and was used as a pyretic (along with *P. vivax* and *P. malariae*) for the treatment of syphilis up until 1955 (18-20). *Plasmodium knowlesi* is found throughout southeast Asia and is the most common cause of malaria in Malaysia. Malaria disease as a result of *P. knowlesi* can be severe, owed in part to its shortened intraerythrocytic lifecycle, with some studies reporting the risk of severe disease to be as high as *P. falciparum*. There is a growing concern about the potential increase and spread of *P. knowlesi*, particularly amongst populations in Southeast Asia and sub-Saharan Africa.

1.1.1.3 Plasmodium malariae

The first species believed to migrate outside of the African continent is *Plasmodium malariae*, which was first formally described in 1890 (1, 21, 22). Its progression was likely initiated by the migration of early hunter and gatherers who travelled to the warmer regions of Europe and Asia. Survival amongst these small early communities would have required the ability to withstand low transmission settings, which include the winter months and low population levels (23). While the disease of *P. malariae* is generally considered mild, with a fever paroxysm every 72 hours (benign quartan fever), prolonged or recurrent *P. malariae* infections have been associated with nephrosis, which would have contributed greatly to malaria mortality rates (24).

1.1.1.4 Plasmodium ovale

Plasmodium ovale was discovered in 1918 and has the most restricted distribution amongst all *Plasmodium spp*. Though *P. ovale* are widely thought to produce hypnozoites, evidence for their existence has been difficult to thoroughly validate (25-27). Two distinct species have recently been identified: *P. ovale curtisi* and *P. ovale wallikeri* (13). Disease of *P. ovale* carries a very low risk of a severe outcome and displays a tertian pattern (every 48 hours, benign tertian malaria). *P. ovale* is present throughout most of sub-Saharan Africa, as well as New Guinea and the Philippines (22).

1.1.1.5 Plasmodium vivax

As one of the first species to be identified and named, *P. vivax* remains the most widely distributed species of *Plasmodium*, and can be found throughout Asia, Latin America, the Middle East, Mediterranean, and parts of Africa (28). *Plasmodium vivax* has a long history of human impact, and is responsible for driving the loss of the Duffy surface glycoprotein on erythrocytes

among West African populations (29). In the absence of the Duffy antigen, parasites are restricted to invading host reticulocytes, limiting their overall pathological impact (30-36). Though it has recently been shown *P. vivax* no longer requires the Duffy antigen for invasion, the parasite is now known to preferentially invade reticulocytes (31).

Plasmodium vivax is able to survive in temperate climates, in part to its ability to form hypnozoites, a form of liver-stage parasites that remain sequestered and dormant for up to six months (37). This allows *P. vivax* to survive through winters wherein Anopheline mosquitoes are not active. Interestingly, it has been found that the proportion of sporozoites which become hypnozoites fluctuates by region, rising in areas that experience cooler winters, and dropping in areas that experience more consistently warm temperatures (38). *Plasmodium vivax* disease is typically mild, and results is a low-grade fever every 48 hours (benign tertian malaria).

1.1.2 Global expansion of *Plasmodium*

The history of malaria and its dominance throughout certain time periods is largely the result of the social, agricultural, and economic changes that were occurring at the time (39). Though it is likely malaria's human origins began in Africa, it's expansion throughout the rest of the world followed the growth of the various human civilizations that were also being developed (1, 39). As new civilizations developed, the land on which these civilizations grew changed dramatically. In some cases, this led to the creation of suitable breeding grounds for Anopheline mosquitoes that were previously absent from the area.

In the 19th century, malaria could be found on every continent (excluding Antarctica), with well over half of the world's population considered at significant risk of disease. During this time, about 10% of those infected with malaria were expected to die (1, 40). Due to human migration

and successful vector adaptations, *P. vivax* and *P. falciparum* spread to the western world, and by the mid 19th century, more deaths were registered due to malaria than any other disease in the region. In areas most afflicted, mortality rates often exceeded 50% (1, 40).

After the introduction of the antimalarial therapy quinine, overall mortality rates dropped to less than one-quarter of those prior to its introduction, further exemplifying the profound effect on global mortality malaria held (1, 40). Throughout the 20th century, malaria is estimated to have been a factor in 2-5% of all deaths, and likely up to 10% of deaths in the early portion of the century (41). A significant contributor to these mortality rates would have been among repeat *P. vivax* infections as well as prolonged infections of *P. malariae*, both of which are associated with nephrosis, and both of which had a strong hold within the Americas and Asia (1, 24).

Specifically looking at Europe and the Americas, as housing and other living conditions improved in the 20th century, so did exposure to mosquitoes (Figure 1A, B). This combined with the readily available quinine started the rapid decline of disease throughout these regions. While the southern United States, southern Europe, Mediterranean, and Balkan countries would continue to experience mortality through mid-19th century, rates of disease and death by the end of the century were far less than that of Africa, Asia, and the Western Pacific (Figure 1A-C). In addition to improved living conditions and therapeutic options, the decline in disease was due to vector-control methods, the significance of which were noted after the discovery of the mosquito's role in transmitting the disease (1).

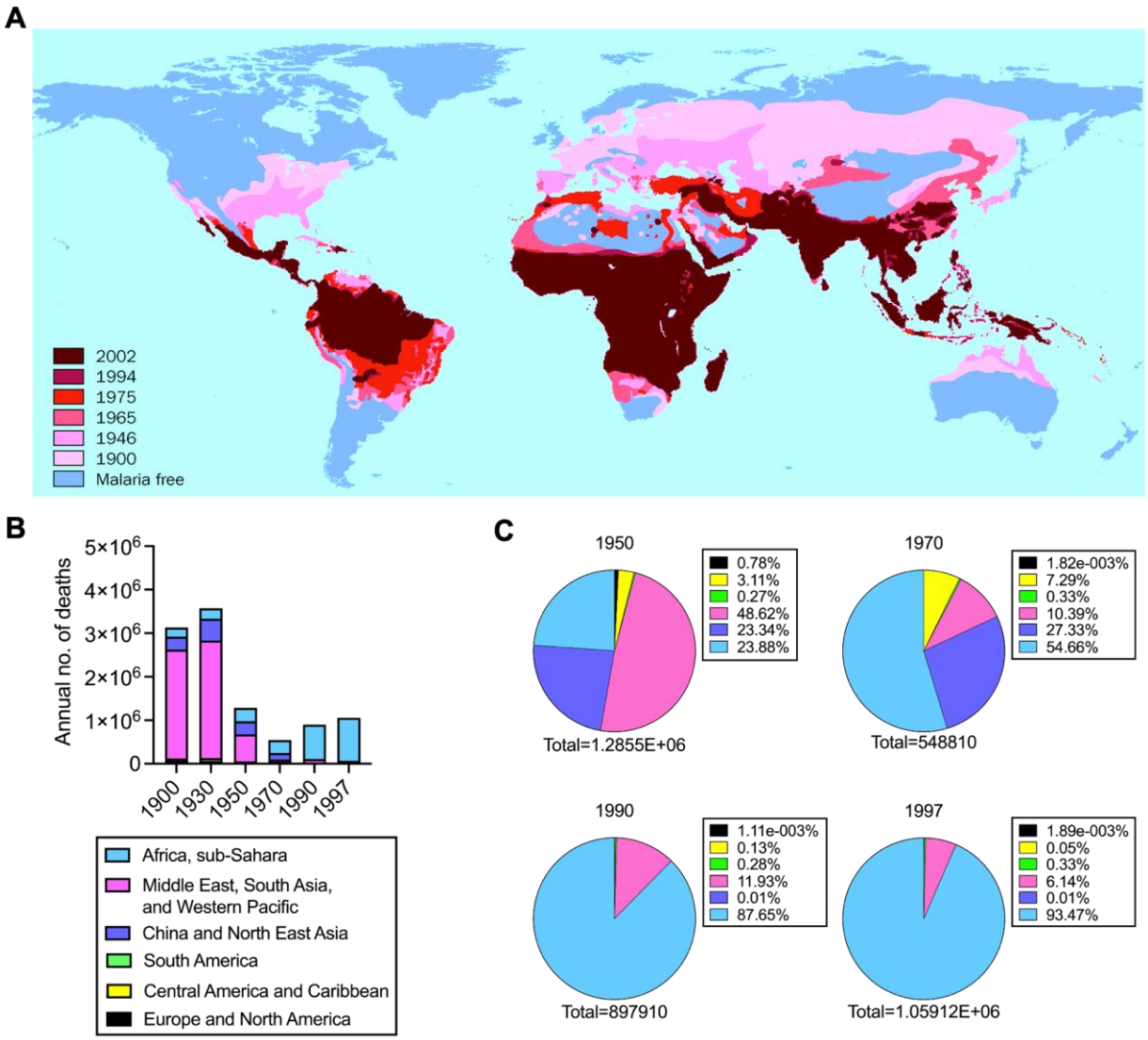

Figure 1. Change in global malaria distribution throughout the 20th century.

(A) Global distribution and populations at risk of malaria through the 20th century. Reprinting with permission from Hay, S. et al (42). **(B,C)** Estimation of the annual number of deaths by region and decade throughout the 20th century (1).

At the beginning of the 20th century, the Middle East and Asia contributed to the most malaria-associated burden of disease (Figure 1B, C). Epidemic malaria in India, political instability, and multiple wars contributed to a strong malaria presence throughout these regions, which lasted throughout the 1950s (41, 43). Between 1950 and 1970, the development of national

control programs which implemented screening programs, as well as the distribution of DDT and the antimalarial chloroquine resulted in an impressive reduction in malaria burden. However, antimalarial and anti-insecticidal resistance then began, causing a resurgence, particularly within China (44). In response, China initiated an impressive campaign combining case detection with treatment, and by the 1990s, with the help of the newly developed antimalarial artemisinin, cases had fallen to just a few hundred a year (Figure 1B, C) (45).

While there has seemingly been much progress in malaria control efforts around the world, populations throughout Africa have had a much longer and much more complicated history with disease. Part of this history includes the evolution of certain populations to avoid or manage infection. As referenced previously, the emergence of Duffy-antigen negativity in response to *P. vivax* amongst West Africans, which likely emerged first, is a mutation that still exists among lineages today (35, 36). While this mutation may be lesser known, likely because of its non-pathological effects in afflicted populations, the effects of *P. falciparum* have been much more profound, as it is widely believed to be the driving force behind the emergence of prominent disease-causing hemoglobinopathies and erythrocyte mutations (46, 47).

Attempts to control malaria throughout Africa did not experience the same level of success as other regions in the 20th century (Figure 1). With the stability and intensity of transmission, along with the recognized immune responses that had been formed among many African populations, it was actually questioned whether an attempt to decrease transmission using DDT could be detrimental (48). Testament to this was a successful malaria-control campaign in Madagascar, which started around 1960 and reduced malaria transmission significantly by 1980. Six years later, however, a malaria epidemic began, which ravaged the population for two years

after all immunity had been lost (49). The effects of this epidemic were a reminder of the devastation that occurs once immune protection is lost in an area where malaria naturally thrives.

1.2 Current epidemiology and outlook of malaria

The first twenty years of the 21st century has been overall successful in the effort to reduce malaria morbidity and mortality, seeing an approximate 40% decrease in disease mortality between 2000 and 2019 (50). In 2015, the World Health Organization (WHO) implemented the Global Technical Strategy (GTS) for malaria, with defined goals for reduction between the years of 2016-2030. Though 46.2% of endemic countries reached the 2020 goal for a 40% reduction in malaria mortality from the 2015 rates, this same goal was not met globally. Notably, though approximately half of the countries reaching the GTS 2020 goal reported zero malaria cases, 20% experienced an increase in cases (Figure 2), and between 2014 and 2019, mortality rates fell on average only 2%, indicating a dramatic plateau in the decrease of change.

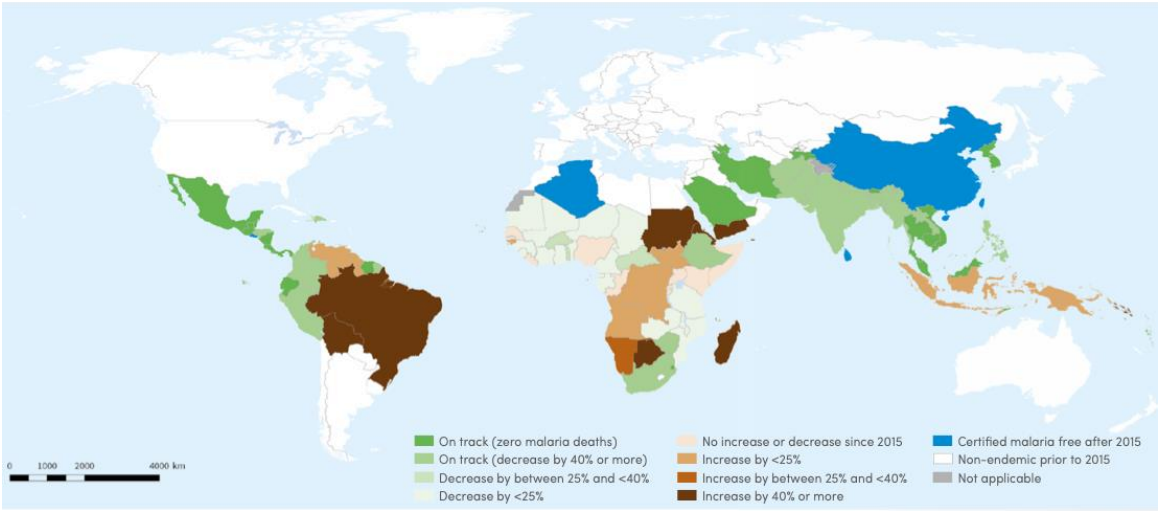

Figure 2. Map of malaria endemicity.

Reprinted with permission from the WHO World Malaria Report 2022 (50).

Current trends indicate that globally, the GTS 2030 goal for case mortality is 89% lower than the current global trajectory (50). The fragility of successful malaria control strategies, which ultimately resulted in the decline between 2000 and 2019 were exposed between 2018 and 2020, as during this time, the global population experienced a 55% increase in deaths attributed to *P. falciparum* (8). This global resurgence of disease and mortality is mostly attributed to obstacles following the COVID-19 pandemic (8, 51, 52). However, with the continued emergence of drug resistant mutations in both *Plasmodium* and Anopheline mosquitoes for nearly all developed therapeutics, new insights and methods into malaria control are long overdue to combat the apparent revival of *Plasmodium spp*., particularly *P. falciparum* (53, 54).

1.3 *Plasmodium* lifecycle

The lifecycle of all human malaria species is complex, as parasites cycle through multiple developmental stages within both a human host and mosquito vector (Figure 3). The length of these lifecycle stages varies between each species of *Plasmodium*, contributing to their unique transmission capacities. For the purposes of this dissertation, focus will remain on the lifecycle of *P. falciparum,* with major differences significantly contributing to species pathogenicity outlined in Table 1.

Table 1. Distinguishing features among the lifcycles of the human *Plasmodium spp. (27, 55-57).*

Species	Liver Stage Time	Intraerythrocytic Cycle Time	Merozoite No.	Gametocyte Maturation Time	Hypnozoites
P. falciparum	7 -10 days	48 hours	16-32	7-10 days	No
P. knowlesi	1 day	24 hours	10-16	1.5-2 days	No
P. malariae	14 days	72 hours	6-14	10-12 days	No
P. ovale	2 days	48 hours	4-16	2 days	Yes
P. vivax	6-8 days	48 hours	12-24	2-3 days	Yes

1.3.1 Human

1.3.1.1 Exo-erythrocytic cycle

Human infection begins when *Plasmodium* sporozoites escape the salivary glands of an infected *Anopheles* mosquito during a blood meal. The sporozoites enter the bloodstream after traversing the dermis, and then quickly migrate to the liver where they begin the hepatic stage of infection (Figure 3A,1). Typically, less than 100 sporozoites are deposited to the host during an infectious bite (58-60). Of these, only 70% successfully make it to the bloodstream (58). The efficient journey to the liver is aided by the circumsporozoite protein (CSP) located on the sporozoite surface. The CSP interacts with heparan sulphate proteoglycans (HSPGs) of hepatic cells, which contain a higher degree of sulphation than HSPGs located in other tissues. This differentiator is thought to enable the specific trafficking of sporozoites to the liver (61-63).

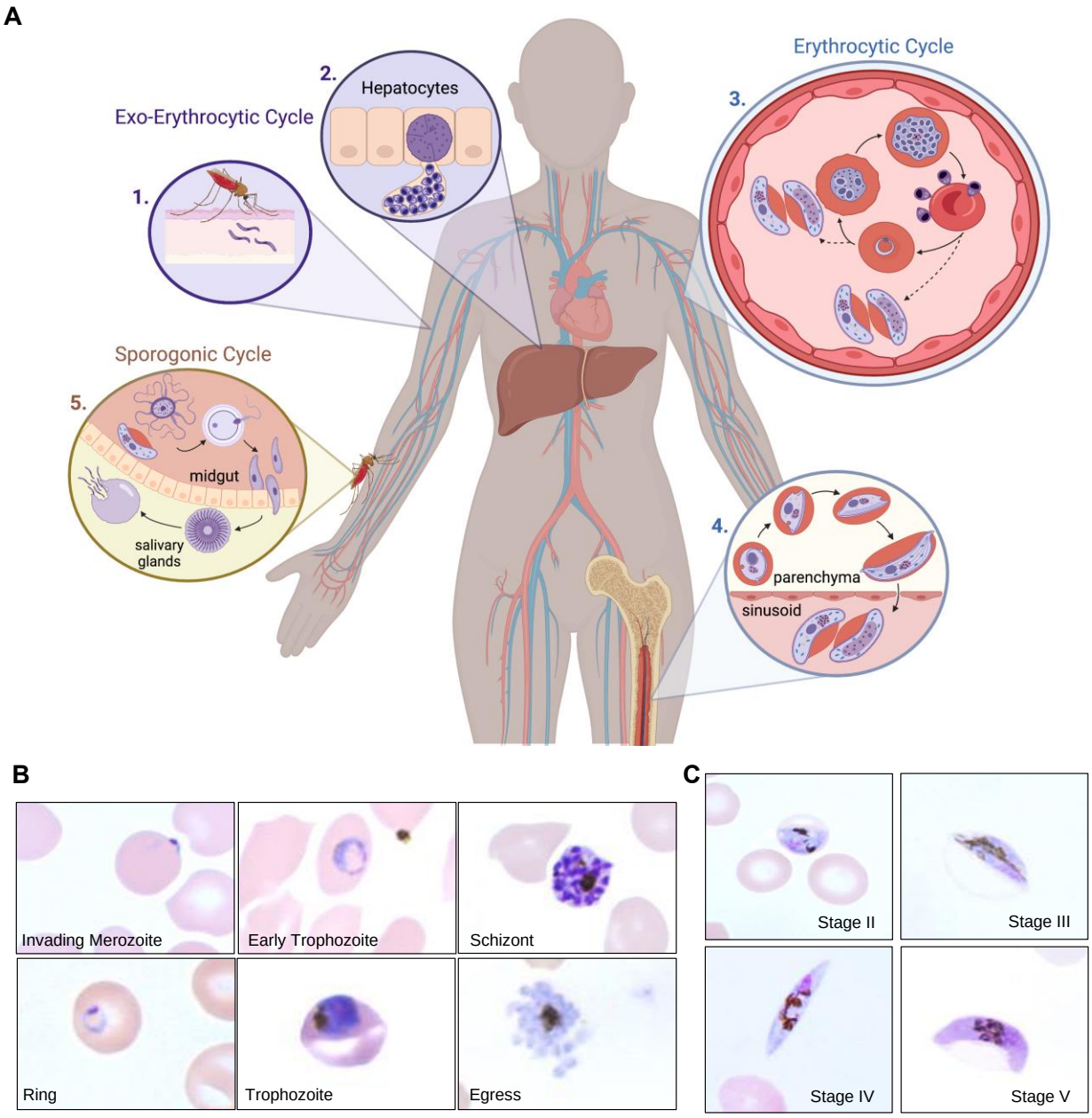

Figure 3. *P. falciparum* lifecycle through human host and mosquito vector.

(A) *P. falciparum* cycle through the exo-erythrocytic (1, 2) and erythrocytic cycle (3, 4) in the human, and the sporogonic cycle (5) in the mosquito. Created with Biorender. **(B, C)** Representative images of aseuxal cycle **(B)**, and sexual cycle **(C)**. Images captured using Olympus Provis microscope (100x) and obtained from blood smears stained with Giemsa.

The entire liver stage of infection in *P. falciparum* occurs over the course of about seven days. After entering the liver parenchyma through liver sinusoidal endothelial cells, *Plasmodium* sporozoites burrow through several hepatocytes prior to establishing a productive invasion, characterized by the formation of a specialized membrane-bound compartment, the parasitophorous vacuole (PV) (Figure 3A,2) (64). Upon an established infection within a hepatocyte, the parasite forms nutrient transport channels, which will support growth and schizogony (65, 66). Hepatic schizogony ultimately results in the production of up to 90,000 daughter merozoites (67).

The liver stage of infection concludes upon the release of merozoites, which will go on to invade erythrocytes in the blood stream. Though the method of merozoite release is commonly referred to in terms indicative of a rupture or burst of the host cell, observations of the process in murine models have revealed a different mechanism (66, 68, 69). Instead, the PV within the hepatocyte ruptures, releasing merozoites into the host cell cytoplasm. This induces the formation of merozoite-filled vesicles, termed merosomes, which bud directly into the liver sinusoid. The merosome is enclosed by the host hepatocyte plasma membrane, which allows the structure to evade host immune mechanisms (70). Each merosome, released into the bloodstream, will rupture upon entering the pulmonary capillaries, releasing anywhere from 10 to 1000 merozoites (66, 71).

1.3.1.2 Erythrocytic cycle

1.3.1.2.1 Invasion

The blood stage of infection begins upon the successful release of merozoites to the bloodstream. Merozoites are the smallest stage of the parasite's lifecycle, averaging 1-2 μM, and have an apicomplexan cell structure, containing an apical complex of secretory organelles and an

apicoplast. The apical complex, which comprises the rhoptries, micronemes, and dense granules, are essential for erythrocyte invasion, a stepwise event involving initial interaction, apical interaction, penetration, and recovery (Figure 3A,3) (72). The process of invasion is rapid, in some cases occurring in as little as one minute (72).

Initial contact with the erythrocyte by the surface of the merozoite is an important first step of the invasion process, stimulating both the erythrocyte and merozoite for invasion. Merozoite surface proteins (MSPs) are among the predominant surface proteins to mediate this phase, causing deformations in the erythrocyte surface through increasing surface area contact (73). The stronger the deformation of the erythrocyte surface during initial contact, the more likely invasion will be successful (72, 74). The contact allows merozoites to reorient or roll until the apical end has reached the erythrocyte surface. Initial interaction is reversable, but upon apical contact, the merozoite will form a tighter attachment to the cell, formally beginning the process of invasion (75-77). This adhesion is mediated by either the erythrocyte binding-like (EBL), Duffy binding-like (DBL) or the reticulocyte binding-like homologous (RBL or Rh) protein families (78-80). These adhesins are likely released from the micronemes upon initial contact with the erythrocyte (72).

The formation of tight junctions follows apical attachment and involves the rhoptry proteins AMA-1 (apical membrane antigen 1) and RON2 (rhoptry neck protein 2) (75, 81, 82). While AMA-1 remains on the surface of the erythrocyte, RON2 is embedded and anchored into the erythrocyte and serves as a receptor for AMA-1 binding. Propulsion into the erythrocyte then occurs via the actomyosin motor as the tight junction formed glides along the perimeter of the merozoite surface until the merozoite is engulfed entirely (75, 77). As the merozoite enters the erythrocyte, it becomes enclosed within a parasitophorous vacuolar membrane (PVM) which will

remain tightly spaced from the parasite's plasma membrane throughout intraerythrocytic development.

1.3.1.2.2 Asexual intraerythrocytic cycle

Once inside the metabolically inactive erythrocyte, the parasite begins a drastic remodel of the cell, which will support its development over the course of 48-hours. The intraerythrocytic cycle is commonly divided into three morphologically distinct phases: the ring, trophozoite, and schizont development phase (Figure 3 A,3, B). The ring stage of infection is typically considered to occur from 0-18-hours post invasion and is characterized by a ring appearance when viewed with Giemsa stain (Figure 3B). This appearance is accomplished by a relatively thin central region, surrounded by a thick ring region, which contains the nucleus and other organelles (83, 84). During the ring phase of infection, the parasite exports proteins to the erythrocyte cytoplasm. In order to transport proteins from the host cell to the parasite, proteins must cross three membranes: the erythrocyte, the PVM and the parasite plasma membrane (PPM). Early after invasion (approximately two-four hours) organelles known as Maurer's clefts are formed in the erythrocyte cytoplasm, which are thought to serve a major role in protein trafficking and sorting (85-94).

In order for nutrient acquisition and the expulsion of waste, the parasite increases the erythrocyte's permeability through the creation of new permeability pathways (NPPs). As a result of these pathways, host cell rigidity drastically increases (95-97). This is a significant change for an erythrocyte, which must be able to pass through small capillaries, and perhaps more importantly, traverse splenic passages. Increased rigidity terminates this ability of the infected erythrocyte and places the cell in danger of elimination by the spleen and immune cells. To circumvent this issue, *P. falciparum* exports two key protein families to the erythrocyte surface: *P. falciparum* erythrocyte membrane protein 1 (PfEMP1), and knob-associated histidine-rich

protein (KAHRP) (98-100). The KAHRPs reorganize the erythrocyte membrane into knob-like structures which serve as an anchor for PfEMP1. PfEMP1 then mediates adherence to endothelial receptors while in circulation, allowing for infected erythrocytes to remain sequestered, ultimately preventing splenic clearance (98, 99, 101-103). For this reason, ring-stage parasites are the only asexual phases that present in *P. falciparum*-infected patients' blood smears (100, 104, 105).

Around 15-hours post invasion, the process of hemoglobin (Hb) digestion begins as the parasite further develops into a trophozoite (Figure 4) (106-108). During this time, which spans approximately through 40-hours of development, as much as 80% of host cell Hb will be consumed (106, 109-111). The digestion of Hb is essential for life within the cell, as it provides essential amino acids to the parasite (112, 113). Hemoglobin acquired from the host cell early after the onset of digestion occurs through cytostomes, which are composed of both the PVM and PPM (114-118). With each engulfment, large volumes of Hb are taken from the cytosol of the erythrocyte. Endocytosed Hb is then assumed to be delivered to the digestive vacuole (119), which originates from the coalescence of previously invaginated cytostomes (107, 120, 121). Uptake of Hb through cytostomes persists throughout the trophozoite phase, though smaller quantities of Hb may be acquired.

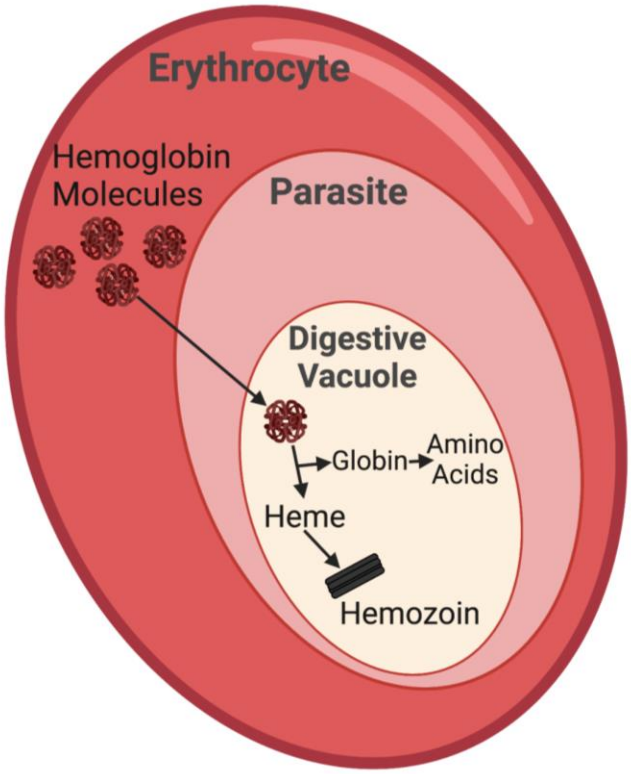

Figure 4. Schematic of Hb digestion and hemozoin formation.

Hb is taken from the erythrocyte into the PV where it is then digested either in the PV or DV. Hb digestion releases the globin chains, which are then recycled for the acquisition of amino acids. The released heme moiety is then polymerized into the inert crystal, hemozoin. Created with Biorender.

Digestion of the acquired Hb may occur prior to reaching the DV, with the first steps of the process mediated by cysteine and aspartic proteases (107, 122-125). The released globin chains are further processed by metalloproteases and aminopeptidases, releasing oligopeptides and dipeptides (126, 127). The digestion of Hb results in the release of heme from the globin portion of the protein (Figure 4). While bound to Hb, heme is responsible for binding oxygen, but when released it is toxic to both *Plasmodium* and humans (107, 124, 128, 129). To detoxify heme, *Plasmodium* polymerize the molecule to form an inert crystal, hemozoin (130). Bond formation between the iron atom of one heme molecule, with the carboxylate group of another form the initial

hemozoin crystal. Formation is continued though the polymerization of dimers via hydrogen bonds (130, 131). Hemozoin formation is facilitated within the DV and requires specific *Plasmodial* proteins (107, 132-134). The process of heme detoxification and formation of hemozoin is required for parasite survival within the erythrocyte. Because of the importance of this processes, Hb digestion and hemozoin formation are prominent drug targets for several antimalarial therapeutics.

Erythrocytic schizogony begins around 36- hours post infection (Figure 3B) (135). During schizogony, parasites undergo multiple rounds of mitotic DNA replication, ultimately resulting in the production of 16-32 merozoites. Hemoglobin digestion may still take place during this phase, providing space for parasite multiplication and growth (110, 136, 137). Tightly packed merozoites remain in the PV until the entire cell ruptures, releasing daughter merozoites to invade new erythrocytes (Figure 3A,3, B). Merozoite egress is a coordinated process, requiring the preemptive remodeling of the erythrocyte membrane. Disintegration of the PVM precedes the rupture of the erythrocyte membrane, which necessitates the action of parasitic enzymes, including the cysteine and aspartic proteases required for Hb digestion (123, 138-142). By the time of PV rupture, the erythrocyte membrane has been primed through the preemptive release of parasite proteases. A pore then forms on the surface of the erythrocyte, leading to a rapid curling of the membrane outward to release the merozoites (143, 144). All released merozoites are capable of infecting new erythrocytes within circulation. Consequently, parasitemia expands rapidly, ultimately resulting in disease.

1.3.1.3 Gametocytogenesis

In order to sustain vector transmission, a subset of asexually replicating parasites become sexually committed and undergo the process of gametocytogenesis. *Plasmodium falciparum* gametocytes mature through five morphologically distinct stages over the course of 7-10 days

(Figure 3A,4, C) (145). Immature micro- and macrogametocyte stages (stage I-IV) are sequestered in the bone marrow during development, while mature stage V gametocytes circulate freely to be transmitted during a blood meal (146-151). The process of asexual parasites becoming sexually committed is essential for vector transmission and will be discussed in greater detail in section 1.4.

1.3.2 Vector

When ingested by an Anopheline mosquito, gametocytes undergo gametogenesis. This process is stimulated by the drop in temperature and increase in pH which occurs upon transfer from the human host to mosquito vector (152). Within the mosquito midgut, microgametocytes undergo three rounds of nuclear replication, resulting in an octoploid genome (153-156). Motile flagella are formed in the cytoplasm during microgametogenesis and will later attach to the nuclear pores to become "sperm". Microgametes then exit the cell in a process termed exflagellation. Microgametogenesis is rapid, with all nuclear replication and exflagellation occurring in as little as 15 minutes post activation (154-156).

Unlike microgametocytes, macrogametocytes do not undergo nuclear replication, and give rise to a single haploid macrogamete. For this reason, sex ratios tend to be skewed towards a higher production of macrogametes, but specific ratios very (157-161). Macrogametes will become fertilized by motile microgametes, which will penetrate the macrogamete surface to form a diploid zygote (Figure 3A,5) (162, 163). Ookinete formation follows meiosis, which occurs over the course of 19-36 hours after the blood meal. Ookinetes are motile, possessing specialized anterior organelles which allow them to traverse the mosquito midgut epithelium to reach the extracellular space in between the epithelium and basal lamina (Figure 3A,5) (164, 165). Surviving ookinetes then become non-motile oocysts, which support sporogony (166). This process closely shadows

hepatic schizogony, but over a much longer period of 10-28 days. Sporogony results in the production of thousands of motile sporozoites, which upon rupture of the oocyst will cross the basal lamina of the mosquito entering the body cavity (Figure 3A,5). Sporozoites then migrate and traverse into the salivary gland epithelial cells and reside in the lumen until the mosquito again takes a blood meal for entire cycle to repeat (167-169).

1.3.3 Lifecycle bottlenecks

The *Plasmodium* lifecycle involves a series of population bottlenecks which are considered appealing targets for therapeutic development. The most severe bottlenecks occur between the transmission of sporozoites from the mosquito to the human, and during the journey of ookinetes from the midgut epithelium to the space between the epithelium and the basal lamina. The former is theoretically associated with a high degree of variability, as a person may receive multiple infectious bites at any point in time. However, infectious mosquitoes have been shown to only contain 2-5 oocysts at one time (170-173). Both of these bottlenecks are followed by the most dramatic increase in population: hepatic schizogony and sporogony, which can result in a 10^8- fold and 10^4- fold increase in parasite populations respectively (174).

These bottlenecks are an important component to the lifecycle and transmission dynamics of all *Plasmodium spp*. The deposit of few sporozoites into the human host though seemingly ineffective or risky, likely works in favor of the parasite to avoid the production of a strong immune response upon infection (58, 175). Likewise, the production of few gametocytes, as well as the timing of gametocyte development, may also serve as a balance of human infection to productive mosquito transfer. Therefore, though these bottlenecks may appear to pose a disadvantage for

parasite survival, the few parasites required in order to sustain transmission is an attuned method to preserve survival and transmission (171, 174).

1.3.4 Maintenance of stage transitions: features of *Plasmodium* gene regulation

Managing and maintaining each stage and phase transition throughout the parasites' lifecycle requires a high degree of gene expression control. Testament to this is the finding that almost 90% of the 5,280 transcribed genes in *Plasmodium* are expressed in a pattern dependent on the lifecycle phase (176). As with other eukaryotes, the mechanisms of *Plasmodium* genetic regulation are multifaceted, involving management at the transcriptional, post-transcriptional, and translational levels. However, several unique features of these individual regulatory mechanisms have been identified.

At the chromatin level, the nucleosome landscape of *Plasmodium spp.* has been shown to play an important role in the first level of gene expression regulation. While nucleosome positioning in eukaryotic organisms is partly determined by GC-content, the same rules cannot be applied to the overwhelmingly AT-rich genome of *Plasmodium spp.* (177-180). Nucleosome occupancy is correlated with differential gene transcription, supporting changes and transitions throughout the lifecycle, though no change in global chromatin structure has been revealed (181-183). In general, higher nucleosome occupancy is found among silenced genes, while nucleosome-depleted regions are found upstream of the transcriptional start site of active promotors. Intergenic regions have been found to have little nucleosome occupancy, but are enriched with certain histone variants (184).

The overall organization of chromatin supports parasite-specific histone modifications. Each precise modification further associates to the parasites' cell cycle progression, transcriptional activity, and DNA replication (185). Specific histone modifications may be associated with euchromatin or heterochromatin, and are reversible and controlled by histone modifiers, writers, and erasers. (184). Other epigenetic regulators such as small and long noncoding RNAs have been an area of increased study in *P. falciparum* and have been shown to regulate gene expression by recruiting chromatin-modifying enzymes to specific locations (186-189).

Interestingly, the predicted number of transcription factors encoded by *P. falciparum's* genome is approximately one-third of the predicted number based on the number of its total genes (190, 191). Of the identified transcription factors, one novel group makes up the vast majority. These are the ApiAP2 family and have been proposed to act as master regulators of transcription during parasite development, wherein hundreds of genes for each specific stage would be under the control of one factor (192, 193). This theory would help explain how such a comparatively small number of transcription factors are able coordinate such convoluted gene expression patterns throughout development (194, 195).

1.4 Malaria pathology

Though there are six recognized species of *Plasmodium* that may cause malaria in humans, *P. falciparum* results in the most severe cases and deaths. Today, most discussion surrounding malaria disease refers specifically to Falciparum malaria, and *P. falciparum* is often regarded as the only species to cause severe disease syndromes (50). Though malaria may cause a wide clinical spectrum, in general, disease is categorized based on severity as either complicated or

uncomplicated. While uncomplicated disease typically manifests with fever, chills, headache, muscle pains, nausea and vomiting, complicated disease describes various severe syndromes, which are all associated with a high mortality and require rapid treatment. The most common manifestations of severe malaria syndromes are cerebral malaria (CM), severe malarial anemia (SMA), placental malaria, and malaria-associated acute respiratory distress syndrome (MA-ARDS). As with the lifecycle, discussion will focus on the features of Falciparum malaria, though insights into other species are highlighted as necessary.

1.4.1 Circulatory pathogenesis

Symptoms of malaria result only from the intraerythrocytic phase of infection, wherein the gradual expansion of blood-stage parasitemia following subsequent rounds of asexual replication gives rise to varying degrees of intravascular hemolysis. As the timing of intraerythrocytic development for each species varies, so does the timing and frequency of symptoms. For example, the 48- hour intraerythrocytic cycle of *P. falciparum* results in symptoms every third day, while *P. malariae*, which has a 72- hour intraerythrocytic cycle, results in symptoms every fourth day.

Following large amounts of intravascular hemolysis, which is consequent of schizont maturation and merozoite egress, patients often become anemic. Further erythrocyte loss then occurs due to increased splenic clearance and a decrease in uninfected erythrocyte half-life (196, 197). It has been estimated that during infection, malaria patients lose eight uninfected erythrocytes for every infected erythrocyte (198, 199). Over subsequent infections, the development of self-reactive anti-phosphatidylserine antibodies contributes to the development of chronic malaria, as reticulocytes produced in response to malarial anemia expose phosphatidylserine on the cell surface early in development (200, 201). Therefore, the unusual abundance of phosphatidylserine

exposed to immune cells during infection, leads to the generation of anti-phosphatidylserine antibodies. The presence of these antibodies may then act against newly produced reticulocytes, further contributing to the development of chronic anemia.

Intravascular hemolysis also leads to the release of Hb, heme, and hemozoin from infected cells. Extracellular Hb is rapidly degraded, further releasing more heme directly into the circulation. As mentioned, heme is toxic, presenting as a danger-associated molecular pattern molecule and a factor for neutrophil activation (202). Resulting oxidative damage has been proposed to contribute to decreased macrophage function and neutrophil exhaustion (203, 204). Interestingly, hemozoin accumulation within phagocytes has been associated with a dysfunction in erythropoiesis, another contributing factor to the development of anemia (205-209).

In Falciparum malaria, parasites express variant surface antigens (VSAs) on the surface of the erythrocyte membrane. The presence of these antigens both contributes to pathogenesis and allows the parasite to evade the host immune system. Multiple gene families comprise VSAs, including the PfEMP1, sub-telomeric variant open reading frames (STEVOR), and repetitive interspersed families of polypeptides (RIFIN) families (210, 211). PfEMP1 is the predominant family responsible for cytoadherence, binding infected erythrocytes to host endothelial receptors to prevent circulation through the spleen (212-214). The role of both STEVOR and RIFIN antigens in cytoadherence is not as well understood, but both are known to mediate parasite rosetting, which occurs when an infected erythrocyte becomes sticky, causing uninfected erythrocytes to adhere to its surface (215, 216). This can lead to blockages in the microvasculature, ultimately contributing to decreased oxygen delivery to tissues (215-219).

1.4.2 Organ and tissue pathogenesis

Though the beginnings of disease occur in the circulation, malaria may progress to affecting organ function. The architecture of blood vessels likely plays a role in this process, as proximity to lymphatics, vessel diameter, and tension between endothelial cells varies throughout the body. Additionally, different variants of PfEMP1 seem to exhibit varying binding affinities to receptors throughout the host. The distribution of the binding may thus determine complicated malaria syndrome development. For example, binding of PfEMP1 to EPCR and ICAM1 leads to sequestration in the brain, ultimately causing CM, while binding of chondroitin sulfate A (CSA) and IgM result in placental sequestration and the development of placental malaria (220-224).

Despite mechanisms to avoid splenic passage, one of the key clinical symptoms of malaria is splenomegaly (225). The increase in splenic size and weight is associated with the expansion of cell populations as a result of the accumulation of both infected and uninfected erythrocytes. As ring stages continue to circulate prior to the expression of KHARP and PfEMP1 proteins, ring stage parasites collect in the spleen, along with uninfected erythrocytes that have become covered in parasite antigens released after schizont rupture. In response to these damaged and infected cells, macrophage populations expand in the red pulp. Furthermore, the white pulp, usually containing designated T and B cell zones, becomes muddled, scattering cell populations throughout the region (225, 226).

Parasite sequestration in the bone marrow, while perhaps not immediately associated with disease phenotypes, has been a more recent and important finding, especially related to the gametocyte transmission stages. Though both asexual and sexual stages have been found to sequester within the hematopoietic niches of the bone marrow, the bone marrow is the only organ shown to contain considerable enrichment of gametocyte stages (227). The specialized

environment of the bone marrow may provide a sanctuary to avoid antimalarial treatment regimens, and an enrichment of cell types suitable for proliferation (*P. vivax*) and sexual development (227-229). The former presents a unique challenge which may be contributing to antimalarial resistance, in that successive exposure to sub-optimal doses of therapeutics may effectively select for resistant phenotypes (230, 231).

1.4.3 Antimalarial therapies

Currently, there are four classes of antimalarial drugs that are able to treat malaria: artemisinin derivatives, quinoline derivatives, antifolates, and aminoquinolines. While each class has a different mechanism of action, interestingly, all are in some way related to heme or hemozoin. While quinoline derivatives and aminoquinolines interfere with hemozoin production, or the detoxification of heme, artemisinin derivatives actually require heme in order to work. When artemisinin comes into contact with heme, it forms free radical intermediates which then generate reactive oxygen species (ROS). The generation of ROS disrupts normal functions and eventually leads to membrane damage (132, 232-234).

The currently available antimalarials may be used in combination or as prophylactic therapy. With the increase in resistance, treatment is increasingly regulated to individuals with severe symptoms, children, pregnant women, and diagnosed travelers. In attempts to circumvent resistance, treatment guidelines are being constantly re-assessed and updated (50).

1.4.4 Mechanisms of immunity for malaria control

Over subsequent infections, it is possible for humans to acquire immunity to malaria. This immunity can be defined on three different levels (anti-disease, anti-parasitic, and anti-infection) and is heavily dependent on the degree of exposure, exposure frequency, and age. Importantly, in the cases of anti-disease and anti-parasitic immunity in which infected individuals experience little-to-no disease symptoms, parasite biomass may remain high. Therefore, a key question throughout malaria infection is how disease is controlled, but still manages to persist.

1.4.4.1 Epidemiology of malaria immunity

The epidemiology of malaria disease exhibits great complexity and heterogeneity between locations. This is, at least in part, due to differing rates of acquired immunity which is dependent on malaria endemicity and transmission. As malaria transmission increases, the age at peak severe malaria incidence decreases (235). Thus, protective immunity is generally acquired at early ages in areas of high transmission (236, 237). In these areas, the risk of severe disease peaks early, and then drops, typically after the first year of life. The risk for uncomplicated disease follows a similar pattern, but over a much longer timeframe, as incidence will increase, eventually hitting a plateau, and then decrease over approximately 10 years of life (236).

The overall risk of developing severe malaria in children younger than 10 years might be lower in areas with high transmission, rather than low-to-moderate transmission (235). This is because older individuals are more likely to develop severe malaria in the absence of immunity, therefore, the delayed acquisition of immunity in low-to-moderate transmission zones may increase the percentage of infections that progress to severe malaria. Indeed, it has been shown that the incidence of severe malaria is spread over a wide age range in areas of unstable

transmission (235, 236). These infections are also associated with multiorgan disease, as opposed to severe malaria in children, which is associated with one single life-threatening syndrome (238, 239).

Interestingly, the observation that immunity to severe malaria can be acquired early in life has led to the hypothesis that there are conserved antigens which may elicit more severe disease. This may include specific parasite strains or parasite phenotypes. This theory is supported by the additional observation that episodes of severe malaria seem to cluster in space and time (240).

1.4.4.2 Immune mechanisms of the human host

Though the pre-erythrocytic cycle of infection does not cause disease, sporozoites are potent stimulators of an innate and adaptive immune response. This finding has made them attractive vaccine candidates, and it has been shown that individuals vaccinated with sporozoites are resistant to malaria infection. However, the feasibility of this vaccine has not been validated (241-245). In mice, this protection was shown to require antigen-specific CD8+ T-cells (246, 247). Injected intra-dermally, sporozoites that do not make traversal to the circulation are found in the subcapsular zones of the skin draining lymph nodes, where they are taken up by resident dendritic cells which will go on to prime CD8+ T-cells (248). Activated CD8+ T-cells then migrate to the liver where they can effectively eliminate infected hepatocytes. Moreover, infected hepatocytes induce innate immune responses, which are reliant on interferon-1 (IFN-1) signaling (249-251). The specific effector mechanisms of CD8+ T-cells are an area of interest, as multiple studies have suggested a multifaceted role of IFN-γ, TNF-α, perforin, and Fas ligand (252, 253).

At early stages of erythrocytic infection, the innate immune response is critical to control the exponential expansion of asexual parasitemia throughout the circulation. Though erythrocytes lack MHC antigen processing and presentation machinery, CD4+ T helper cell responses are

launched and are important to produce proinflammatory cytokines which activate macrophages. Monocytes and neutrophils function to phagocytose merozoites and infected erythrocytes (254-258). The immune response early upon onset of the erythrocytic cycle has been shown to predict the outcome of infection in mouse models (259). *In vivo* studies of African children have shown that severe malaria syndromes are associated with elevated inflammatory mediators, including TNF-α, which is released by macrophages upon recognition of pathogen associated molecular pattern molecules (PAMPs) (260). During malaria infection, predominant PAMPs include glycosylphosphatidylinositol and hemozoin. While excessive inflammatory mediators seem to cause severe disease, moderate levels of both IFN-γ and TNF-α are predicted to provide a more controlled response, limiting parasite replication without promoting disease (240). Additionally, a high ratio of IL-10:TNF-α has been shown to be associated with a reduced risk of SMA (261). Importantly however, too much of an anti-inflammatory response early in infection may result in suboptimal control of parasite replication, ultimately resulting in a high-density infection and mortality (262).

The main virulence factors of *P. falciparum* are the VSAs located on the erythrocyte membrane (predominantly, PfEMP1, RIFIN, and STEVOR). Each of these families is capable of polymorphic gene expression with PfEMP1 encoded by 60 copies of *var* genes, RIFIN encoded by 200 copies, and STEVOR encoded by 30 copies (263-265). PfEMP1, known to be responsible for parasite sequestration in the endothelium, is also known to reduce dendritic cell induced T-cell proliferation through binding of CD36 (266). Moreover, in monocytes, PfEMP1 has been shown to induce a weaker expression of cytokines (267). The RIFIN family of proteins also play an immuno-modulatory role through binding of a group of receptors which are broadly expressed on myeloid cells and lymphocytes (268-272). The binding of these receptors can downregulate cell

activation through the antagonization of activation signals (273-276). Cellular responses against gametocyte-infected erythrocytes have also been observed, but less is understood about these mechanisms. There is evidence that phagocytosis occurs for stage I and early-stage II gametocytes, though interestingly not for later stages of development (258, 277).

Antibodies play a key role in acquired immunity to the pre-erythrocytic, erythrocytic, and gametocyte stages of malaria infection. Antibodies against CSP function in preventing sporozoite invasion of hepatocytes, activating complement, and initiating an antibody-dependent cell-mediated elimination mechanism of infected hepatocytes by Kupffer and NK cells (278). Antibodies formed against merozoites function primarily in opsonization, invasion inhibition, and complement-mediated elimination (279). Adhesion and sequestration of infected erythrocytes and gametocytes can be inhibited and blocked by antibodies, ultimately leading to elimination in the spleen. Effector functions of antibodies against gametocytes and asexually replicating parasites also include complement activation and neutralization of released toxins from infected cell rupture (279, 280). Additionally, many studies have recognized the ability of human-derived antibodies to effectively prevent sexual replication after mosquito transfer (257, 281-284).

In 2021, the WHO recommended the implementation of the RTS,S vaccine, also known as Mosquirix (285). This subunit vaccine, which has been in development since the 1980s, combines a recombinant portion of the CSP protein with the hepatitis B antigen and an adjuvant, AS01B. RTS,S has been shown to stimulate both cellular and humoral responses. The cellular response, mediated by T cells, recognize hepatocytes which have been infected by sporozoites surviving the humoral response (286, 287). The humoral response, mediated by B cells, is active against the CSP, functioning to prevent hepatocyte invasion (288, 289).

The frequency of malaria exposure and age have been found to influence the efficacy of RTS,S. In a four-year phase III trial, young children, between the ages of 5-17 months, were found to experience greater efficacy compared to young infants, those between 6-12 weeks. Efficacy over the first year with a primary 3-dose administration was found to be 55% in the older age group. As efficacy was previously shown to wane, a fourth booster dose was administered, providing up to 36% protection from symptomatic malaria and 29% protection against severe malaria through four years (290, 291).

1.4.4.3 Immune mechanisms of the mosquito vector

As humans have developed mechanisms to control *Plasmodium* infection, Anopheline mosquitoes, which are also burdened by parasite infection, are likewise equipped with immune defenses against the pathogen (292-294). Anopheline mosquitoes contain cellular and humoral responses which primarily act against ookinete traversal of the midgut and sporozoite migration to the salivary glands (295). The mosquito hemolymph contains thioester-containing proteins, which serve a complement-like role in ookinete control (296). Hemocytes, the primary immune cells of mosquitoes, function in phagocytosis, secretion of antimicrobial peptides, and encapsulation nodule formation leading to melanization (292, 297).

Hemozoin has also been shown to activate key genes involved in the mosquito immune response (298, 299). As asexual parasites are not excluded during the feeding process, a blood meal on an infected individual likely contains asexual parasites, as well as, in the case of active transmission, gametocytes. Though the asexual parasites are a source of hemozoin, depending on the state of the infection, so is the whole blood, as hemozoin is released upon schizont rupture. Interestingly, hemozoin has been shown to negatively impact the development of oocysts in mosquitoes and has been proposed to interfere with digestion of the blood meal, causing

mosquitoes to feed more frequently (300). Therefore, as the generation of hemozoin is a sign of effective proliferation, the parasite is further able to promote its own transmission through dysregulation of the mosquito's feeding patterns.

1.5 Transmission and gametocytogenesis

As *Plasmodium spp.* have repeatedly been able to successfully navigate host and vector defenses as well as pressure exerted by antimalarial therapies, the focus on host-to-vector transmission as a means of malaria elimination has increased. In order to sustain vector-transmission, and thus the parasite's lifecycle, a subset of intraerythrocytic asexually replicating parasites undergo gametocytogenesis, a process which gives rise to gametocytes which then sexually reproduce within the mosquito. Commonly used antimalarials do not immediately eliminate gametocytes, meaning even after successful treatment addressing asexual parasitemia within a human host, the infected individual is likely still capable of spreading gametocytes for at least two weeks after the start of treatment (301). Only two gametocytes, a microgametocyte (male) and a macrogametocyte (female), within the approximate 4 μl volume of the mosquito gut are required to sustain the transmission cycle.

As discussed previously, *P. falciparum* gametocytes mature over the course of 10-12 days within the human, and progress through five morphologically distinct stages (Figure 3C). Immature stages (stages I-IV) are sequestered primarily in the bone marrow, while mature stage V parasites circulate freely for around six days (145-151, 302). Transmission dynamics and gametocyte conversion rates (GCRs) are known to vary depending on a variety of factors, both environmental and social. Recently, much progress has been made in uncovering the known

molecular mechanisms of gametocyte conversion, environmental signals that have been found to increase gametocyte conversion, and evolutionary theories that help define and predict gametocyte conversion patterns.

1.5.1 Molecular mechanisms of sexual conversion

On a molecular level, the process of initiating gametocytogenesis, or the process of parasites becoming sexually committed, is reliant on a master-regulating transcription factor of the ApiAP2 family of transcription factors, AP2-G (Figure 5) (194, 303, 304). During asexual replication, the gene locus of *ap2-g* remains silenced by the epigenetic reader heterochromatin protein 1 (HP1), which is associated with histone 3 lysine 9 trimethylation (H3K9me3) (305, 306). H3K9me3 represses sub-telomeric gene families and other genes that have been associated with sexual conversion (307). Histone deacetylase protein 2 (Hda2) contributes to the epigenetic silencing of the *ap2-g* locus, presumably by reducing histone acetylation during DNA replication, ultimately enabling methylation and gene repression during the next generation (308, 309). De-repression of *ap2-g* results after the removal of HP1 by gametocyte development protein 1 (GDV1), which has been shown to be regulated by an anti-sense RNA (306-308, 310). Field studies have shown allelic variance in *gdv1*, indicating changes in transmission dynamics between the populations (311, 312).

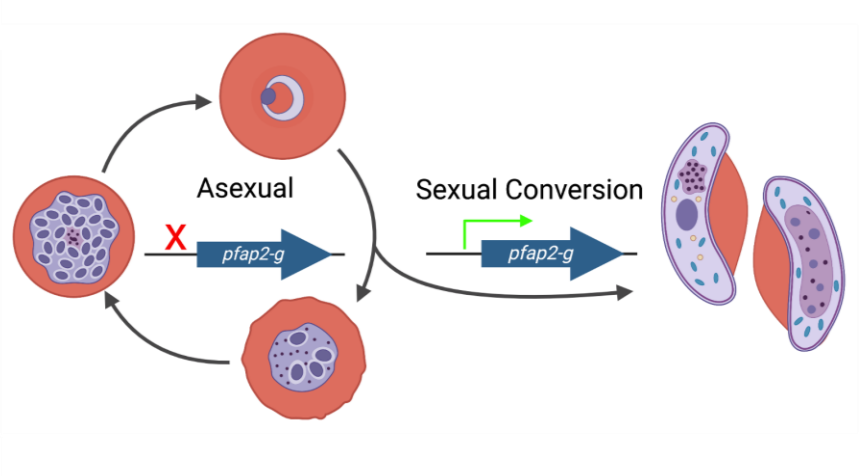

Figure 5. *ap2-g* control of gametocyte conversion.

Schematic of transcriptional control of sexual conversion by ap2-g. Created with Biorender.

Upon expression, AP2-G acts as a transcriptional switch, inducing sexual commitment and the activation of other gametocyte-specific genes, ultimately resulting in sexual conversion (303). The transcription factor AP2-G recognizes the GNGTACNC motif, which is found in the upstream region of many early-expressed gametocyte genes (194, 313). Interestingly, this motif is also found upstream of *ap2-g*, indicating de-repression and expression of *ap2-g* leads to a positive feedback loop which enhances its own expression (194, 313-315). Expression of *ap2-g* during the ring stage of the intraerythrocytic cycle (0-20 hours post invasion) results in sexual differentiation of the parasite in that single cycle. Expression of *ap2-g* after 20 hours post invasion results in one additional cycle of asexual replication, with all resulting merozoites sexually committed and beginning the process of differentiation upon invasion of an uninfected erythrocyte (316).

1.5.2 Environmental factors influencing sexual conversion

As the process of sexual conversion is essential to sustain parasite transmission, factors that initiate commitment have become of increasing interest. To date, only one specific environmental stimulant has been firmly established, while other more general environmental conditions have been proposed (227, 317-321). While field studies to determine gametocyte carriage are usually completed among symptomatic patients, more investigation into potential gametocyte carriage among asymptomatic infections is currently being undertaken (322-326). A recent study found median gametocyte density and median peak gametocyte density was significantly higher among chronic infections compared to incident infections. This was further correlated to higher mosquito infectivity, even after adjusting for gametocyte density (327). Furthermore, a systematic review completed in 2016 found that among 48,840 uncomplicated malaria patients, 12.1% contained gametocytemia by microscopy (328). Considering transmission is still possible below the microscopic-detection range, this number is likely a conservative representation of an infectious reservoir among non-severe and asymptomatic cases.

Both of these studies found higher gametocyte presence among younger ages, with one going on to determine young children had a longer duration of gametocyte carriage (327, 328). Ultimately, this may indicate acquired immune factors play a role in gametocyte clearance. Interestingly, another study found an association between gametocyte prevalence in familial groups, suggesting a genetic contribution to gametocyte production. Associations were only found among asymptomatic *P. falciparum* infections, while no association was found with symptomatic infections (329).

As gametocytes are not immediately eliminated by antimalarials, much interest has been placed on the effect of antimalarial therapy on gametocytogenesis, especially in the context of

usage on a preventative level. Though there have been some variable results, in general it is accepted that antimalarials actually promote gametocyte carriage. However, there is an important distinction to be made in that there is no evidence to support a direct effect of any antimalarial in the promotion of gametocytogenesis. Rather, it is assumed that while antimalarials have little to no gametocidal effect, their effect on asexual parasites sends stress signals which promote gametocytogenesis (319, 328, 330). Though a recent study did find increased *pfap2-g* expression after artemisinin-based treatment in two different locations, which was supported by increases in several other early gametocyte genes, the direct effects have yet to be proven (331).

Other field studies have found an association between gametocyte carriage and Hb concentration. However, as previously discussed, two characteristics of malaria pathogenesis are anemia and dyserythropoiesis. Therefore, it is difficult to determine whether anemia is a causal factor in gametocytogenesis, or if, over the course of infection, both are normal consequences of prolonged infection. Interestingly Hb variation, which may cause anemia in affected individuals, has also been associated with gametocyte production (321, 332-334). This phenomenon will be discussed in greater detail in section 1.6.

During *in vitro* culture, it is generally accepted that sexual conversion is induced by physiological stress on the cultures (309, 335, 336). This has been demonstrated with the use of spent media, allowing cultures to reach a high parasitemia, a decreased hematocrit, and with the use of sub-optimal levels of antimalarials (314, 319, 337, 338). It is known these stress mechanisms induce reactive oxygen species, alter the availability of required nutrition, and effect the levels of metabolic signaling molecules and metabolites, but a specific pathway has yet to be elucidated (318, 339-342).

Recently, the depletion of a specific metabolite, lysophosphatidylcholine (LysoPC), was found to be the first factor to induce gametocytogenesis (343). LysoPC was further shown to be the parasite's primary source of choline, of which depletion also induces sexual conversion (343). *Plasmodium spp.* metabolize choline to phosphatidylcholine (PC), which is a major component of phospholipid membranes in all eukaryotic cells. During schizogony, PC is required for the production of merozoites, and as such, after the depletion of LysoPC, parasites were shown to survive for only one additional asexual cycle prior to displaying an increased rate of sexual conversion (343). Interestingly though, in the complete absence of LysoPC, sexual conversion rates did not exceed 30%, meaning 70% of parasites from each generation continued to replicate asexually. Therefore, though a factor for sexual conversion, alternate mechanisms must exist.

1.5.3 Vector dynamics of transmission

Malaria was once transmitted freely in northern Europe and throughout North America, which was only possible through the adaptation of *Plasmodium*, as well as the adaptation of feeding mosquitoes. As populations and ecologies changed throughout western development, so did the ability of several *Anopheles spp.* to both feed from humans and live in human ecologies (39). It is known today that approximately 70 of the over 500 recognized species of *Anopheles* are capable of malaria transmission, and at least one of these species is present on every populated continent (344). While the average female Anopheline mosquito will survive for only 10-21 days, it has been estimated that only around 10% will survive long enough to complete a 14-day sporogonic cycle (345). Though seemingly detrimental to the transmission cycle, the high densities and intricate feeding patterns among circulating mosquitoes adapt for the varying ecological conditions and human patterns.

The likelihood of malaria transmission from one human to another human is relative to the proportion of human blood contained in one mosquito. This metric is also known as the human blood index (HBI) (346). *Anopheles spp.* present outside of Africa are zoophilic rather than anthropophilic, meaning they prefer to feed on animals rather than humans (1, 347). This greatly affects the HBI, and thus the malaria transmission capabilities of the mosquitoes (1, 347-349). In most parts of the world, the anopheline HBI has been shown to be less than 50%, and more typically less than 10-20% (347). The HBI of *Anopheles gambiae*, the most prominent malaria vector in Africa, is often over 80%, sometimes reaching 100% (1, 347, 350). The anthropophilic adaptation of *Anopheles spp.* in Africa has been hypothesized to be a consequence of the adoption of an agricultural lifestyle by human populations. This lifestyle both increased the human population density and created new aquatic habitats for mosquito propagation (39). Importantly, this change in lifestyle did not include the simultaneous domestication of several different animal species as it had in other regions of the world (1, 351). Therefore, in the absence of other potential vectors, *Anopheles spp.* became anthropophilic.

The distribution and implementation of long-lasting insecticidal nets has had an important impact on preventing malaria transmission in many regions throughout sub-Saharan Africa. However, the rise of insecticide resistance among the Anopheline populations not only diminishes their elimination capability, but also contributes to the alteration of feeding dynamics, wherein outdoor feeding has been shown to increase after net dissemination (348, 349, 352, 353).

Field studies have demonstrated differences among transmission rates among seasonal changes in different areas. For example, one study completed in coastal Ghana, showed that gametocyte carriage increased during the dry season in urban areas, but not in rural areas (354). Another study conducted in western Kenya compared gametocyte levels between the dry and wet

season and interestingly found levels to be higher following the dry season. This is curious because prevalence of asexual parasitemia and clinical disease is higher during the wet season, largely due to the ready availability of vector breeding grounds (355). Ultimately, this indicates parasites may be able to alter the investment of asexual and sexual stage development to balance potential vector capabilities. Moreover, genetic analysis of samples collected within low- and high- transmission regions has revealed higher expression of early gametocyte genes in the low transmission regions (356). One intriguing hypothesis that has emerged to explain this phenomenon is that parasites may be able to sense the number of mosquito bites an infected individual receives. However, there is no direct evidence for this association in humans or in rodent malaria models thus far (357, 358).

1.5.4 Evolutionary theories of reproductive control

As it has been discussed, through the course of a typical *P. falciparum* infection, it is known that only a small fraction of parasites under gametocytogenesis to produce gametocytes. The fraction or rate of parasites which eventually become gametocytes, or the GCR is not a static value. Rather, the ability to change the rate of parasites that undergo gametocytogenesis is an important survival adaptation among *P. falciparum,* ultimately which ensures a balance between the parasite's survival within a host (intra-host) and further transmission among different hosts (inter-host). If all intraerythrocytic parasites underwent gametocytogenesis simultaneously, intra-host survival would cease. Moreover, if the rate was consistently higher, there would likely be a more pronounced immunologic response within the host against the gametocytes, thus threatening any further transmission. Therefore, the adaptation which provides a lower rate of sexual conversion serves to provide a continuous supply of transmittable gametocytes for mosquito consumption.

Life history theory, which was originally developed for multicellular taxa to understand how organisms achieve balanced reproductive success, can be applied to *P. falciparum,* to understand variation in sexual conversion rates (SCR) (359, 360). The conclusion that *Plasmodium spp.* can in fact respond to environmental signals and change the allocation of reproductive effort is an example of phenotypic plasticity, allowing a continuous optimization of fitness (359-361).

In applying life history theory to *Plasmodium spp.*, reproductive investment is based on parasite "state" or physiological condition. For example, during conditions of extreme stress, *P. falciparum* has been shown to invest more highly in sexual conversion as opposed to asexual replication (318, 360, 362, 363). This aligns with the terminal investment hypothesis, wherein organisms sacrifice intra-host survival for inter-host transmission (Figure 6) (360, 364-366). Conversely, during environments of manageable stress, *P. falciparum* have been shown to exhibit reproductive restraint, wherein diverting all resources into salvaging intra-host survival "pays-off" by increasing the likelihood of greater inter-host transmission in the future (309, 360, 365, 366).

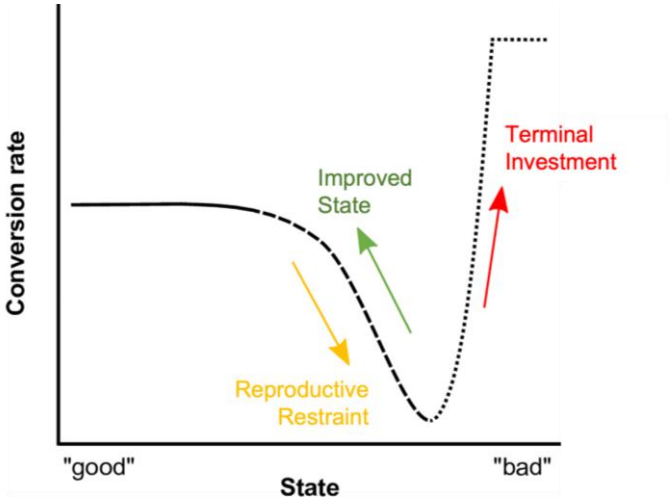

Figure 6. Model of reproductive plasticity in *Plasmodium.*

Modified and reprinted with permission from Schneider, P. et al. (366).

Understanding reproductive management of *P. falciparum*, and the external and internal factors that shape or drive each strategy, could lead to the development of preventative measures which cease human to host transmission of malaria. As current therapies focus solely on responding to disease, rather than preventing disease, the potential in this approach provides a new genesis for malaria control.

1.6 Hemoglobin and hemoglobinopathies: the malaria hypothesis

In the absence of effective ways to control malaria disease and transmission, its long history and continued burden has taken a drastic toll on the human population. Monogenic disorders of the Hb protein, which are now the most common monogenic disorder throughout the world incur a significant public health burden to the countries with the highest incidence (367-369). Predominantly affecting sub-Saharan Africa, these genetic polymorphisms have been driven by the persistence of malaria within these regions (Figure 7). The most profound evolutionary changes have occurred amongst populations with the most consistent history of battling *P. falciparum* (368). This phenomenon, when first propositioned in 1948 by J. B. S. Haldane, was titled the "malaria hypothesis", a phrase which has been adopted widely to explain the human genetic polymorphisms that have been selected with high frequency because of the protection they provide against malaria disease (370).

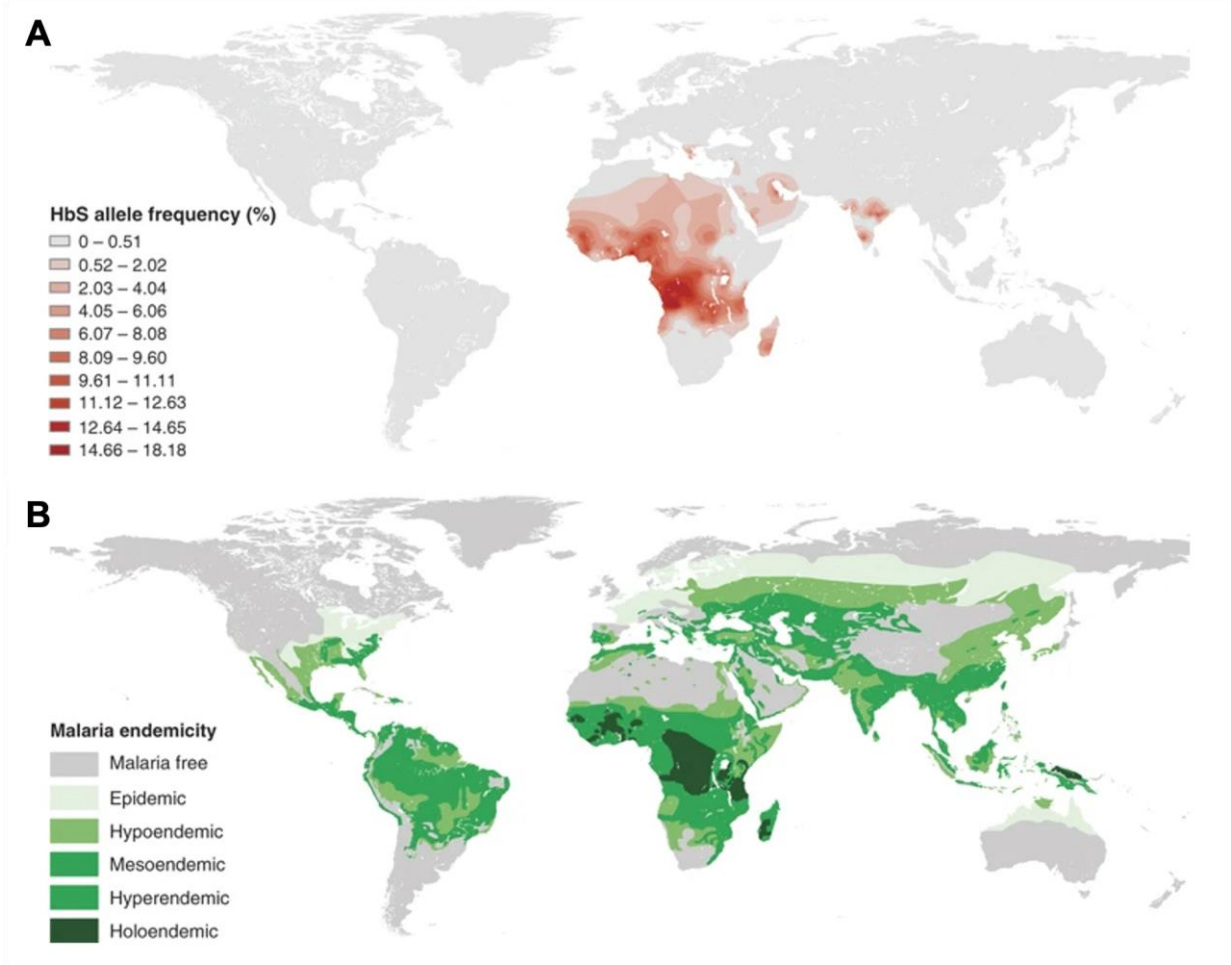

Figure 7. Hb S allelic frequency and its overlap with malaria endemicity.

(A) Hb S allele frequency and **(B)** the historical map of malaria endemicity. Reprinted with permission from Piel, F. et al. (368).

Though the malaria hypothesis theoretically applies to any genetic mutation which provides an advantage against malaria disease, most identified mutations directly affect erythrocytes. These include enzymatic mutations, such as glucose-6-phosphate dehydrogenase (G6PD) deficiency, membrane disorders, such as hereditary elliptocytosis, and a broader category of hemoglobinopathies, which defines any mutation among the Hb protein (371-373). Hemoglobin is composed of four subunits of globin proteins, with the most common form, Hb A, being a combination of two α- globin and two β-globin chains. Each one of the globin proteins contains a

heme moiety which is responsible for binding the oxygen that is carried in the erythrocyte (374, 375). Through the course of erythrocyte maturation, major organelles, including the nucleus and mitochondria, are lost. This ultimately allows for over 90% of the total protein composition within a mature erythrocyte to be entirely Hb.

The globin chains of Hb are encoded by genes located on separate chromosomes (α-globin on chromosome 16, and β^A-globin on chromosome 11) (376, 377). The β^A-globin gene locus is developmentally regulated, resulting in the expression of three β-like globin proteins throughout the course of embryonic to fetal to adult development, a phenomenon known as Hb switching (378-380). During fetal development, the β-like globin, γ–globin, and α–globin together form fetal Hb (Hb F) which predominates throughout the rest of fetal development. Around six months of development, γ-globin begins to decrease, while β^A-globin begins to increase. The ratio of Hb F to Hb A then continues to shrink as development continues, until about six-months post birth in which >10% of total Hb will be Hb F (381-384).

1.6.1 Pathologies of hemoglobin

Genetic mutations of the Hb protein are among the most common inherited diseases around the world. Though detrimental to human health, these mutations have been widely retained in malaria endemic areas due to the protection they afford against the disease (46, 385-387). Interestingly, major protective hemoglobinopathies are hypothesized to have arisen within the human population somewhere between 13,000-8,000 BCE, indicating the presence malaria from at least this time (388). Over 1100 individual Hb variants have been identified, and it is estimated that regional prevalence ranges from 3-40%, with approximately 7% of the world's total

population carrying a single variant (367, 369, 389). Hemoglobinopathies are further divided into two sub-categories, mutations that effect the production of Hb, the thalassemia's, and mutations that effect the structure of Hb (390). The severity of disease that may arise from each individual hemoglobinopathy is as diverse as each mutation.

1.6.1.1 The sickle mutation

The most common hemoglobinopathy in *P. falciparum* endemic areas is the sickle cell mutation, wherein a change in one single nucleotide of the β^{A}-globin chain results in β^{S}-globin and Hb S, rather than Hb A. The missense mutation replaces a negatively charged glutamic acid for a hydrophobic valine (E7V substitution) (391). This change to valine results in an alteration of total protein charge, as well as a hydrophobic interaction between the exposed valine upon deoxygenation (392-395). The hydrophobic interaction causes the formation of long fibers between Hb S molecules, eventually deforming the erythrocyte into the characteristic sickle shape which severely impairs rheology (396). The pathological symptoms of homozygous β^{S}-globin inheritance (sickle cell anemia, SCA), manifests as vaso-occlusion, endothelial dysfunction, and sterile inflammation. Clinically, these pathological symptoms result in acute and chronic pain, multi-organ dysfunction, and eventually organ failure (202, 397). Interestingly, Hb F ameliorates Hb S polymerization, and inducing its expression has become a therapeutic intervention for individuals with SCA (398-402).

While SCA specifically describes the homozygous inheritance of β^{S}-globin, sickle cell disease (SCD) is a general term for the inheritance of one β^{S}-globin allele along with the inheritance of another mutant allele which may cause similar clinical features to that of SCA (390). Other common mutations resulting in SCD include β^{C}-globin (E7K substitution, Hb C), β^{E}-globin

(E26K, Hb E), and β-thalassemia (367, 390, 403). In the case of β-thalassemia, two major subtypes are recognized, β-thal^{+} or β-thal^{0}. The former results in a reduced production of β-globin and the latter results in little or no β-globin production. Individuals heterozygous for β^{S}-globin (Hb AS) account for an estimated 10-30% of the population in certain malaria-endemic regions, 6-9% of the African American population in the United States and exceed 300 million globally (368, 404, 405). Usually, the concentration of Hb S will be lower than that of Hb A, though it is known to exhibit variability between 30-40% (382, 406, 407). Heterozygous inheritance of β^{S} is typically benign, but associations to certain conditions such as papillary necrosis, splenic infarction, exertion-related rhabdomyolysis, venous thromboembolism, and renal disease have been established (406, 408-410).

1.6.2 Protective advantages afforded against malaria

The protective effects of the sickle cell mutation on the pathogenesis of malaria was most prominently confirmed by AC Allison in 1954, though speculations of the association had been made for at least six years prior (46). Since this finding, it has been broadly corroborated that individual with sickle cell trait (SCT, Hb AS) are resistant to mortal complications of malaria by up to 90% (1, 46, 411, 412). Mechanisms of this protection have not been entirely elucidated but include the following factors:

1) Increased phagocytosis and elimination of intraerythrocytic ring-stage parasites: the Hb S present within Hb AS- containing erythrocytes (Hb AS-Ery) also polymerizes, leading to deformation of the cell into the characteristic sickle shape. Sickle-shaped erythrocytes are more easily recognized by the immune system, and are thus removed,

ultimately leading to faster clearance of infected erythrocytes and a limitation on the parasite's ability to maintain a stable parasitemia (254, 413-416).

2) Decreased invasion: altered remodeling of the erythrocyte cytoskeleton resulting from deformation of Hb alters the membrane composition of the cell. This makes the cell more rigid, and more hostile to parasite invasion (417-419).
3) Reduced cytoadherence and elimination of intraerythrocytic trophozoite and schizont-stage parasites: after successful invasion, the altered remodeling of the cytoskeleton effects the parasite's ability to export proteins to the erythrocyte surface. These exported proteins include the VSAs, which are crucial for cytoadherence to the vascular endothelium to evade the host immune system (420-422).
4) Impaired parasite growth: impaired protein export, as well as polymerization of Hb needed for digestion may limit the availability of nutrients required for parasite survival, thus reducing parasite growth and proliferation (415, 420, 423-425).
5) Increased tolerance to extracellular heme: individuals with SCT contain increased levels of heme scavenging molecules compared to individuals with Hb A (412, 426). This is due to an increase in the amount of free heme resultant from erythrocyte lysis during steady-state conditions (427-429). As malaria results in the release of massive amounts of heme, the ready availability of these scavenging molecules, as well as a system already accustomed to handling increased heme concentrations, has been proposed to be a contributing mechanism of disease tolerance (412, 426).

While other hemoglobinopathies have also been shown to provide some kind of protection against malaria disease, no identified combination has proven to be as effective as SCT. Rather, it appears as though each offers a distinct facet of protection against a specific component of disease. For

example, the heterozygous combination of Hb A with Hb C (Hb AC) provides protection specifically from clinical malaria, by impairing the lysis of mature schizonts and reducing expression of PfEMP1 (386, 430, 431).

On the other hand, those with SCA have been shown to be at the same level of risk for acquisition, and at an even greater risk of severe forms and mortality from malaria disease (432, 433). In fact, antimalaria prophylaxis for individuals with SCA is an established policy in some countries in sub-Saharan Africa (434). One study observed that children with malaria and SCA were 10x more likely to die from malaria disease than non-SCA counterparts (433). This is explained by a variety of mechanisms. On the pathogen side, though there is some degree of invasion protection afforded by Hb S, the parasite is still capable of invading circulating RBCs, which increases stress on an already severely stressed system (432).

While Hb S trait provides a survival advantage against malaria disease, it is important this is distinguished from sterilizing protection. Individuals with SCT still carry the parasite and can therefore still transmit the parasite to its mosquito vector, as do those with SCA (46). In fact, several studies have found that high SCT prevalence is associated with a higher prevalence of asymptomatic and clinical malaria (435-437). In one specific community, a 25% increase in malaria prevalence translated to a 4.3% increase in individuals with SCT, and this was additionally found to increase with age (435). Similarly, other hemoglobinopathies when acquired on a heterozygous level, though known to confer protection against clinical or severe disease, do not protect against parasite carriage (387, 438, 439).

1.7 Remaining gaps in knowledge

It is known the degree of Hb variant carriage among individuals within a malaria-endemic community has an effect on the rate of clinical malaria experienced within the community itself (368, 435, 440). Though there is a well-established relationship between Hb variants and a protective effect against asexual parasitemia, the degree of this protection is not perfect. Considering life history theory of reproductive investment, when asexual proliferation suffers to an extreme degree, investment into gametocyte stages should increase. Curiously though, the relationship between gametocytogenesis and Hb variants remains to be defined.

Recent research efforts have focused greatly on environmental determinants of sexual conversion, but thus far no experimental studies have explored the potential effect of Hb variation. Interestingly, epidemiologic reports have noted a positive relationship between Hb variation and gametocyte carriage (332-334, 386, 441). Specifically, the following has been shown:

1) During low transmission seasons, individuals with Hb SC experienced a higher frequency of gametocyte carriage, while Hb AS and Hb CC individuals showed higher gametocyte densities (333).
2) Hb CC individuals have been shown to have higher rates of gametocyte carriage (332).
3) Individuals with Hb C (one copy of Hb C) have been associated with more frequent gametocyte carriage (334).
4) Mosquito infection rates, *in vivo*, were found to be higher among Hb CC and Hb AC individuals, compared to Hb AA.
5) Mosquito infection rates *ex vivo* were higher among Hb AS, Hb AC, and Hb CC individuals compared to Hb AA. This led to higher oocyst densities in *ex vivo* experiments among these Hb variants (332).

Though most of the studies addressed have found the associations with Hb C, this phenomenon has never been studied experimentally. Furthermore, and still relevant to theories of reproductive investment, a potential link exists between the inhibition of parasite growth in Hb variant erythrocytes and growth inhibition among cultures treated with antimalarials. While both conditions have been shown to have an impact on parasite growth, the latter has been shown to positively influence gametocytogenesis among *P. falciparum*. Moreover, as most antimalarials function through perturbation of the Hb digestion process, this is ultimately suggestive of a relationship between Hb digestion and sexual conversion.

2.0 Hypothesis and Specific Aims

2.1 Hypothesis

Human infection and disease of *P. falciparum* is maintained through the expansion of blood-stage parasitemia, which develops from subsequent rounds of intraerythrocytic asexual replication. As discussed, transmission between the human host and the mosquito vector requires the formation of sexually competent gametocytes, which arise from these asexually replicated parasites in the human bloodstream. Rates of sexual commitment are known to exhibit seasonality, vary between parasite strains, and in response to certain environmental stimuli (227, 309, 317-321, 343, 355, 442). Though it is assumed that a low fraction of asexual parasites become sexually committed during each replication cycle, it has been widely demonstrated *in vitro* that during conditions of extreme stress, *Plasmodium spp*. invest more highly in sexual conversion as opposed to asexual replication (309, 318, 360, 363, 364).

Interestingly, individuals who have certain pathologies of Hb have been shown to carry a greater number of mature gametocytes when compared to Hb A counterparts (332-334, 386, 441). Though genetic mutations of Hb have been widely retained in malaria endemic areas due to the protection they afford against the disease, it is important this defense is distinguished from complete sterilizing protection, in which no infection would occur (46, 385-387). Considering the essentiality of Hb to the intraerythrocytic cycle, the epidemiology of Hb variants, and the potential significance in transmission potential of increased gametocyte carriage among individuals with hemoglobinopathies, this dissertation research tests the **overall *hypothesis* *that "Hemoglobin***

variants influence sexual differentiation of P. falciparum". The approach to test this hypothesis is outlined in Figure 8.

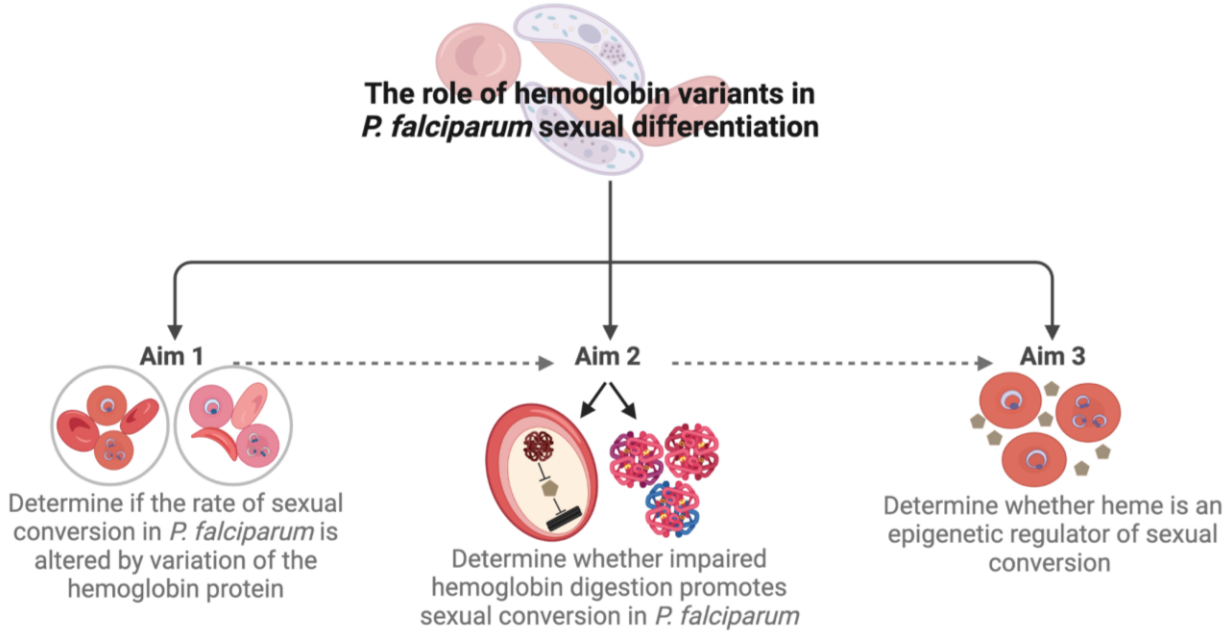

Figure 8. Graphical representation of specific aims.

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2.2 Specific aims

2.2.1 Aim 1: determine if the rate of gametocyte conversion of *P. falciparum* is altered by the Hb S protein

Culture methods to synchronize parasite stages *in vitro* have been well established (337, 443). Using a combination of these methods, it is possible to isolate specific stages of parasites cultured in Hb A containing erythrocytes (Hb A-Ery) and sub-culture the parasites in Hb S

erythrocytes (Hb S-Ery) (444). This method has been used previously to study differences in parasite invasion among Hb S-, Hb AS-, and Hb A-Ery cultures (419). In this aim we developed a similar *in vitro* culture system to study the development of gametocytes and used this method to test the *hypothesis that erythrocytes containing Hb S stimulate increased rates of gametocyte conversion.* This aim determines a proportional difference in sexual stage differentiation between Hb A- and Hb S-Ery.

2.2.2 Aim 2: determine whether impaired Hb digestion promotes gametocyte conversion in *P. falciparum*

Findings from Aim 1 reveal significant differences in the rate of gametocytogenesis between Hb A- and Hb S-Ery cultures. As digestion of Hb within erythrocytes is required for parasite survival, we next focused on potential differences in Hb digestion between Hb variants. Prior research has shown certain Hb variants to be resistant to digestion by *P. falciparum* hemoglobinases. Moreover, therapeutic agents that inhibit this process, such as chloroquine diphosphate (CQ), have been shown to increase gametocytogenesis *in vitro* (445-447). Digestibility of Hb S by *Plasmodium spp.* enzymes has never been investigated (448, 449). Thus, it is reasonable to consider the potentiality that Hb S is resistant to parasite digestion, which may then stimulate gametocyte conversion. This aim tests the *hypothesis that hemozoin formation efficiency and gametocyte conversion are inversely correlated*. This aim examined whether [1] there is a dose-dependent effect of hemozoin formation and gametocyte conversion, [2] whether Hb S is resistant to hydrolysis by parasite hemoglobinase proteins, and [3] whether the digestibility of variant Hb proteins contributes to high sexual conversion. Contrary to our initial hypothesis, we find Hb S to be significantly more readily hydrolysable by a *P. falciparum* hemoglobinase than

Hb A. Interestingly, because Hb digestion results in the release of heme, a faster rate of Hb S hydrolysis would result in a faster rate of heme release. We then go on to determine CQ exposure increases sexual conversion and find a negative correlation between the formation of hemozoin and the sexual conversion rate.

2.2.3 Aim 3: determine whether heme concentration affects gametocyte conversion in *P. falciparum*

The digestion of Hb by *Plasmodium spp*. results in the intracellular release of free heme. While a decrease in Hb digestion would result in a decreased amount of free heme, an increase in Hb digestion would result in a greater amount of free heme release. The mechanism of action of CQ involves inhibiting the Hb – hemozoin formation process, which somewhat counterintuitively results in an increase in intracellular heme concentration. Therefore, if an association exists between Hb digestion and sexual conversion, this relationship can be further explored and supported by examining sexual conversion under conditions of altered heme concentration. This aim will determine the *hypothesis that excess heme concentration suppresses sexual conversion of P. falciparum*. By culturing *P. falciparum* in conditions of increasing heme concentration, we find sexual conversion to increase, ultimately indicating heme is a promoter of sexual conversion.

3.0 Materials and Methods

3.1 Parasite culture

P. falciparum strain NF54 were obtained from the Malaria Research and Reference Reagent Resource Center (MR4) and cultured in O+ erythrocytes. Cultures were maintained in complete parasite media (CPM), containing RPMI with 25 mm HEPES, L-Glutamine, and 50 mg/L hypoxanthine (KD Medical), and supplemented with 0.25% $NaHCO_3$ (Gibco), 2.5 mg/ml gentamicin (Gibco), and 10% heat inactivated fresh human serum (Zen-Bio). All cultures were gassed with 5% CO_2, 5% O_2, and 90% N_2 (Matheson). Human blood for cultures was purchased from authorized collection facilities (Zen-Bio and Innovative Research) or collected from steady state SCD patients in accordance with the guidelines set by the Institutional Review Board at the University of Pittsburgh and the Declaration of Helsinki. All blood was washed in incomplete parasite media (IPM), CPM without fresh human serum, and stored at 50% hematocrit (HCT) in CPM. Hemoglobin type was determined by Hb electrophoresis (Helena). All synchronization was performed by sorbitol lysis, wherein cultures were resuspended in 5% sorbitol (Sigma), incubated for 10 minutes at 37°C, and washed once with CPM. Synchronizations were performed 18-20 hours apart to achieve 6-4-hour development windows respectively.

3.2 Hemoglobin electrophoresis

To verify the Hb type of each whole blood sample received from patients and collection facilities, Hb electrophoresis was performed. Hemoglobin determination was performed prior to

use for parasite culture. During the washing procedure, a small aliquot (5-10 μl) of whole blood was taken and immediately frozen on dry ice. Samples were removed from dry ice and quickly thawed, ensuring complete lysis of erythrocytes. In a fresh tube, 2 μl of the lysed whole blood was mixed with 6 μl of cystamine hydrochloride solution (Sigma). Samples were incubated at room temperature (RT) for 20 minutes and then applied to a cellulose acetate membrane plate (Helena), along with two standards containing Hb A, F, S, A_2, and C (Helena). The plate was then placed in an electrophoresis apparatus and run for 30 minutes at 350 V. Upon visual confirmation of protein separation, the plate was removed from the apparatus and placed in Ponceau S stain (Sigma) for 5 minutes. The plate was then washed twice in 5% acetic acid, and twice in methanol for 3 minutes each. After washing, the plate was placed in Clear Aid (Helena) for 15 minutes. Final determination of Hb type was completed by comparing each sample against the standards.

3.3 Gametocyte conversion assays

3.3.1 Culture preparation for gametocyte conversion between Hb A-, Hb AS, and Hb S-Ery cultures

For comparison of gametocyte conversion between Hb A-Ery, Hb AS- Ery, and Hb S-Ery cultures, a large feeder culture was produced within Hb A-Ery. The feeder cultures were synchronized to a six-hour development window by sorbitol lysis, prior to magnetic separation of mature schizonts using MACS LS columns (Miltenyi). Columns were pre-rinsed with IPM prior to loading with pre-synchronized schizont cultures. The flow-through containing uninfected erythrocytes and early-stage parasites was discarded while the schizonts remaining on the column

were eluted with CPM. Eluted schizonts were then re-applied to a freshly prepared column, to ensure complete isolation from uninfected erythrocytes and early-stage parasites. Schizont isolation was confirmed with a blood smear prior to inoculating Hb A-Ery, Hb AS-Ery, or Hb S-Ery cultures at a 6% HCT, and to a final parasitemia of 0.3% in T75 flasks. Media was changed daily, with a double volume added on day three, to create a 3% HCT culture. Every other day, cultures were sampled for flow cytometry and blood smear analysis. Conversion rates were determined by two methods described in the following sections.

3.3.1.1 Determination of gametocyte conversion rate: method 1

The first method for determining the rate of gametocyte conversion was based off the method of inducing sexual commitment in Hb A-Ery cultures through a high parasitemia. After inoculation, cultures were allowed to stabilize and proliferate for 72 hours, allowing for one complete replication cycle prior to doubling media volume (Figure 9). This created a 3% HCT environment, which when initiated at the trophozoite stage creates a source of stress that is continued through day seven. Peak parasitemia is then reached at day seven, after the additional round of replication (443, 450, 451). N-acetylglucosamine (NAG, Sigma) was added to cultures at a concentration of 50 mM on day seven to prevent further asexual replication. Therefore, the highest level of parasitemia is reached on day seven.

Cultures were monitored for an additional seven days to allow gametocyte development from the last possible point of conversion and to deplete the culture of residual asexual parasites. Parasitemia was carefully monitored over the 14-day culture period by flow cytometry and blood smear analysis. Gametocyte conversion rates were then determined based on gametocytemia on day 14 from total parasitemia on day seven, as the parasitemia measurement on day seven

represents the absolute peak of proliferation. Blood smears on day 14 were used to confirm the presence of mature gametocytes and absence of asexual parasites in all cultures.

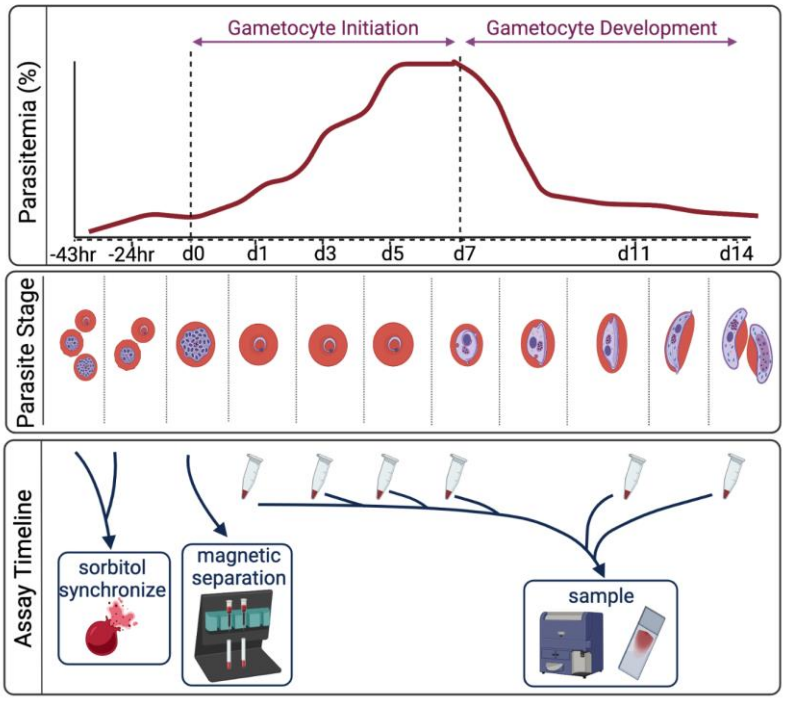

Figure 9. Diagram of method 1 for determining gametocyte conversion.

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3.3.1.2 Determination of gametocyte conversion rate: method 2

The second method used to determine the GCR establishes the rate of sexual conversion from the first replication cycle (Figure 10). Thus, no gametocyte induction occurs amongst any cultures. This method heavily relies on microscopic analysis of blood smears. To provide a measure of validation for microscopic analysis, total parasitemia was measured and compared by both microscopy and flow cytometry on days one, four, and seven. For all microscopy analysis, slides were examined on three separate occasions with gametocytemia determined based on the count of 2,000 uninfected erythrocytes.

For quantification of sexual conversion, blood smears were analyzed on days four and seven for the presence of stage II and III gametocytes respectively. The rate of gametocyte conversion was then determined by dividing total stage II/III gametocytemia on day four/seven by total parasitemia on day one.

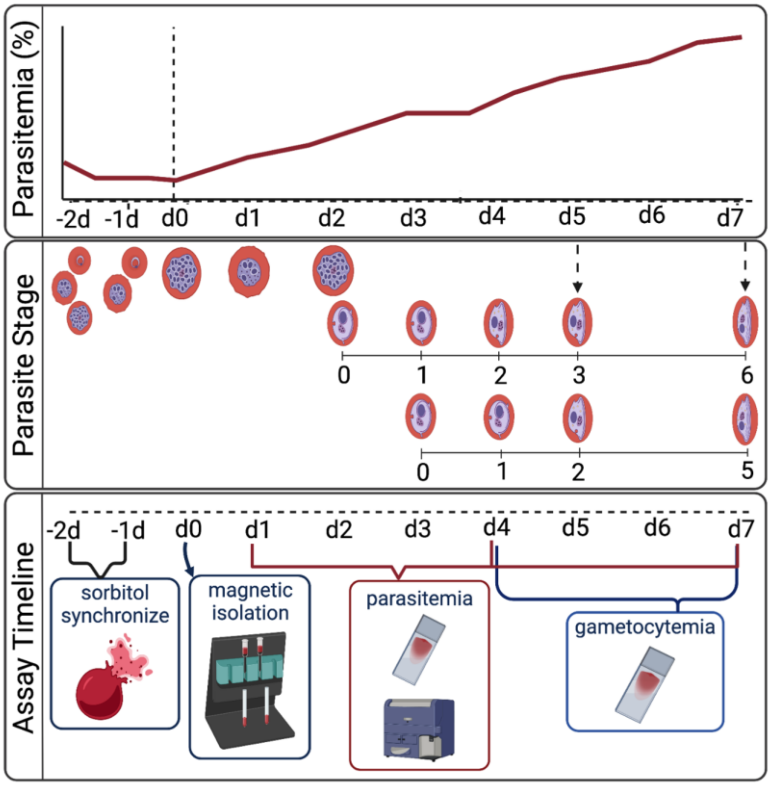

Figure 10. Diagram of method 2 for determining gametocyte conversion.

Created with Biorender.

3.3.2 Culture preparation for gametocyte conversion between Hb A-Ery cultures exposed to CQ or hemin

For comparison of gametocyte conversion between Hb A-Ery cultures exposed to either CQ or Hemin, a feeder culture was again used to begin all cultures from the same synchronized

culture. For these assays, the feeder cultures were synchronized to a more stringent four-hour development window by sorbitol lysis. Magnetic isolation of schizonts was not needed for these assays, as all cultures to be inoculated contained Hb A-Ery. Cultures were plated at 2% HCT to a final parasitemia of 0.5% late-stage schizonts in T75 flasks or 12- well plates. For experiments involving hemin exposure, hemin (Frontier) was prepared by dissolving in NaOH (Sigma) and adjusting to a pH of 7.52 with HCl. On day two of the assay (48- hours) NAG was added at a concentration of 50 mM to prevent further asexual replication. The cultures then reach their peak parasitemia on day two, with only gametocyte development occurring between days two through days seven. For all cultures, blood smears from day seven were used to confirm the presence of mature gametocytes and absence of asexual parasites. Gametocytemia was then determined for gametocyte conversion analysis to be completed by dividing day seven gametocytemia by day two parasitemia.

3.4 Exflagellation assays

To test the exflagellation capabilities of mature microgametes, exflagellation assays were performed between days 13-15 of the sexual conversion assay. Small aliquots of culture were taken and placed in pre-warmed Eppendorf tubes. Samples were spun down at 4000 g for 5 seconds and supernatant was removed. Equal parts (10 μl) pre-warmed fresh human serum was then added, and samples were incubated at RT for 10 minutes. Samples were then transferred to a hemacytometer to monitor and record exflagellation activity. Samples were monitored for 10 minutes to observe all potential activity within each quadrant.

For video analysis, exflagellation was observed with a Nikon Eclipse Ti Live Cell Microscope. For imaging, 10 μl of sample containing serum were placed in 35 mm Matek dishes with a 12 mm cover slip (Electron Microscopy Sciences). Samples were allowed to settle to a monolayer prior to imaging at 10 second intervals for 2 minutes.

3.5 Standard curve generation for micro- and macrogametocyte quantification

Micro- and macrogametocyte quantification was attempted using a RT-qPCR standard curve assay. To prepare the standard curve, genomic DNA was isolated from cultures stored in TRIzol Reagent (ThermoFisher). DNA was then solubilized, and the total yield was determined. DNA amplicons for Pfs25, Pfs230p, and 18S rRNA (micro-, macrogametocyte, and human reference gene respectively) were produced by end point PCR, using specific primers tailed with T7 promoter sequences to enable *in vitro* RNA transcription. Total RNA was then transcribed using the T7 RNA Polymerase Kit (Promega). DNase treatment (Promega) was then performed, and RNA was confirmed free of gDNA using qPCR for the specific target genes. RNA concentrations were then quantified by nanodrop, and cDNA was produced using High-Capacity cDNA Reverse Transcription Kit (Life Technologies).

Ten-fold dilutions of each target were then prepared to obtain standard curves. Experimental samples were prepared as detailed in section 3.6. Primers and sample preparation for analysis are detailed in Table 2.

3.6 RT-qPCR for gene expression

For gene expression analysis, 5-8 mL whole culture were pelleted, lysed in 0.05% saponin (Sigma), and resuspended in Qiazol (Qiagen), before frozen at -80°C. Upon thaw, chloroform was added, followed by centrifugation at 12,000 g for 15 minutes. The aqueous portion of the samples was then mixed with 70% ethanol. RNA was isolated with Qiagen RNeasy Mini columns with on-column DNase digestion (Qiagen). Samples were then reverse transcribed (Applied Biosystems), diluted and analyzed for gene expression on Applied Biosystems StepOnePlus Real-Time PCR System using FAST Sybr Green Master Mix (Applied Biosystems). Primers for analysis are listed in Table 2. All data is calculated using the relative quantity method ($2^{-\Delta\Delta Ct}$) and presented in fold-change relative to the reference gene *seryl-tRNA synthetase*.

Table 2. RT-qPCR primers.

qRT-PCR		
Gene ID	**Primer ID**	**Sequence (5'-3')**
PF3D7_1031000	T7Pfs25 Fw	TCTGAAATGTGACGAAAAG
	Pfs25 Rev	TAACAAGGATTGCTTGTATC
PF3D7_0209000	T7Pfs230p Fw	TTATGAACGTACATCAGGAGAAGAT
	Pfs230p Rev	ATTTGGTAAGAGGCACCGAT
100008588	18s Fw	GGAACTGAGGCCATGATTAAGA
	18s Rev	ATCGCTCCACCAACTAAGAAC
PF3D7_1031000	Pfs25 Fw	TGGAAATCCCGTTTCATACGC
	Pfs25 Rev	ACCGTTACCACAAGTTACATTCT
PF3D7_0209000	Pfs230p Fw	CCCAACTAATCGAAGGGATGAA
	Pfs230p Rev	TGTTGTTCGATTCCAGTTGGT
PF3D7_1218600	tRNA synthetase Fw	AGCTAAAGAGATGCATGTTGGTCATT
	tRNA synthetase Rv	GAGTACCCCAATCACCTACATGA
PF3D7_0935400	gdv1 Fw	TAGGCGTCGAAATAGTGCTAGTAGAAA
	gdv1 Rv	GTCCTCACAACCAGCATCATTAGTA
PF3D7_1222600	ap2g Fw	TGGTGGTAATAAGAACAACAGAGGT

	ap2g Rv	CCATCATAATCTTCTTCTTCGTCG
PF3D7_1335000	msrp1 Fw	TACCAGGTGCCTTATCAAGTG
	msrp1 Rv	CTTGGTTGTGATTCGGTTGATG
PF3D7_0936600	gexp5 Fw	GTGGTTGTTTGAGAAGTGGTGA
	gexp5 Rv	ACAGAATCCGTTTGAGATGATGA
PF3D7_0113600	surfin 1.2 Fw	TTTTTCCCTCGATCTCCGCG
	surfin 1.2 Rv	GGGTTTGGCCGTACTCTACT
PF3D7_1301800	surfin 13.1 Fw	ACCCGAAGTGACAACATCTCC
	surfin 13.1 Rv	TCTCCACGAGTTCCAAGTTTT
PF3D7_1115700	fal2 Fw	GCTTGTAGGTTTT GGTATGAAAGAA
	fal2 Rv	AGATAGGTCCCTTTTTAAAATACTATTGAC
PF3D7_1446800	hdp Fw	AACCAACGACCCAGAAAGTATAA
	hdp Rv	TCCATGTTGTTACCTGTAGGATG

3.7 Flow cytometry

All flow cytometry measurements of parasitemia and gametocytemia were conducted through flow cytometry on a BD LSR Fortessa. For each sample, 10 µl of whole culture was resuspended in 20 µM Hoechst (Thermo Scientific). Whole culture contains only uninfected erythrocytes and infected erythrocytes, as all blood is washed of white blood cells prior to culture. Samples were incubated for 30 minutes at 37°C and immediately analyzed. For each sample, 100,000 events were collected and gated based on FSC and SSC, followed by doublets exclusion (FSC-H vs FSC-A) (Figure 11). Parasitemia was then determined by Hoechst-positivity, as mature erythrocytes do not contain nuclei. Excitation of cells for Hoechst was performed with a UV laser (355 nm) and band pass filter 450/50 nm. To control for the potential of reticulocytes, particularly among the Hb S-Ery cultures, samples were normalized to a sample of uninfected erythrocytes, which also served as a negative control.

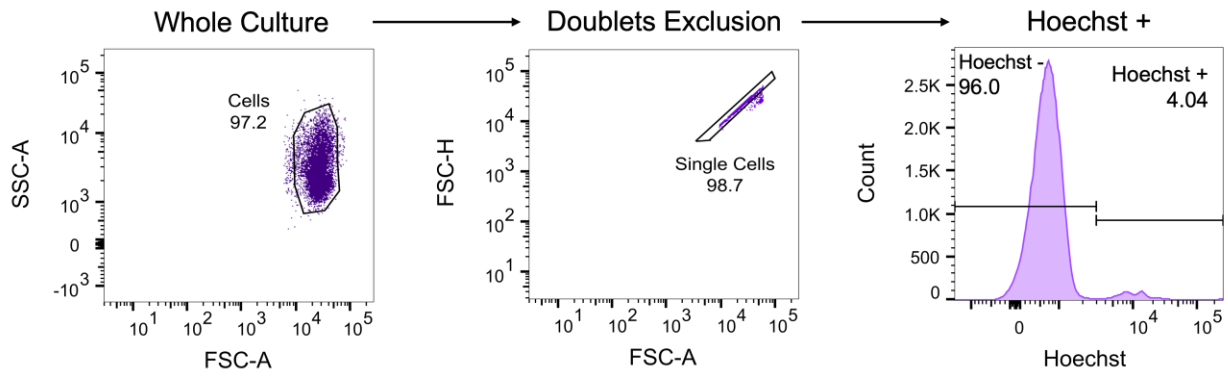

Figure 11. Flow cytometry gating strategy for measuring parasitemia.

The main erythrocyte population (containing both parsitized and non-parasitized cells) was selected based on fsc versus ssc (left panel), followed by doublets exclusion based on fsc-h versus fsc-a (middle panel). Hoechst positivity was then determined using a histogram in which positive populations can be clearly distinguished from negative populations. Hoechst positivity indicates parasite presence within a cell, as mature erythrocytes do not contain nuclei. Therefore, the representative plot indicates a parasitemia of 4.04%.

3.8 Hemozoin quantification

In order to verify CQ treatment, hemozoin was quantified among each experimental group. For each treatment group, 2 mL of culture was collected, lysed, and washed in a 1% final concentration of Triton X-100 (Sigma). Pellets were centrifuged at 13,000 rpm for 45 minutes at 4°C and then washed three times in molecular grade H_2O. Each hemozoin pellet was then solubilized in a solution of NaOH (Thermo Fisher), pyridine (Sigma), and water (1:2:8 v/v).

A 75 µl aliquot of each sample was plated in duplicate on a 96-well plate. For determination of oxidized and reduced heme, 10 µl of 2.5 mM potassium ferricyanide (Sigma) and sodium hydrosulfite (Sigma) were added respectively. Absorbance was measured at 560 nm with hemozoin calculated by subtracting the OD of the oxidized sample (potassium ferricyanide-treated) from the reduced sample (sodium hydrosulfite- treated). Hemozoin content was then determined according to a hemin standard curve, which was prepared in parallel.

3.9 Production of recombinant falcipain-2

For analysis of Hb digestion, recombinant falcipain-2 was produced. Production of the protein is detailed in the following sections, and as described (453-455).

3.9.1 Confirmation of sequence

E. coli containing the plasmid construct for falcipain-2 (FP2) was obtained by the generous donation of Philip Rosenthal. Prior to producing the protein on a large scale, the construct was confirmed. DNA was isolated using Qiagen MiniPrep Kit and sequenced by GeneWiz. Primers for sequencing are listed in Table 3 and were based on the expected orientation of the *fp2* gene in the pQE-30 expression vector (Figure 12).

Table 3. Falcipain-2 sequencing primers.

Sequencing Primers	
T5 Promoter	GCTTTGTGAGCGGATAAC
5' No. 1	AAACACAGATTTGCC
Lambda t0	GGAGTTCTGAGGTCA
3' No. 1	GAATGCATCAGTACC
5' No. 2	GTGGATCTTGCTGGG
3' No. 2	GATCACCACATTCTCC

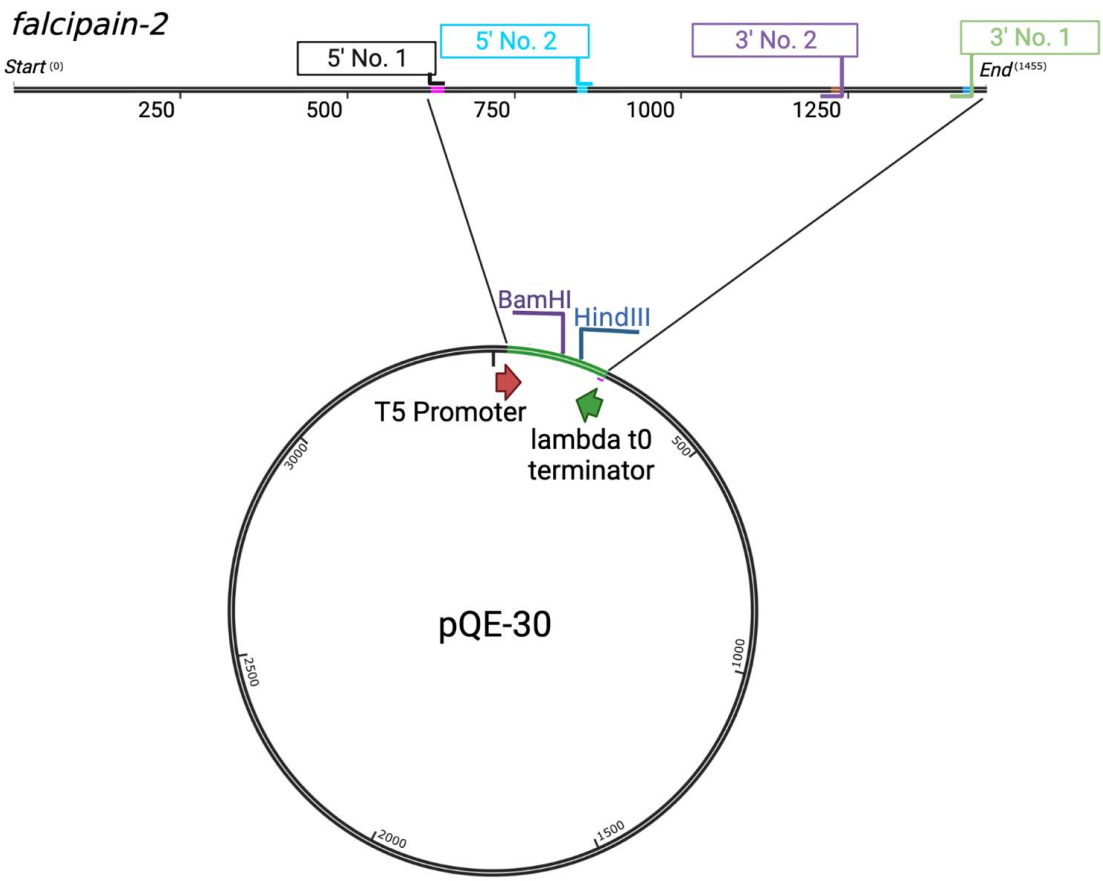

Figure 12. Diagram of pQE-30 expression vector containing *fp2* construct.

FP2 was cloned into the pQE-30 expression vector using BamHI and HindIII. For confirmation of sequence, primers were developed spanning the T5 promoter, lambda t0 terminator, BamHI and HindIII restriction sites, as well as four primers along the length of the expressed portion of the gene. Created with SnapGene.

3.9.2 Protein generation and purification

For full-scale production, bacteria were grown to mid-log phase and induced with IPTG (Sigma) for 3 hours at 37°C. Cells were then harvested and washed in cold Tris-NaCl (20 mM, 150 mM). Samples were sonicated for 4 minutes (10 seconds on, 10 seconds off) and centrifuged for 30 minutes at 17,000 rpm. Remaining pellets were solubilized in urea solution (8 M urea (Fisher), 10 mM Tris (Tocris Biosciences), 200 mM Imidazole (Sigma-Aldrich), pH 8.0). The insoluble material was then separated by centrifugation at 17,000 rpm for 30 minutes. Protein was

purified from the supernatant using nickel-nitrilotriacetic acid (Ni-NTA+) resin (Qiagen). Bound protein was eluted (8 M urea, 10 mM Tris, 500 mM Imidazole), and elution fractions were visualized on a protein gel followed by quantification with nanodrop.

3.9.3 Protein refolding

After confirming isolation and protein quantity, eluted protein was refolded into its active form. Protein refolding was performed overnight in folding buffer (100 mM Tris-HCI, 30% glycerol (Sigma), 250 mM arginine (Sigma), 1 mM EDTA (Sigma), 1 mM GSH (Sigma), 1 mM GSSG (Sigma), pH 9.2) at 4°C. Refolded protein was then concentrated using 50 mL, 10K centrifuge units (Thermo Scientific).

3.9.4 Confirmation of falcipain-2 activity

Activity of concentrated FP2 was verified with the substrate N-carbobenzyloxy; 7-Amino-4- methylcoumarin (Z-LR-AMC (R&D Systems)). After concentration was determined, 200-500 nM FP2 in folding buffer was combined with 0.05 μg/μl Z-LR-AMC in activity buffer (10 mM DTT (Sigma), 100 mM NaOAc (Sigma)). The assay was run at a pH of 5.5 and 9.0, with BSA used as a negative control. The assay plate was incubated for 10 minutes at 37°C, with three reads in total from 10-30 minutes. The plate was read on a fluorometer wherein the release of 7-amino-4-methyl coumarin (AMC) was monitored over the course of 30 minutes (excitation, 355 nm; emission, 460 nm).

3.10 Hemoglobin isolation

Hemoglobin was isolated from Townes mice (The Jackson Laboratory), which express exclusively human Hb. Procedures were reviewed and approved by the University of Pittsburgh IACUC. For Hb A, Hb S, and Hb AS samples, blood was collected from adult mice by cardiac puncture. The sample containing Hb A, Hb S, and Hb F was obtained from a pooled blood sample collected from two-day old pups, which still express high levels of Hb F, as well as Hb A and Hb S. All blood was thoroughly washed with PBS, followed by lysis in water. Lysed blood was then centrifuged at 13,000 g for 30 minutes prior to filtering and concentrating (Millipore). Hemoglobin concentration was then determined with QuantiChrom Hemoglobin Assay Kit (BioAssay Systems), which measures Hb concentration based on the Triton/NaOH method (BioAssay Systems). In this method, Triton is used to lyse any remaining cells, and NaOH lowers the pH. The lowered pH oxidizes the Hbs in the reaction so they emit one single peak, instead of the multiple peaks that would be seen with differing Hb forms (456).

3.11 Hemoglobin hydrolysis assay

To assess Hb degradation, 50 μM of FP2 and 0.12, 0.24, 0.36, and 0.5 μg/μl Hb, buffered in 100 mM NaOAc, and 1 mM GSH, pH 5.5 in a 250 ml volume, was placed in a 96-well plate and incubated for 24 hours at 37°C. Spectrophotometric measurements were taken at 410 nM at 0, 3, 6, and 24 hours of incubation. Samples were compared between Hb types, as well as to a no-enzyme control. At 24 hours, 50 μl of each sample was removed and used for total hemoglobin quantification by QuantiChrom Hemoglobin Assay Kit (BioAssay Systems).

3.12 Western blot of digested hemoglobin

In order to obtain a better visualization of the digested Hb, digested samples and their original counterparts were run on a western blot. Samples were prepared in 20 µl volumes containing 10 µg Hb protein with 4x sample buffer (BioRad) containing beta-mercaptoethanol (Sigma). Samples were incubated for 3 minutes at 90°C and cooled prior to loading onto 10-well, 30 µl AnyKD gels. The gel apparatus was run for 5 minutes at 70 V, followed by 40 minutes at 100 V. Gels were then transferred to an Immobilon-P^{SQ} transfer membrane for 40 minutes at 100 V at 4°C.

After transfer, membranes were blocked for 1 hour in 5% milk in tris-buffered saline with Tween 20 (TBST) (BioRad) at RT. Primary antibodies (Table 4) were incubated overnight at 4°C, followed by three washes in TBST and incubation with secondary antibodies (Table 4) at RT for 1 hour. Membranes were washed of secondary antibodies and imaged on a ProteinSimple (bio-techne) using Super Signal West Pico Plus Chemiluminescent Substrate (ThermorScientific).

Table 4. Western blot antibodies.

Western Antibodies					
Target	**Manufacturer**	**Dilution**	**Secondary**	**Manufacturer**	**Dilution**
α-globin	Novus Biologicals: NB110-41083	1:2000	Anti-Goat	Vector Laboratories: PI-9500	1:2000
β-globin	Santa Cruz: sc21757	1:1000	Anti-Mouse	Vector Laboratories: PI-2000	1:2000

3.13 RNA- fluorescent *in situ* hybridization

In order to analyze gene expression on the single-cell level, the utility of an RNA- based fluorescent in situ hybridization (248) assay was determined. Approximately 5 ml whole culture was collected for each experimental sample. Cultures were spun down to a packed volume of 100 µl in 1.5 mL tubes provided in ThermoFisher Scientific's PrimeFlow Assay Kit. Samples were fixed (PrimeFlow RNA Fixation Buffer 1) for 30 minutes at 4°C, inverted, and then centrifuged at 800 g for 5 minutes. The supernatant was discarded, and samples were permeabilized (PrimeFlow RNA Permeabilization Buffer). Samples were subsequently re-fixed (PrimeFlow RNA Fixation Buffer 2) and incubated at RT for 1 hour in the dark. After centrifuging at 800 g for 5 minutes, all but 100 µl of the supernatant was removed, and cells were resuspended in the residual volume by a brief vortex.

At this stage, samples were split between additional tubes, each of which would be used for experimental target probe combinations. Samples were washed twice (PrimeFlow RNA Wash Buffer), with all but 100 µl of the supernatant removed after each wash. Probe sets were diluted to the working concentration in PrimeFlow RNA Target Probe Diluent. For each target probe mix, 100 µl was added directly to the appropriate cell suspension. Samples were briefly vortexed and incubated for 2 hours at exactly 40°C. After incubation, cells were washed twice and then stored overnight at 4°C.

On the following day, samples were brought to RT and incubated with PrimeFlow RNA PreAmp Mix for 1.5 hours at exactly 40°C. Samples were then washed three times, followed by incubation with PrimeFlow RNA Amp mix for 1.5 hours at exactly 40°C. After incubation, samples were washed twice, and then labeled with PrimeFlow RNA Label Probes, and incubated

for an additional 1 hour at exactly 40°C. Label probes and corresponding fluorophores are listed in Tables 7 and 8. After final incubation, samples were washed twice with wash buffer, followed by an additional wash with flow cytometry staining buffer (ThermoScientific). All but 100 μl supernatant was removed.

Single-color compensation controls were prepared using the PrimeFlow Compensation Kit (ThermoScientific). Briefly, one drop of UltraComp eBeads microspheres (ThermoScientific) were placed in each compensation tube. For each tube, 5 μl of the appropriate fluorophore was added. Controls were incubated for 30 minutes at 4°C in the dark. Controls were then washed with flow cytometry staining buffer and resuspended in IC fixation buffer (ThermoScientific). Controls were again washed in flow cytometry staining buffer prior to run on a BD LSR Fortessa cytometer. Controls were kept for no longer than three days.

Compensation controls were analyzed first and applied to subsequent sample runs. Fluorescent-minus one (FMO) controls were additionally analyzed in order to set the upper boundary for any background signal. An asynchronous reference culture was used as a negative control, as there should not be consistent enough gene expression among the experimental genes within these samples.

Table 5. Target probe fluorphores.

Target	Gene ID	Fluorophore
actin-1	PF3D7_1246200	Alexa Fluor- 750
falcipain-1	PF3D7_1115700	Alexa Fluor- 488
ap2-g	PF3D7_1222600	Alexa Fluor- 647
gexp5	PF3D7_0936600	Alexa Fluor- 568

Table 6. RNA-FISH label probe sequences.

Target: *actin-1*	
Function	**Sequence**
LE	tgggagaagaagatgttcaagctttagtt
LE	gttgacaacggatcaggtaatgtaaaagca
LE	ggagttgcaggagatgatgc
LE	acctcgttccgtttttccaagtata
LE	gtaggaagaccaaagaatccaggaattat
LE	ggttggtatggaagagaaagatgcat
LE	ttgttggtgatgaagcacaaaccaaga
BL	gaggtatattaacattaaagtatccaataga
BL	atgatatggaaaaaatatggcatca
LE	cacttttataatgaattaagagctgctccagaa
LE	gaacacccagtgttattaacagaa
LE	gctcctttaaatccaaaaggaaatcgt
LE	gagaggatgacacaaattatgtttgaatctttt
LE	aatgtaccagctatgtatgttgctattcaagct
LE	gttttatccttatattcttctggtcgtaccactg
LE	gtattgtgttagatagtggagat
LE	ggtgtatcacacactgttcca
LE	atttatgaaggttatgctttaccaca
BL	tgcaattatgagattagatttagctggta
BL	gagatttaactgaatatttaatgaaaat
LE	tcttcatgaaagaggttatggatttt
LE	caacatcagcagaaaaagaaattg
BL	ttagagatattaaagagaaattatgttatattg
LE	cattaaattttgatgaagaaatgaaaa
LE	catctgaacaaagcagtgatattga
BL	aaaatcatatgaattaccagatggaaat
BL	attattactgtaggtaatgaaagatttaga
LE	tgtccagaagctttattccaaccat
LE	ccttcttaggaaaagaagcagc
BL	aggaatccacacaactactttcaa
BL	ctctattaaaaaatgtgatgtggatattc
LE	gtaaagatctttatggaaatatcgttttat
LE	ctggaggtactactatgtatgaaggt
BL	ataggagaaagattaactagagatattacaa
LE	cccttgcaccatcaaccatgaaaatt
LE	aaagttgttgcaccaccagagagaa

LE	aatactcagtctggataggaggttc
LE	tatcttatcatctctttctacctttc
LE	aacaaatgtggatcacaaaagag
LE	gaatacgatgaatcaggacc

Target: *falcipain-2*

Function	**Sequence**
LE	ctccaaattctagaaaaagtgatttatttaa
LE	aaactcttcagttgaaaataataatg
BL	atgactatataataaatagcttgctaaaaa
LE	gccctaatggcaagaaatttatcgtctcaa
LE	aaattgatgaagccttatcattctat
LE	gatagtaaaaagaatgacata
LE	aataaatacaacgaaggtaat
LE	aacaacaataatgctgactttaaaggtctta
LE	gcttatttaaagaaaacacaccatcaaa
BL	taattttattcataataaagattattttataaat
BL	ttattaaaactaataataaacaatataattctcc
LE	aaatgaaatgaaggaaagatttca
LE	agtattcttacaaaatgcacacaaagta
BL	aatatgcataacaataataaaaatagttta
BL	tataaaaaagaattaaacagatttgccg
LE	atttaacttatcatgaatttaaaaacaaa
LE	tatcttagtttaagatcttcaaaaccatt
BL	aaagaattctaaatatttattagatcaaatgaa
BL	ttatgaagaagttataaaaaaatataaagg
LE	aaatgaaaattttgatcatgcagctt
LE	atgattggagattacatagtggtgt
BL	aacacctgtaaaggatcaaaaaaat
LE	tgtggatcttgctgggcctttagta
LE	gtataggttccgtagaatcaca
BL	atatgctatcagaaaaaataaattaataacc
LE	ttaagtgaacaagaattagtagattgtt
LE	catttaaaaattatggttgtaatggagg
BL	tctcattaataatgcctttgaggatat
BL	gattgaacttgggggtatatgt
LE	acagatgatgattatccatatgtaagtgat
LE	gctccaaatttatgtaatatagatagatgtac
LE	tgaaaaatatggaatcaaaaattatttat
LE	ccgtaccagataataaattaa

BL	aagaagcacttagattcttggg
BL	acctattagtattagtgtagccgtatcagatg
LE	attttgctttttacaaagaaggtattttcgat
LE	ggagaatgtggtgatcaattaaatcatgc
LE	cgttatgcttgtaggttttggtatgaaagaaattgt
BL	taatccattaaccaagaaaggagaaa
BL	aacattattattatataattaagaactcatgg
LE	ggacaacaatggggagaaagaggtttcataaa
LE	tattgaaacagatgaatcaggattaatgagaaaat
LE	gtggattaggtactgatgcattcattccattaatt
Target: *ap2-g*	
Function	**Sequence**
LE	gttcccatatcaaggctatttga
LE	ttttattttggtagttataacatatgtgc
BL	tcattacattgttttgttatattatgattaa
BL	ctttctttatctgttataatattattatatatattg
LE	ttttgttgaatcattatcattactttct
LE	catcgtcatcatttaacattgattg
BL	attatttaattttatattatccaatttatcag
BL	tgaaatctaagataattttcatgtaatgtt
LE	ttacatctttttcaagaacgttaggt
LE	tcattgtcatatgtttattaccaccat
BL	ttatagaattatgcttgaaattatctatatatc
BL	ctagatatattatacaaacggttactattattac
LE	tttttctttaaactattaagtgcctca
LE	attttgtaacctcatataaagtgagtca
BL	tgttatgaccactggaaaaagatataat
BL	aaattatttatatttataaccttaatctttgtat
LE	aatttttaaatttatttgcattattattg
LE	taggtgcattcatattatccatattta
LE	ccacattgaggtttgttactccat
LE	tcttcttttactacttcattgtttatgc
LE	catttatatttaggcaagtattagttttc
LE	aattttgtatgggaatgtatttgct
LE	ggcttcccatttttgatacatttc
LE	cattattttccttctgattaatatttcta
BL	cattatgagaattatcagattcaatatatacta
BL	attattctcaaaactgttattattattattatta
LE	tttggacttacatagcaacagcat

LE	tgttcattctattttgattcttaacatt
BL	tctttattttcctctttacgaaatagat
BL	atttatattttggttattataattgttcatat
LE	aatattatgcgtcaatggataaatatt
LE	tgattcaatatatggttaacaatgga
BL	ttgttctgtattttcattgtaataggaa
BL	tatgaacaattcatattattaacatctgat
LE	ggcatgttaccattaatcgattca
LE	tatgtatcacacgattgatcatttatt
BL	atcattatatgtatcattatattcatcattg
BL	tcattagtaattccatttaatgtatcatatag
LE	tgcatttgtaggtgtaatatatggtgta
LE	acattaattcttcataagaaaggttaca
BL	aatatatattattgttattttgcattttgt
BL	tttactatttacataagtattgatatttgtgac
LE	ttgtaacagcaacatttgaaaatatatt
LE	tattacaagcatgtaaatcagtatcgt

Target: *gexp5*	
Function	**Sequence**
LE	gaaagatcagattgaatcaa
LE	gtttatattcccagaggaccaaa
LE	attagtgctgaggataagg
LE	attcatttagttgtagaaaat
LE	ctagtccttttaacaaagagact
BL	aggaaggaaaaaagtttgagtgtg
BL	tttattgaatttttatatatataatggtaattg
LE	taatgtagtagaaggtacca
LE	ttggtcataaaagaaagctgtc
LE	tgaattagtagactccaaaagtg
LE	gttgtttgagaagtggtgaaaat
LE	gaaacgaatgtaaaaagttcatc
LE	atctcaaacggattctgtattaag
LE	cagtaataatttgacagatt
BL	tctataattcatataatgaattta
BL	atattaaagattaacttcttta
BL	catgataaatattatgattt
LE	aacaaaacagtggagtgatgaaa
LE	aaattgaagagattataaactctttattgga
BL	tattccaaataaatttgatgtacttactata

LE	tggatgcaagtccgaggtagtga
LE	gaaattgaagatgcataatatgttg
BL	tatggtttaagattattatataaa
BL	gaattaattaaaataatatt
BL	aatttccttgacttaactac
LE	taaagaaatattgacgcgtaca
LE	gattttatagactttattaaagaca
LE	cgaaaaataaattcaacaaattaa
LE	gaaatgaattaagagaaact
LE	gtaaggaaagatttaataga
*** LE- Label Extender, BL- Blocking Probe**	

3.14 Chromatin immunoprecipitation with qPCR

Two methods of chromatin immunoprecipitation (457) analysis were performed to optimize the process and determine the most appropriate method. Both protocols were performed as described (458).

3.14.1 Native ChIP

3.14.1.1 Nuclei isolation

Whole cultures (5-8 mL) were transferred to a 50 ml conical tube and pelleted at 3000 rpm for 5 minutes at 4°C. Samples were washed once with cold PBS prior to resuspension in chilled 0.05% Saponin solution (Sigma). Samples were inverted 5-6 times and placed back on ice for 5 minutes, or until cultures were visibly lysed. Samples were then spun down for 10 minutes at 600 g at 4°C. Pellets were washed with PBS and resuspended in cell lysis buffer (10 mM Tris-HCI pH 8.0, 3 mM MgCl2 (Sigma), 0.2% Nonidet P-40 (Sigma), and protease inhibitor cocktail (Roche)).

Samples were then gently homogenized using a glass homogenizer. Homogenate was layered onto cell lysis buffer containing 0.25 M sucrose and centrifuged for 600 g for 10 minutes. The pellet was then resuspended in cell lysis buffer containing 0.25% glycine and immediately frozen at -80°C.

3.14.1.2 Chromatin digestion

Nuclei pellets were resuspended in digestion buffer (50 mM Tris–HCl pH 7.4, 4 mM $MgCl_2$, 1 mM $CaCl_2$, 0.075% Nonidet P- 40, 1 mM DTT (Sigma), protease inhibitor cocktail) with micrococcal nuclease (Mnase) and exonuclease (NEB) for 10 minutes at 37°C. Reactions were then quenched with quenching solution (2% Triton X-100, 0.6% SDS, 300 mM NaCl, 6 mM EDTA) and sonicated in sonication buffer (25 mM Tris pH 7.4, 1% Triton X-100, 0.3% SDS, 150 mM NaCl, 3 mM EDTA, 2 mM $MgCl_2$, 0.5 mM $CaCl_2$, protease inhibitor cocktail) for 3 minutes (15 seconds on, 15 seconds off). Samples were then centrifuged at 9600 g for 10 minutes prior to storage at -20°C.

To assess digestion efficiency, an aliquot of each sample was analyzed by gel electrophoresis. DNA was purified (Qiagen) and quantified using Nano-drop. Each sample (200 ng) was then examined on a 2% agarose gel, wherein sizes were expected to range from 200-800 bp.

3.14.1.3 ChIP

After ensuring digestion efficiency by gel electrophoresis, 500 ng DNA was incubated with Rabbit IgG, anti-H3, anti-H3K9me3, and anti-H3K9Ac (Table 5). Antibodies were incubated with each sample for a minimum of 12 hours at 4°C while under constant rotation. Protein A and Protein G Dynabeads (ThermoFisher) were added to each reaction and incubated for an additional 2 hours.

Samples were then washed as follows: two washes with 20 mM Tris–HCl pH 8.0, 2 mM EDTA, 1% Triton X-100, 0.1% SDS, 150 mM NaCl, two washes with 20 mM Tris pH 8.0, 2 mM EDTA, 1% Triton X-100, 0.1% SDS, 500 mM NaCl, and two washes with 10 mM Tris-HCl pH 8.0, 1 mM EDTA, prior to elution with elution buffer (1% SDS, 0.1 M $NaHCO_3$). Samples were then de-crosslinked (10% SDS, 1 M $NaCO_3$, 5 M NaCl) overnight at 45°C, followed by DNA purification with Qiaquick PCR purification kit (Qiagen). Primers for qPCR analysis are listed below (Table 6). The amount of target DNA recovered after immunoprecipitation was directly compared to a 2% input sample of DNA and analysis was performed using the percent input method.

3.14.2 Cross-linked ChIP

3.14.2.1 Cross-linking and nuclei isolation

Cultures were pelleted prior to resuspension in 1% formaldehyde for cross-linking. Samples were then incubated for 15 minutes at 37°C in a shaking water incubator and subsequently quenched with 0.125 M glycine. Samples were washed with PBS and lysed in 0.05% Saponin solution. After an additional wash, isolates were gently homogenized in cell lysis buffer (10 mM Tris-HCI pH 8.0, 3 mM $MgCl_2$, 0.2% Nonidet P-40, protease inhibitor cocktail (Sigma)), and separated on a 0.25 M sucrose gradient as described in section 3.13.1.1.

3.14.2.2 Chromatin digestion

Nuclei pellets were resuspended in digestion buffer and digested as detailed in section 3.13.1.2. To assess digestion efficiency, an aliquot of each sample was taken and de-crosslinked (10% SDS, 1 M $NaCO_3$, 5 M NaCl) overnight at 45°C. DNA was then purified (Qiagen), quantified

using Nano-drop, and run on an electrophoresis gel to determine digestion efficacy as performed previously in section 3.13.1.2.

3.14.2.3 ChIP

After ensuring digestion efficiency by gel electrophoresis, 500 ng DNA was used for ChIP analysis as detailed in section 3.13.1.3. After washing and elution of beads, samples were de-crosslinked (10% SDS, 1 M $NaCO_3$, 5 M NaCl) overnight at 45°C, followed by DNA purification with Qiaquick PCR purification kit (Qiagen). Resulting DNA was used for qPCR analysis and quantified using the percent input method.

Table 7. ChIP antibodies.

ChIP Antibodies		
Target	**Manufacturer**	**Concentration**
Rabbit IgG	Millipore-Sigma, 12-370	2.5 mg
Anti-H3	Abcam, ab1791	2.5 mg
Anti-H3K9me3	Millipore-Sigma, 07-442	5 mg
Anti-H3K9Ac	Sigma-Aldrich, 06-942	5 mg

Table 8. ChIP primers.

ChIP qPCR Primers		
Gene ID	**Name**	**Sequence (5'-3')**
PF2D7_1246200	actin-1 Fw	AGCAGCAGGAATCCACACA
	actin-1 Rv	TGATGGTGCAAGGGTTGTAA
PF3D7_1222600	ap2g- Fw	TGGTGGTAATAAGAACAACAGAGGT
	ap2g- Rv	CCATCATAATCTTCTTCTTCGTCG
PF3D7_0412400	pfemp1- Fw	ACCGCCCCATCTAGTGATAG
	pfemp1- Rv	CACTTGGTGATGTGGTGTCA

3.15 Image analysis

Blood smears were prepared using 5 μl whole culture and stained for 10 minutes in 10% Giemsa stain (Sigma). All images were taken on an Olympus Provis microscope at 40x-100x magnification. Gametocyte quantification was performed using ImageJ Fiji software wherein color channels were segmented, the threshold adjusted, and image subtraction performed to determine gametocyte versus uninfected erythrocytes. Particles were then analyzed and quantified to calculate gametocytemia.

3.16 Statistical analysis

GraphPad Prism 8.0 software was used for all statistical analysis. For comparison of significance between three or more means, a one-way ANOVA was used. In these analyses, if groups were compared to one control group (e.g. Vehicle vs 12 nM, Vehicle vs 30 nM, etc.), correction for multiple comparisons was completed using the Dunnett test. If groups were compared to every other group (e.g. Hb A vs Hb AS, Hb AS vs Hb S, etc.), correction for multiple comparisons was completed by Tukey's honestly significant difference test (HSD). For experiments with repeated measures (i.e., measurements across time), in which certain values were missing for random reasons, a mixed-effects analysis was completed, as repeated measures ANOVA cannot handle missing values. For these analyses, correction for multiple comparisons was completed by Tukey's HSD, and results are interpreted like repeated measures ANOVA. For comparison of multiple group means between two factors in which measures were not repeated, data were analyzed by an ordinary two-way ANOVA. For comparison of multiple group means

between two factors in which measures were repeated, data were analyzed by repeated measured two-way ANOVA. For both of these data, correction for multiple comparisons was completed by either the Tukey HSD, or Dunnett test as distinguished above. For all data, statistical significance was based on a p- value of less than 0.05.

The correlation between hemozoin and sexual conversion rate was determined by Pearson correlation analysis. Gametocyte quantification was completed through linear models generated from the resulting qPCR Ct values versus the log-transformed number of molecules of DNA (calculated from the pcr-generated DNA). Gene expression analysis through qPCR is calculated using the relative quantity (ΔΔCt) method, wherein the ΔΔCt was determined by the difference between the average control values subtracted from the difference between the average experimental values. The resulting ΔΔCt was then log transformed ($2^{-\Delta\Delta Ct}$), providing the fold change levels in gene expression (459).

4.0 Results

4.1 Hemoglobin S-Ery promote gametocyte conversion in *P. falciparum* in NF54 parasites *in vitro*

The production of gametocytes in Hb A-Ery cultures has been well established, with proportion survival after NAG treatment used to determine the GCR (337, 338, 460, 461). By using this method which induces sexual commitment in Hb A-Ery cultures, we compared the corresponding GCR occurring in Hb S- and Hb AS-Ery.

4.1.1 Gametocyte conversion assays can be adapted for the culture of Hb variant erythrocyte samples

The gametocyte assay used in this research is primarily based on methods used in Fivelman, et al. (337, 338). Some modifications were made to ensure assay sustainability in Hb S- and Hb AS-Ery. All Hb S and Hb AS blood samples were received from steady-state sickle cell patients or volunteers with Hb S-trait. To reduce the amount of sample needed from all volunteers, the total amount of Hb S- and Hb AS-Ery used for these experiments was limited to the fullest extent possible. In doing so, one "feeder" culture was used to inoculate each experimental culture at the beginning of each assay (Figure 13A). The feeder cultures were maintained in O+ Hb A-Ery blood received from FDA authorized collection facilities and synchronized to a six-hour development window through sorbitol lysis (Figure 13B). Late-stage schizonts (40-48 hours of

intraerythrocytic development) were then magnetically isolated from the feeder culture and used to inoculate each experimental group (Figure 13C, D).

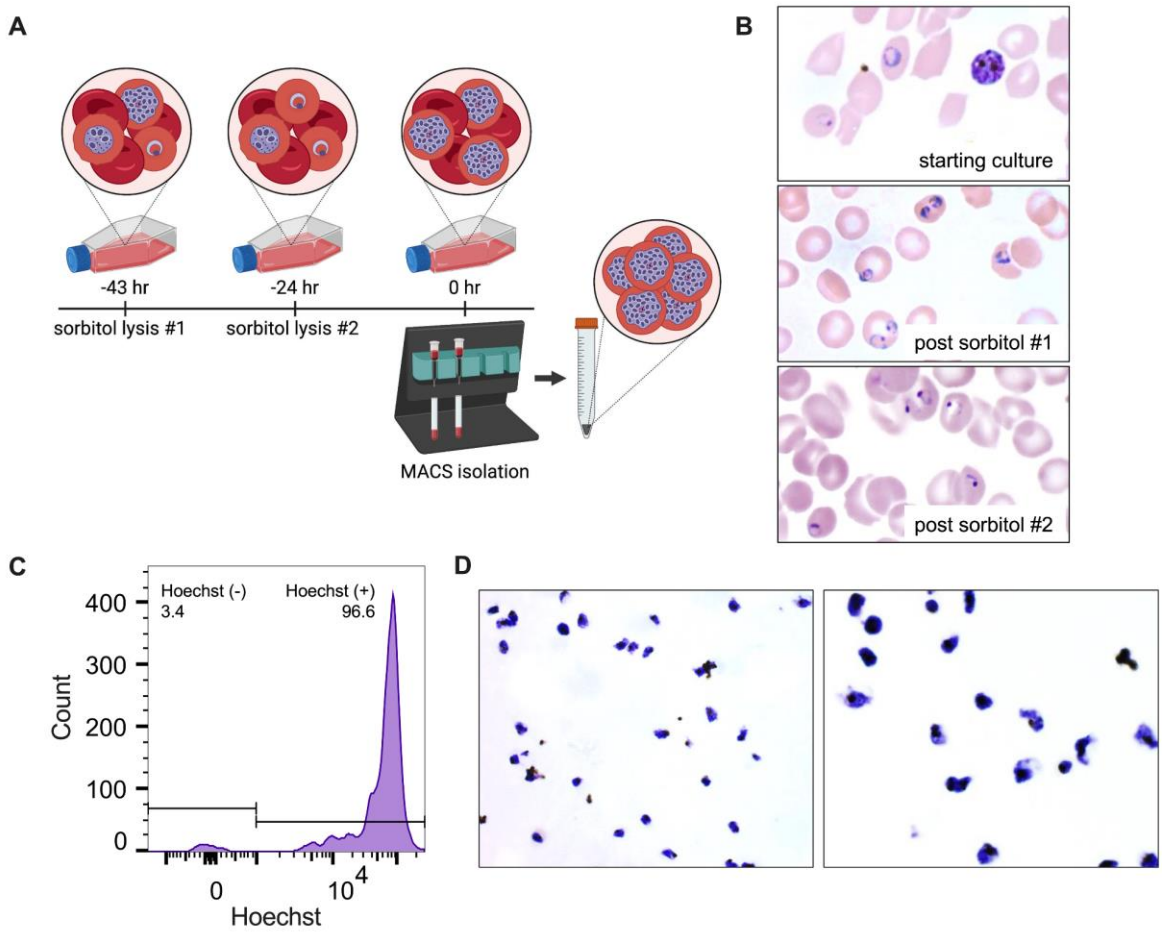

Figure 13. Isolation of mature schizonts for gametocyte conversion assays.

(A) Timeline of *P. falciparum* NF54 culture synchronization and schizont isolation using the MACS magnetic separator. **(B)** Representative images of cultures throughout the synchronization process, showing a homogeneous population of rings after the second sorbitol synchronization (bottom panel). **(C)** Representative flow plot of magenetic isolation of schizonts. Hoechst positivity indicates parasite-positive erythrocytes **(D)** Representative images of isolated schizonts. All images were captured using an Olympus Provis microscope at either 60x (left) or 100x (right) and obtained from blood smears stained with Giemsa.

Late-stage schizonts were used for inoculation because they can be efficiently grown to a synchronous level and then isolated from uninfected erythrocytes. We found approximately 900,000 late stage schizonts were needed for each 2 mL culture. This number reliably produces a

starting parasitemia of 0.3% on day 0, and an overall parasitemia of approximately 1-2% on day one of the assay. All schizont preparations isolated from feeder cultures were counted with a hemacytometer prior to inoculating fresh cultures. To obtain this number of synchronous, magnetically isolated schizonts, large culture volumes were needed. For each experiment, we expanded cultures to 60-80 mL total volume at a 4% HCT, with each T75 culture flask containing 20-25 mL.

Two sorbitol synchronizations were necessary to ensure a six-hour development window, as each sorbitol treatment synchronizes parasite cultures to approximately 0-20 hours of intraerythrocytic development. Sorbitol synchronization functions through osmotic lysis of later stages (21-48 hours of intraerythrocytic development), as the parasites develop NPPs (Figure 13B) (462). The transfer of sorbitol through these NPPs leads to hypotonicity-induced cell lysis, enriching the culture of early, ring-stage parasites (i.e. parasites approximately 0-20 hours of intraerythrocytic development) (463, 464). Therefore, by performing these synchronizations 18-hours apart, we were able to produce a six-hour development window, by sequentially removing the older stages.

We found it best to perform the first synchronization step on cultures that contained at least 3% ring-stage parasites, at no higher than 6% total parasitemia. This was crucial because it provided enough parasites to sustain the multiple synchronizations, without producing too many parasites which would overly-stress the culture prior to beginning the assay. Isolation of mature schizonts was performed by magnetic separation 24 hours after the second sorbitol lysis, on cultures that were typically between 1-2% late-stage schizonts. Prior to isolation, cultures were carefully analyzed by blood smear to ensure gametocyte numbers were no greater than 0.05%. Magnetic isolation of parasites is possible because of hemozoin which, as explained in section

1.3.1.2, is produced by the breakdown of Fe- containing Hb. The ferric state of iron-containing hemozoin contains paramagnetic properties that are not present in uninfected erythrocytes or early-stage parasitized erythrocytes (465-467). As more hemozoin is produced throughout the intraerythrocytic cycle, the magnetic properties of the cell become greater. To ensure complete separation of mature schizonts, the cultures were double purified, wherein the resulting isolates from the first magnetic column were placed through a fresh magnetic column. Confirmation of purity was performed through microscopic examination and flow cytometry, which showed this method to be approximately 97% effective in complete isolation of parasitized erythrocytes from uninfected erythrocytes (Figure 13C, D). This isolation technique was critical to perform, as the invasion rate between Hb A and Hb AS-Ery has previously been shown to differ, in favor of Hb A-Ery (417, 419).

4.1.1.1 Flow cytometry provides an accurate method for determining parasitemia among *P. falciparum* cultures in Hb variant erythrocytes

As very low levels of parasitemia were expected among the Hb AS- and Hb S-Ery cultures, to ensure parasitemia could be accurately measured by flow cytometry, we first validated the method of measurement with low values.

A culture of 1% parasitemia was used to prepare serially diluted samples between 0.03 – 1% parasitemia. Samples were prepared in triplicate and were verified by microscopic quantification. For validation by microscopic quantification, an average of three reads from 2000 uninfected erythrocytes was used. Samples were then stained with Hoechst and quantified by flow cytometry. Resulting values were compared to expected values, with no significant variance in measurement detected (Figure 14). Therefore, we determined flow cytometry to be effective in

parasite quantification down to at least 0.03% parasitemia, which is below that of our expected range.

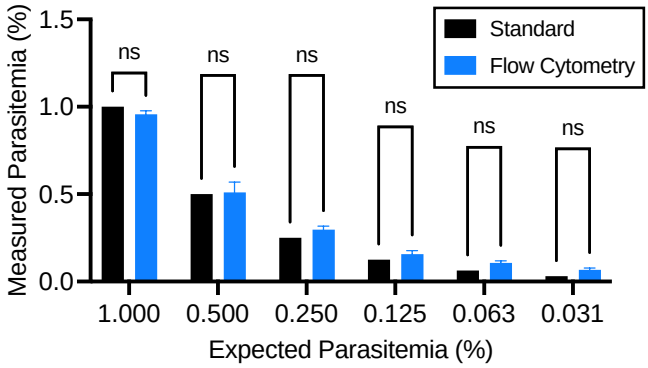

Figure 14. Validation of flow cytometry for measurement of low parasite quantities.

Sensitivity of flow cytometry in measuring low parasite densities in *P. falciparum* cultures. Standards were generated through serial dilutions of samples, confirmed with three microscopic readings. Experimental samples were then compared to the standard to determine sensitity.

4.1.2 Gametocyte conversion rates are significantly increased among Hb S-Ery

The rate of gametocyte conversion was determined via two methods as outlined in section 3.1.1. Ultimately, both methods resulted in similar trends among the Hb A-, Hb AS-, and Hb S-Ery cultures tested. The rationale and specific results of each method are described in the following sections.

4.1.2.1 Hb S-Ery promote gametocyte conversion: method 1 for determination of GCR

The first method for determining GCR was based off the rationale of inducing sexual commitment with a high culture parasitemia (Figure 15A). While the Hb A-Ery cultures proliferated as anticipated, it was apparent overall proliferation within the Hb AS- and Hb S-Ery cultures was limited. Notably however, the cultures did sustain and consistently maintained

between a 1-2% parasitemia for each donor sample analyzed (Figure 15B). After NAG addition on day seven, all experimental groups experienced a drop in total parasitemia. Ultimately this indicates that asexual replication was occurring amongst all experimental cultures.

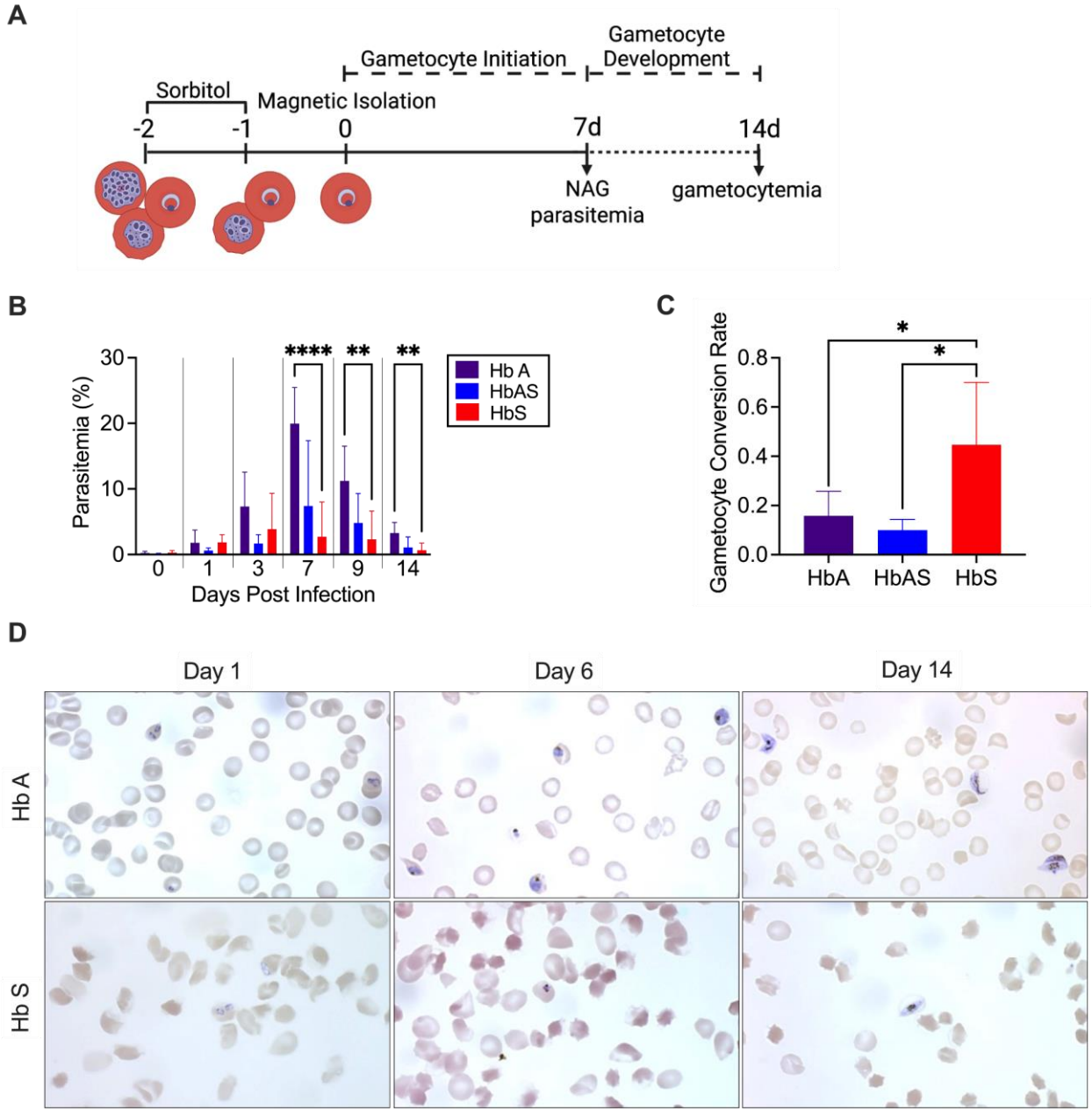

Figure 15. Determination of gametocyte conversion, method 1.

(A) Schematic of method 1 for gametocyte conversion. **(B)** Parasitemia or gametocytemia measured by flow cytometry by day throughout the course of the assay for Hb A-, Hb AS-, and Hb S-Ery (n=6). Days 0-9 represent mixed populations of asexual and sexual stages, while day 14 represents gametocytemia after clearance of asexual stages by NAG treatment. **p<0.01, ****p<0.0001, mixed-effects analysis. **(C)** Accompanying sexual conversion rate calculated by total gametocytemia at day 14 obtained from total parasitemia on day 7. *p<0.05, one-way ANOVA. **(D)** Representative images throughout culture period. Images captured using Olympus Provis microscope (100x) and obtained from blood smears stained with Giemsa.

The rate of gametocyte conversion was determined based on the ratio of day 14 gametocytemia obtained from the day seven total parasitemia (Figure 15B, C). Blood smears on day 14 were used to confirm the presence of mature gametocytes and absence of asexual parasites in all cultures to ensure a clean population of gametocytes were being measured (Figure 15D, left panels). While total gametocytemia was found to be significantly higher among the Hb A-Ery cultures than the Hb S-Ery cultures, the resulting GCR was found to be higher among the Hb S-Ery cultures (Figure 15C). This indicates Hb S-Ery promote sexual commitment at a higher rate than Hb A-Ery.

4.1.2.2 Hb S-Ery promote gametocyte conversion: method 2 for determination of GCR

The second method for determining GCR bases the rate from the first replication cycle (Figure 16A). As noted in method 1, though parasitemia within Hb AS- and Hb S-Ery cultures was overall lower than the Hb A-Ery culture over the first replication cycle, notably, the cultures did sustain, and consistently maintained between a 0.5-2% parasitemia over the seven-day period for each donor sample analyzed (Figure 16B-D). Additionally, blood smears show normal development in all cultures through day two of culture (Figure 16E). Though total parasitemia was significantly different on both day four and day seven, a non-significant difference in gametocytemia was observed between all groups (Figure 16C, D). From the stage II gametocytemia measurement on day four, we found a significantly increased rate of gametocyte conversion among Hb S- Ery cultures compared to Hb A-Ery (Figure 16C). This difference was

sustained through day seven (Figure 16D). Moreover, as the GCR did decline amongst all groups between day four and day seven, upon comparing the day four rates to the day seven rates, no significant difference in rate was observed between any group (Figure 16F). This indicates that though there was gametocyte loss, the rate of loss was not due to any variant tested.

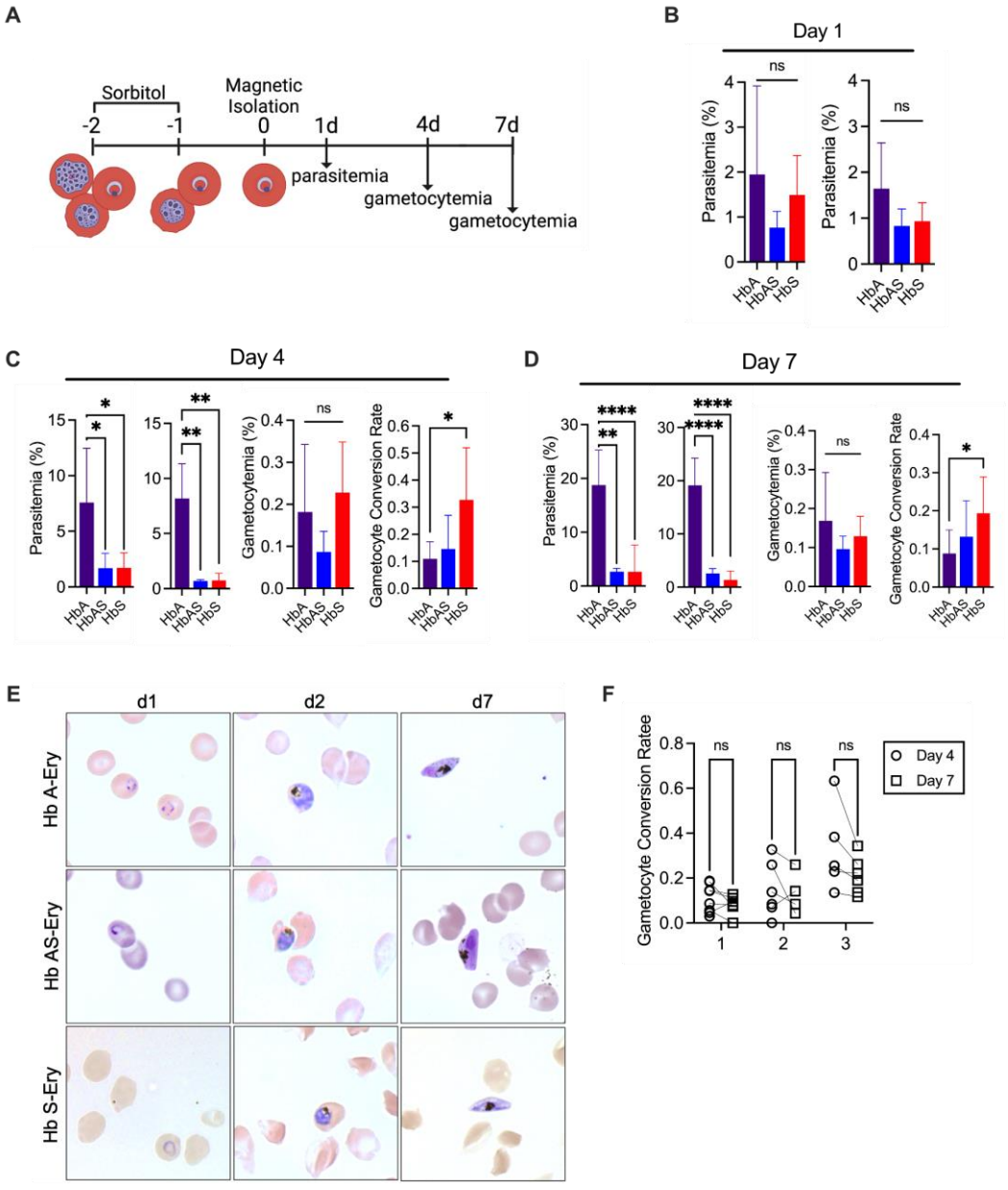

Figure 16. Determination of gametocyte conversion, method 2.

(A) Schematic of method 2 for gametocyte conversion. **(B)** Day one parasitemia measured by flow cytometry (left) and microscopy (right) 24 hr after inoculation (n=6). one-way ANOVA. **(C)** Day four parasitemia (by flow cytometry, left, and microscopy, right), and stage II gametocytemia measured by microscopy. Resulting GCR (right), calculated from microscopy measurements of parasitemia at day one and stage II gametocytemia on day four (n=6) *p<0.05, **p<0.01, one-way ANOVA. **(D)** Day seven parasitemia (by flow cytometry, left, and microscopy, right), and stage III gametocytemia measured by microscopy. Resulting sexual conversion rate calculated from microscopy measurements of parasitemia at day one and stage III gametocytemia on day seven. (n=6), *p<0.05, ****p<0.0001, one-way ANOVA. **(E)** Representative images throughout culture period. Images captured using Olympus Provis microscope (100x) and obtained from blood smears stained with Giemsa. **(F)** GCR between day four and day seven in Hb A-, Hb AS-, and Hb S-Ery (n=6). Two-way ANOVA.

4.1.3 Equating the low parasitemia of Hb S-Ery to Hb A-Ery results in decreased rates of gametocyte conversion

In both methods, we found the cultures produced within Hb S-Erys to significantly increase the rate of gametocyte conversion. As the level of parasitemia was also found to be significantly different, with the Hb AS- and Hb S-Ery cultures displaying significantly lower levels compared to that of the Hb A-Ery cultures, we sought to verify whether the observed change in gametocyte conversion was due to low levels of total parasitemia or due to an effect of the Hb protein.

Additional Hb A-Ery cultures were prepared as previously described and inoculated with dilutions of the isolated schizont pellets. This generated varying levels of parasitemia on day one, represented as “Low”, “Medium”, and “High,” meant to mimic the different parasitemia levels observed among the Hb S- and Hb AS-Ery cultures (Figure 17A). We then used both methods to determine the rate of sexual conversion (Figure 17A-B).

For both methods, we observed the opposite trend in gametocyte conversion, as the low parasitemia group resulted in a lower rate of sexual conversion. For method 2, no gametocytes were detected by microscopy in the low parasitemia group (Figure 17B). Together, these results indicate the higher gametocyte conversion observed among the Hb AS- and Hb S-Ery cultures was not due to the low parasitemia achieved by both cultures. It also indicates there was no bias in the

gametocyte conversion results obtained from microscopic counting or flow-cytometry, due to the low parasitemia.

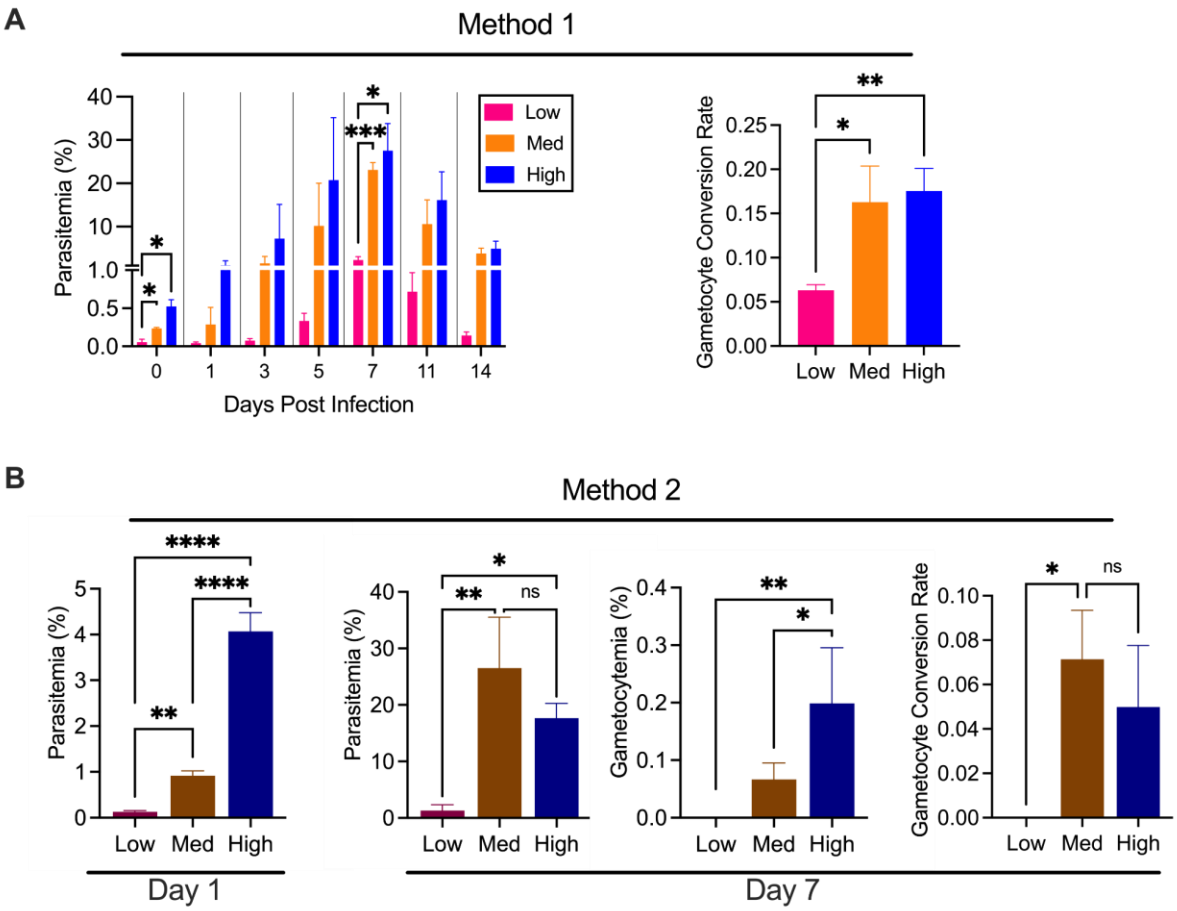

Figure 17. Validation of determined GCRs with parasitmia-adjusted Hb A-Ery culture.

(A) Method 1 for gametocyte conversion among Hb A-Ery cultures only, started at low parasitemias. Total parasitemia measured by flow cytometry by day throughout the course of the assay (left), and resulting gametocyte conversion rate (right). Low, Medium, and High cultures started with 0.1%, 0.5%, and 1% schizonts respectively (n=3). Days 0-11 represent mixed populations of asexual and sexual stages, while day 14 represents gametocytemia after clearance of asexual stages by NAG treatment. **p<0.01, ***p<0.001, mixed-effects analysis. Resulting gametocyte conversion rates from parasitemia at day seven and gametocytemia on day 14. (n=3), *p<0.05, **p<0.01, one-way ANOVA. **(B)** Method 2 for gametocyte conversion among Hb A-Ery cultures only, started at low parasitemias. Day one parasitemia (left-most panel) and day seven parasitemia, gametocytemia, and resulting GCR. Day one parasitemia measured by microscopy 24 hr after inoculation. Day seven parasitemia (left), and stage III gametocytemia (middle) measured by microscopy. Resulting gameotycte conversion rate (right) calculated from microscopy measurements of parasitemia at day one and stage III gametocytemia measurement at day seven (n=3). *p<0.05, **p<0.01, ****p<0.0001, one-way ANOVA.

4.1.4 Hb A- and Hb AS- Ery cultures display similar exflagellation rates among mature microgametes

In attempts to quantify microgamete viability, we performed exflagellation assays between days 13-15 of Hb A, Hb AS, and Hb S-Ery gametocyte cultures. While we found microgametes produced in Hb A and Hb AS-Ery cultures to exflagellate as expected, no exflagellation of microgametes was observed among the Hb S-Ery (Figure 18A, Hb S not included). However, considering the low gametocytemia obtained from the Hb S-Ery cultures, this finding wasn't unexpected, as other reports have indicated a gametocytemia of approximately 4-6% is necessary to reliably quantify exflagellation (468).

In attempts to automate exflagellation analysis, videos were collected during early experimentation on Hb A and Hb AS-Ery cultures (videos not included). When it was determined Hb S-Ery exflagellation would not contribute significantly to overall conclusions, no further analysis was pursued.

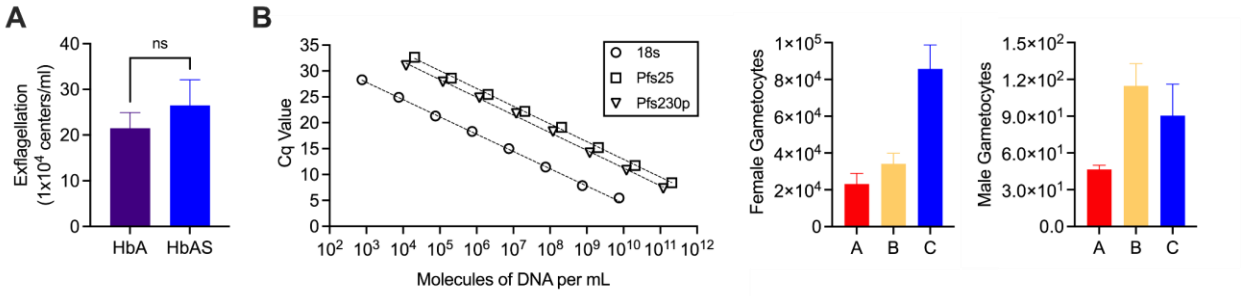

Figure 18. Quantification of exflagellation, and micro- and macrogametocytes.

(A) Exflagellation comparison between Hb A- and Hb S-Ery cultures. The number of exflagellation centers were recorded over the course of 10 minutes to determine microgametocyte viability. No difference in viability was determined between the groups (n=5). **(B)** Gametocyte quantification by sex-specific qPCR wherein Pfs25 and Pfs230p were used to quantify macrogametocytes and microgametocytes respectively. Average Cq are plotted versus the determined molecules of DNA present (per mL), with the resulting curve used for female and male quantification from three different cultures.

4.1.5 Gene expression can be used to determine gametocyte sex ratios in Hb A-, Hb AS-, and Hb S-Ery cultures

The ratio of micro- to macrogametocytes has previously been reported to be a significant factor for successful transmission, as there is a small window of time in which both must be consumed by a mosquito during a blood meal (159, 161, 469). In order to determine whether there was an influence of the Hb variants on the ratio of micro- to macrogametocytes, and thus a potential difference in transmission efficiency, we prepared a qPCR standardization curve to quantify micro- and macrogametocytes (Figure 18B) (470, 471).

Micro- and macrogametocyte specific genes *Pfs230p* and *Pfs25* were used for quantification respectively. Serial dilutions prepared from known quantities of amplified DNA for each gene were prepared and plotted against the resulting qPCR Ct value. While generation of the standard curves were successful, their utility for quantification of mature gametocytes produced within the Hb A-, Hb AS-, and Hb S- Ery cultures was ultimately not feasible due to the low gametocyte yield and/or culture volume required to complete this analysis. To generate reliable quantities of RNA for use in the standard curve, we would have needed approximately 10 mL of whole culture. Those quantities would only be possible through the pooling of samples, which would ultimately not be suitable for these experiments.

4.1.6 Summary

These results show that erythrocytes containing Hb S induce a greater rate of gametocyte conversion compared to erythrocytes containing Hb A. Though the rates observed in method 2 were overall lower than those observed in method 1, the overall observed trends were consistent

between methods. Interestingly, though the change in GCR between the Hb A- and Hb AS-Ery cultures were not significant, rates were higher among the Hb AS-Ery culture groups, landing in between that of Hb S and Hb A. This gives the appearance of a dosing effect of Hb S on sexual conversion.

While we did observe a significant difference in parasite proliferation between Hb A- and both the Hb AS- and Hb S-Ery cultures, intraerythrocytic development did not appear significantly impaired through the first replication cycle in which GCR was determined. Ultimately, these results indicate that Hb S is a driver of gametocyte conversion, modifying *P. falciparum's* reproductive investment strategy.

4.2 Efficiency of FP2-mediated Hb hydrolysis varies among Hb variants

The change in reproductive strategy witnessed among the Hb S-Ery cultures illustrates a shift in investment from intra-host survival to inter-host transmission. Though we show the condition of low parasitemia was not the factor which initiated the change in sexual conversion, the alteration in reproductive approach is indicative of a factor restraining the success of intra-host survival. As we did observe a significant change in parasite proliferation over the culture period, it is likely these two events, increased GCR and decreased asexual proliferation, are not independent.

Interestingly, we note Hb S to have a dosing effect on gametocyte conversion, wherein the Hb AS-Ery cultures resulted in a GCR in between that of Hb A- and Hb AS-Ery. As the digestion of Hb is a vital part of the intraerythrocytic cycle, providing the parasite with amino acids needed for protein synthesis and preventing the premature lysis of infected cells, this prompted the

question of a direct effect by the protein (110, 111, 137, 453-455). It has previously been hypothesized that Hb S may be resistant to parasite-mediated digestion, ultimately limiting the parasite's ability to sustain life within these cell environments (391, 417). Because of the importance of the Hb protein to intraerythrocytic survival, we next looked at the process of Hb digestion as a potential stressor that may prompt the observed change in reproductive investment.

To investigate Hb digestion between Hb A, Hb AS, and Hb S, we analyzed Hb hydrolysis with an *in vitro* assay (454, 455). By combining a high pH, physiologically relevant reducing agent, and recombinant *P. falciparum* enzyme, falcipain-2 (FP2), this assay provides a clean examination of Hb digestion, while mimicking the essential components of the digestive vacuole environment.

4.2.1 A recombinant cysteine protease effectively digests Hb *in vitro*

The bacterial containing the plasmid construct of FP2 was received by the generous donation of Phillip Rosenthal and produced using an *E. coli* expression system. FP2 is a papain-like cysteine protease containing eight cysteine residues and four disulfide bonds (454). Cysteine proteases play a central role in the digestion of Hb, and inhibitors which block cysteine proteases block Hb hydrolysis, ultimately disrupting parasite development (454, 472-475).

Falcipain-2 was cloned into the pQE-30 expression vector and transformed in M15 *E. coli* (454). An interesting feature of FP2 among other papain family proteases is its lack of a need for a prodomain for proper refolding (476-478). Instead, FP2 contains an amino-terminal extension which mediates folding, while the prodomain, as with other papain-family proteases, contains a conserved region which serves as an inhibitor of the mature protease (479, 480). For this reason, only 35 amino acids of the C terminus of the prodomain were necessary to produce the protein.

4.2.1.1 Sequencing of FP2 plasmid confirms protein identity prior to large-scale production

In order to confirm the correct protein construct was received, the plasmid was sequenced. Primers were designed based on the cloned FP2 sequence, as well as the T5 promoter and lambda t0 terminator regions of the pQE-30 vector (Table 3). Primers were premixed with isolated DNA and sent to GeneWiz for sequencing. All sequences, except "5' No. 1", passed quality control based off the quality score or contiguous read length. In the case of the 5' No. 1 sequence, it is assumed both the primer length and GC content did not allow for appropriate annealing. All other contiguous read lengths were 900+ bases. These sequences were then aligned against FP2 (PF3D7_1115700) which returned a high (97-99%) identity in the expected regions (Table 9, Figure 19).

Table 9. NCBI nucleotide blast results for confirmation of *fp2* identity.

Primer	Max Score	Total Score	Query Cover	Percent Identity
T5 Promoter	1432	1432	60%	97.47%
3' No. 1	1384	1384	56%	98.47%
3' No. 2	1243	1243	51%	98.31%
5' No. 2	907	907	36%	99.01%
Lambda T0	883	883	37%	97.67%

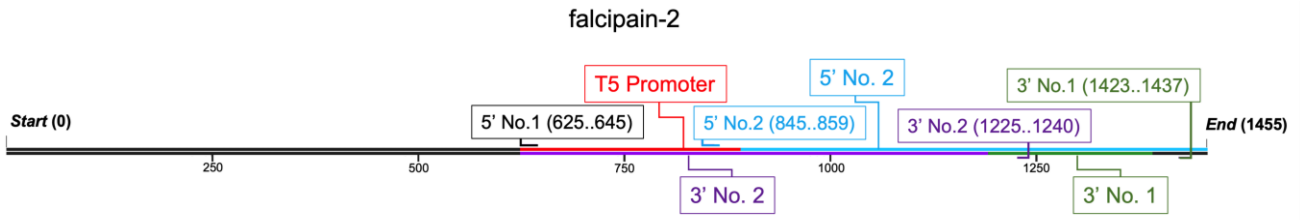

Figure 19. Alignment of each primer along *fp2* sequence.

Falcipain-2 gene with the location of each primer alignment indicated by color. Returned sequences were blasted against the *fp2* gene to confirm alignment and gene identity (Table 9).

4.2.1.2 Recombinant FP2 can be efficiently isolated and refolded into an active protease

After confirming the construct, we proceeded with the generation of recombinant FP2. A full, detailed description of this process is available in Section 3.9. Briefly, the *E. coli* containing the plasmid construct were grown to mid-log phase and induced with IPTG. Cells were harvested, washed, and sonicated to release inclusion bodies. After separating out the inclusion bodies, the pellets were solubilized, and the protein was column purified on a Ni-NTA resin (Figure 20A). Column fractions were run on a protein gel and stained with Coomassie blue to confirm protein isolation (Figure 20B). As revealed on the gel, much protein is lost during the wash and refolding stages. However, upon literature review, it appears this loss is normal, requiring even larger starting quantities to generate enough folded protein (454).

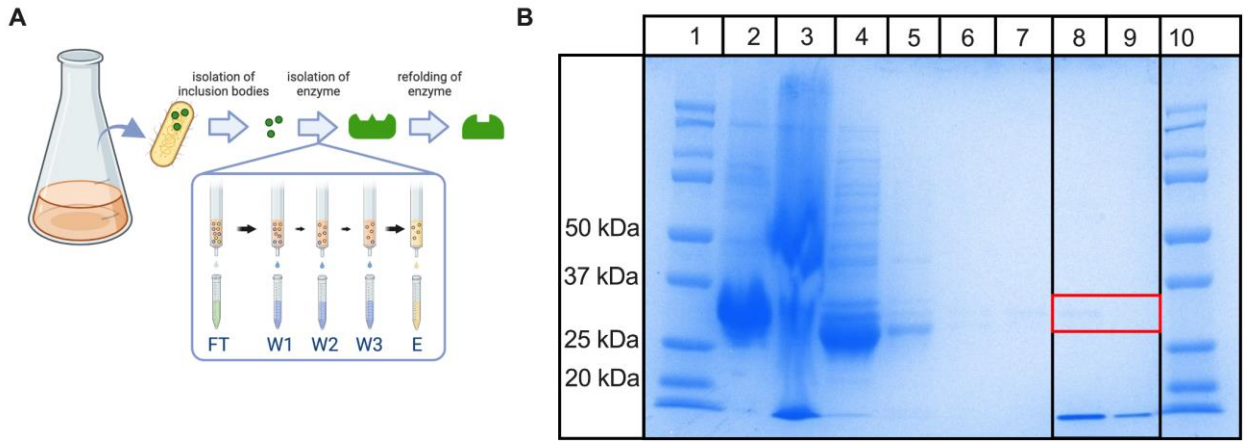

Figure 20. Generation of recombinant FP2.

(A) Diagram of recombinant protein generation, isolation, and extraction from *E. coli.* **(B)** Representative protein gel stained with coomassie blue. Lane 1, 10: Ladder, Lane 2: isolated inclusion bodies prior to solubilization, Lane 3: solubilized inclusion bodies, Lane 4: flow through after column incubation, Lane 5-7: washes 1-3, Lane 8-9, elution fractions. Red box around faint bands in lane 8 and 9, indicative of FP2 protein.

The production of functional proteins containing disulfide bonds can be difficult, as this post-translational modification requires the oxidative folding of cysteine pairs. In natural systems, this oxidative environment is typically sequestered to specialized compartments of the cell. As the cytoplasm in both eukaryotic and prokaryotic cells is a reducing environment due to the presence of reductases such as thioredoxins and glutaredoxins, special refolding conditions are needed to not only ensure cysteine bond formation, but proper cysteine bond formation *in vitro* (481).

Here, the solubilized protein was first reduced with DTT to cleave previously formed disulfide bonds and maintain cysteines at a reduced state. To ensure proper refolding, glutathione in both oxidized and reduced forms was used at a 1:1 ratio to provide a redox environment which would allow for the mutual reshuffling and formation of disulfide groups (482-484). The use of L-arginine suppresses protein aggregation, and glycerol promotes protein stabilization (482, 485-489). Together, these components were found to provide a suitable environment for FP2 refolding (454).

To ensure successful refolding of FP2, activity was measured using the fluorogenic peptide substrate, Z-LR-AMC. With this peptide substrate, the highly fluorescent AMC group remains quenched by the arginine residue of the Z-LR. An enzyme capable of cleaving this bond results in a fluorescent signal due to the release of AMC (Figure 21). As expected, fluorescent signal increased steadily over time, at the low pH condition, with little change occurring at the high pH condition (Figure 21). The pH is a crucial component for FP2 activity as the food vacuole in the natural system is highly acidic. BSA had no effect on Z-LR-AMC. These results indicate the proper refolding and activity of FP2.

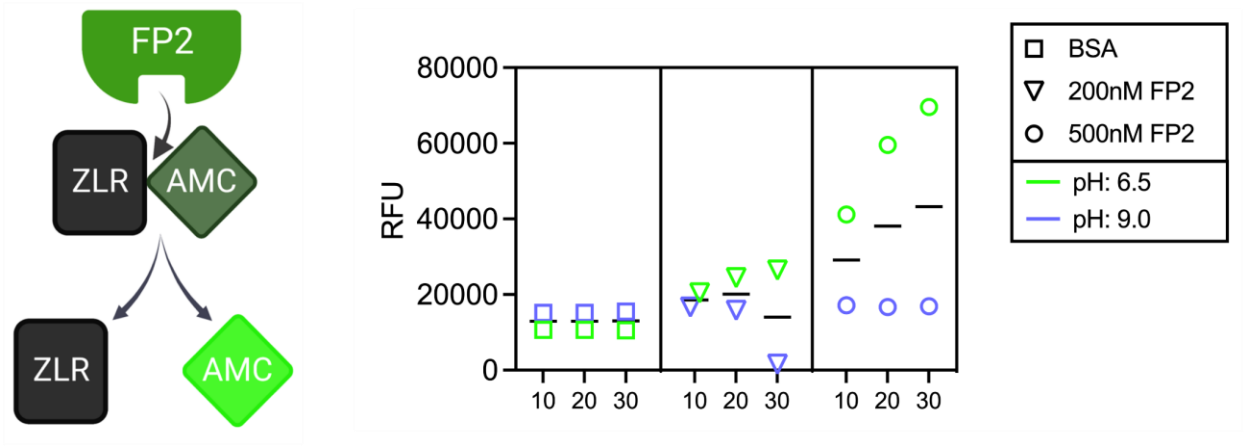

Figure 21. FP2 activity assay confirms FP2 refolding and activity.

Diagram of ZLR-AMC activity assay for determining proper folding and activity of FP2 (left), and activity assay of FP2 confirming the proper refolding of the enzyme. Comparison of negative control BSA (left panel), 200 nM FP2 (middle panel), and 500 nM FP2 (right panel) after 10, 20, and 30 minutes incubation.

4.2.2 Hb S is more efficiently digested by FP2 than Hb A

Using the prepared recombinant FP2, we reveal significant disparities in hydrolysis efficiency between the Hb variants tested (Figure 22). Overall, we found Hb S to experience the highest rate of hydrolysis and a sample containing Hb A, Hb S, and Hb F (Hb ASF) to experience

the most resistance to hydrolysis. The Hb ASF sample was included in analysis because Hb F has been reported to be resistant to *P. falciparum* hemoglobinase activity, and therefore served as a control demonstrating a degree of resistance (490).

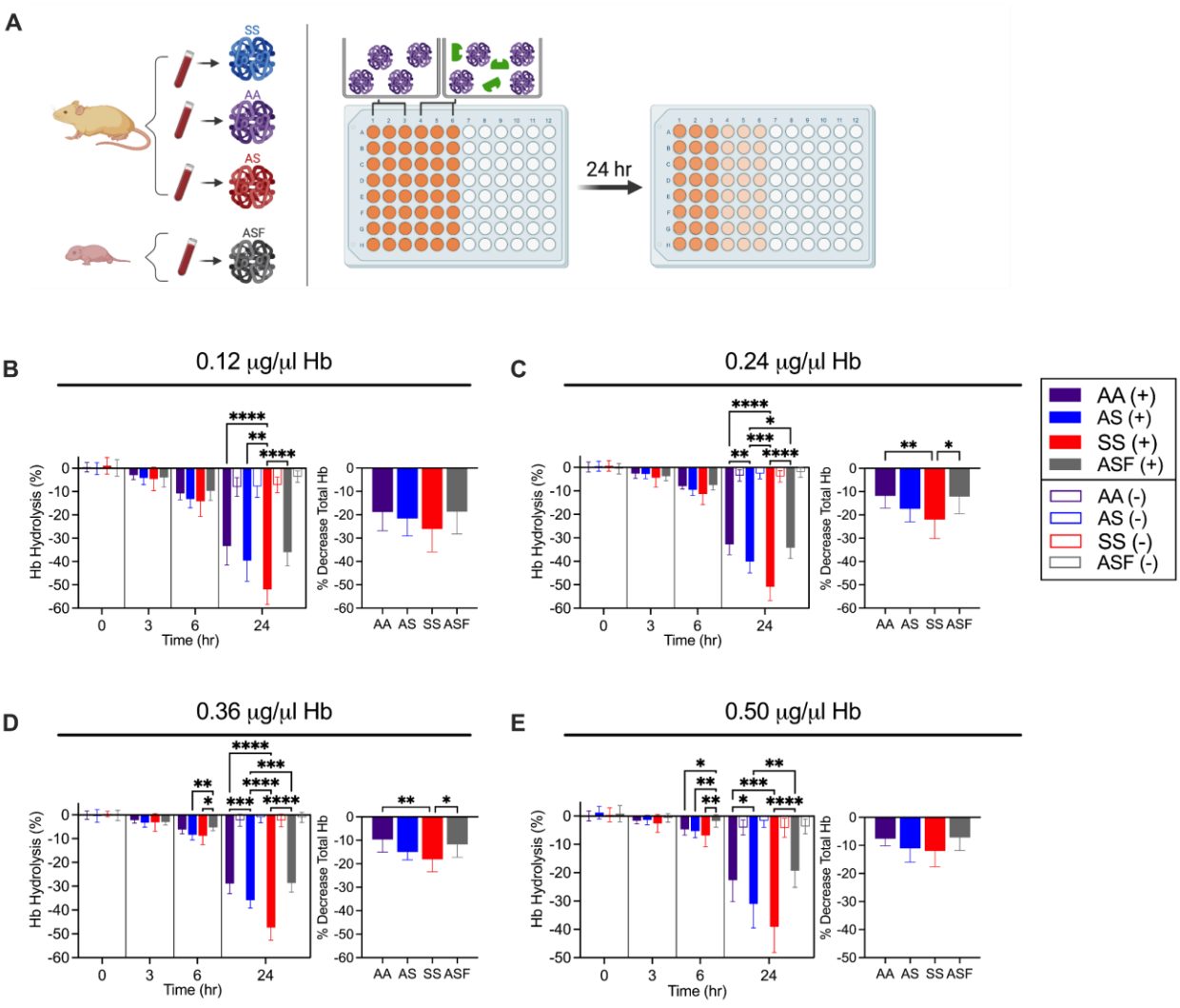

Figure 22. Efficiency of FP2-mediated Hb hydrolysis varies by Hb type.

(A) Diagram of Hb retrieval and assay set-up. **(B-E)** Hemoglobin hydrolysis (left) and percent decrease in total Hb concentration (right) by 50 µM FP2 for **(B)** 0.12, **(C)** 0.24, **(D)** 0.36, and (**E**) 0.5 µg/µl Hb (AA, AS and SS, n=14) (ASF, n=11), *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, mixed-effects analysis and one-way ANOVA, respectively.

Notably, these results were consistent over four different concentrations and confirmed by total Hb quantification at 24- hours (Figure 22B-E, left panels). Importantly, negative controls for all samples were used to verify the hydrolysis displayed was due to the action of FP2. As Hb S is known to be unstable at low concentrations, and the reducing environment of the buffer will cause spontaneous hydrolysis of all Hbs, these data confirm FP2 had the most hydrolytic activity on Hb S (491-494)

4.2.2.1 Visualization of Hb hydrolysis with western blot corresponds to previously recognized alterations in digestion among Hb variants

To additionally visualize the degradation of Hb subunits, aliquots from each digestion assay were analyzed by western blot. Gels were probed with antibodies against Hb α and β–chains to reveal unequal digestion among the chains, consistent with what was observed among the plate assays (Figure 23).

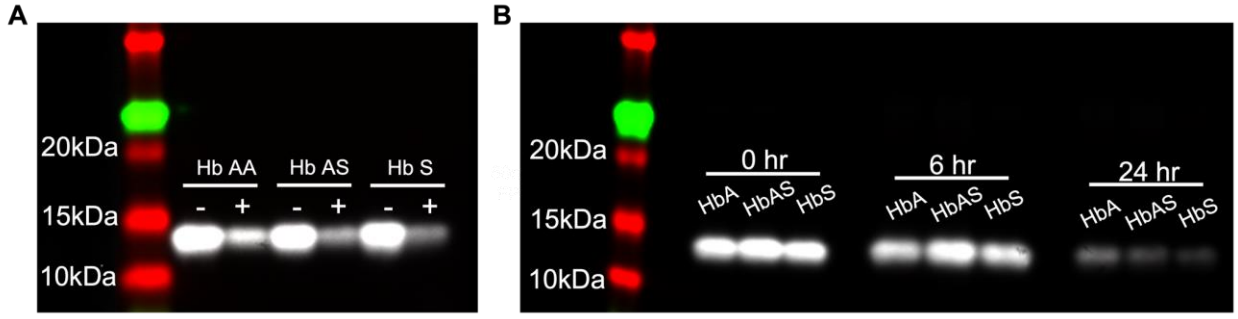

Figure 23 Visualization of Hb subunit digestion with western blot.

(A) α-globin of Hb A, AS, and S 24-hours after digestion with 50 nM FP2, 50 mg protein. **(B)** β-globin of Hb A, AS, and S at baseline, 6- , and 24-hours after digestion with 50 nM FP2, 50 μg protein.

4.2.2.2 Increasing Hb concentration relevant to physiological conditions results in Hb precipitation

To make the Hb hydrolysis assay as physiologically relevant as possible, we attempted to increase the starting Hb protein concentration. The normal physiological concentration of Hb is approximately 340 μg/μl, and at this concentration in the assay buffer, the Hb S in particular "gels". We then increased the Hb concentration above the current assay conditions (0.5 μg/μl) and found the Hb S and Hb ASF to precipitate in the buffer. Therefore, because of the precipitation, increasing the Hb concentration was not feasible to accurately determine Hb hydrolysis.

4.2.3 Summary

These results demonstrate that the efficiency of Hb S degradation by FP2 is more rapid than Hb A. As Hb digestion in the parasite's digestive vacuole results in the release of free heme, an increased rate of Hb hydrolysis would lead to a faster rate of heme release. Interestingly, Hb S cells are known to contain higher concentrations of heme at baseline (427, 429, 495, 496). As heme is a powerful oxidant, known to be toxic to both the human host and parasite, we questioned whether this alteration of Hb digestion and heme concentration may be a relevant factor to the previously observed change in sexual conversion.

4.3 Identification of heme as a common feature between increased Hb hydrolysis and CQ exposure

To better understand the relationship between heme release and gametocyte conversion, we therapeutically altered Hb digestion using the antimalarial CQ. Chloroquine functions by preventing *P. falciparum* from detoxifying heme released during Hb digestion (108, 232, 497, 498). Suboptimal doses can measurably limit heme detoxification without eliminating parasite viability. Interestingly, CQ has also been shown to increase rates of sexual conversion (319, 499-501). We sought to further investigate this relationship using a modified version of our gametocyte conversion assay combined with gene expression analysis.

4.3.1 Chloroquine diphosphate promotes gametocyte conversion

Cultures were treated with CQ at doses based on previous reports for 48 hours, followed by the addition of NAG to prevent further asexual growth (Figure 24A) (319). The rate of sexual conversion was calculated using the total gametocytemia captured on day seven from the point of NAG addition on day two.

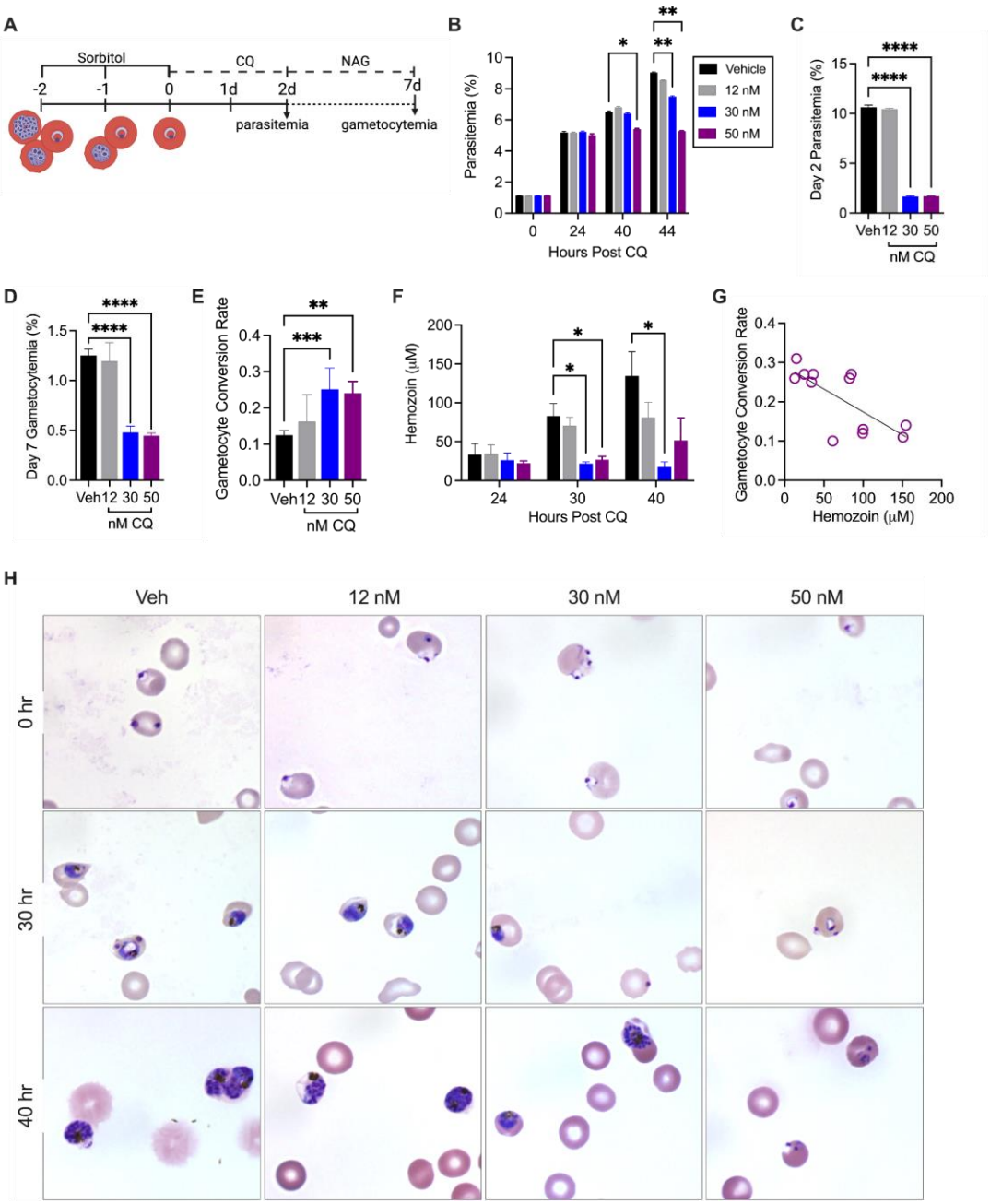

Figure 24. Gametocyte conversion rate is increased after treatment with CQ.

(A) Schematic of experimental design. Hb A-Ery cultures were synchronized to a four- hour window and plated during the early ring stage (0-4 hrs post infection). Media was changed daily, with NAG addition at 48 hrs. Created with BioRender.com **(B)** Parasitemia measured by flow cytometry through 48- hours of CQ exposure. *p<0.05, **p<0.01, two-way ANOVA **(C)** Day two parasitemia and **(D)** day seven gametocytemia measured by flow cytometry. **(E)** Corresponding GCR calculated from flow cytometry measurements of parasitemia at day two and gametocytemia measurement at day seven (n=6), **p<0.01, ***p<0.001, ****p<0.0001, one-way ANOVA. **(F)** Quantification of hemozoin content after CQ treatment in Hb A-Ery cultures (n=3), *p<0.05, mixed-effects analysis. **(G)** Correlation between hemozoin content and SCR. Pearson r= -0.7244, **p<0.01. **(H)** Representative images of culture development taken at 24-, 30-, and 40- hours post CQ exposure. Images captured using Olympus Provis microscope (100x) and obtained from blood smears stained with Giemsa.

No differences in total parasitemia were observed between all treatment groups through 40- hours of treatment (Figure 24B). Though the 30 nM treatment group appeared morphologically normal through 40- hours, total parasitemia was significantly lower at the point of NAG addition (Figure 24C). Total gametocytemia was also significantly decreased among the 30- and 50 nM treatment groups at day seven (Figure 24D). However, similar to what was observed among the Hb S-Ery cultures, the calculated rates of sexual conversion reveal a significant increase among the 30- and 50 nM treatment groups compared to that of the vehicle (Figure 24E). Furthermore, quantification of hemozoin content after CQ treatment revealed an overall negative correlation between hemozoin production and the rate of gametocyte conversion (Figure 24G). Interestingly, though appearing morphologically normal, the 30 nM treatment group displayed the largest decrease in total hemozoin content and the highest rate of sexual conversion (Figure 24G, H).

Expression of early gametocyte specific genes were additionally analyzed at 24-, 30-, and 40- hours post CQ exposure (Figure 25) (106, 319, 499, 500, 502). Gene expression analysis of early gametocyte genes support the observed increase in gametocyte conversion, while analysis of genes involved with Hb digestion support a disruption of the Hb degradation process (Figure 25).

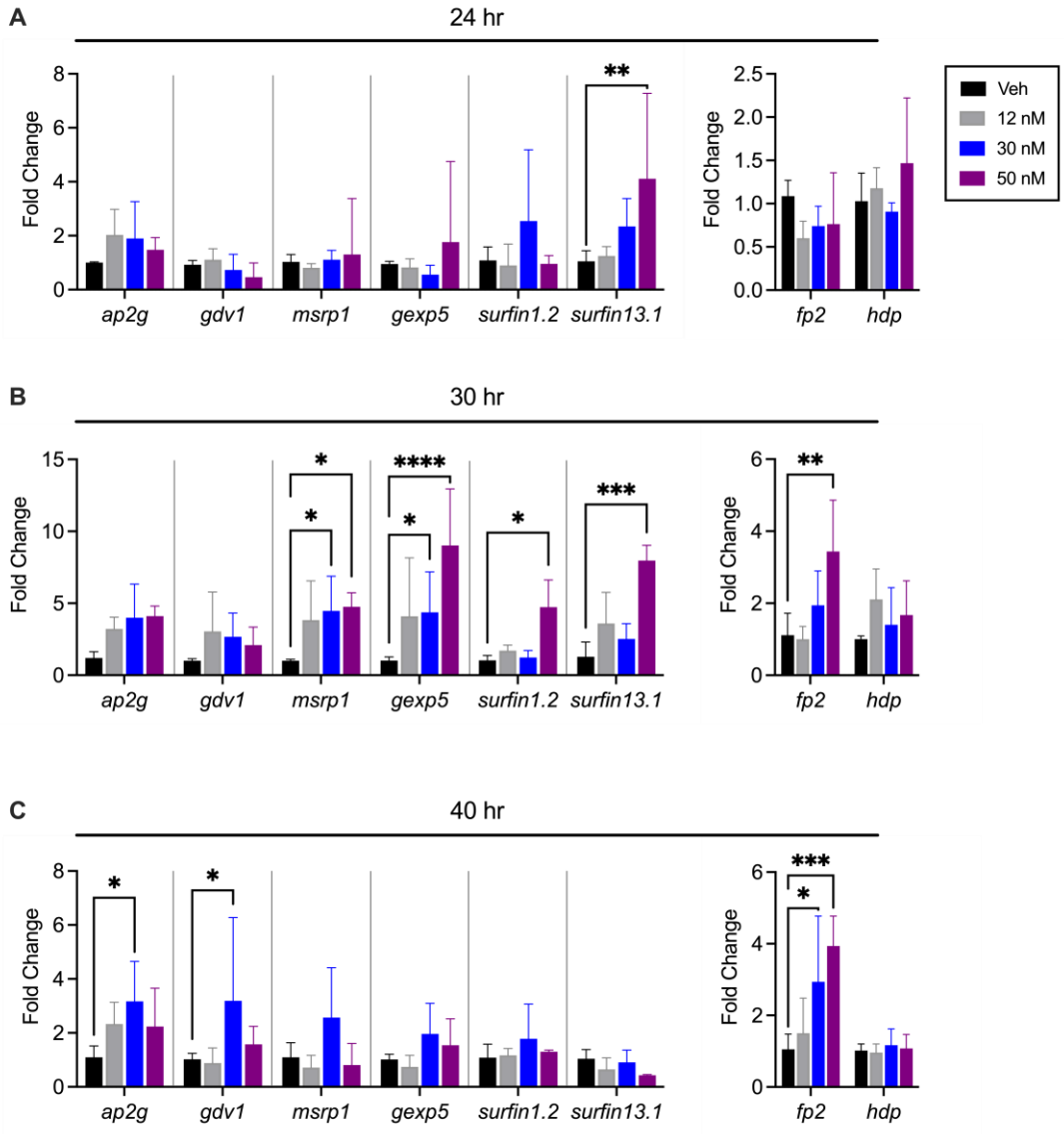

Figure 25. Gene expression of CQ-exposed cultures.

Early gametocyte genes *ap2-g*, *gdv1*, *msrp1*, *gexp5*, *surfin 1.2*, and *surfin 13.1* (left), and genes relevant for the process of Hb digestion, *falcipain-2* (*fp2*) and *heme detoxification protein* (*hdp*) (right) **(A)** 24- hours, **(B)** 30- hours, and **(C)** 40- hours post CQ treatment in Hb A-Ery cultures *p<0.05, **p<0.01, ****p<0.0001, two-way ANOVA.

4.3.2 Gene expression analysis of low-proliferative cultures is more feasible through qPCR than by RNA-FISH

Large cultures are required to generate sufficient quantities of RNA for gene expression analysis. Moreover, as discussed in Section 1.3.4, gene expression of *P. falciparum* is known to

be tightly regulated. Therefore, it was necessary for parasites to be synchronized to a close developmental window, to ensure an accurate analysis of gene expression changes. For experiments involving CQ treatment, cultures were synchronized to a four-hour window, which calls for at least two synchronizations, 20- hours apart using the sorbitol method. To ensure enough parasite quantities remained after both synchronization steps, careful culture preparation prior to the first synchronization is needed. For these experiments, optimal conditions were obtained when four T75 culture flasks, containing 20- 25 mL of culture were grown to about 6-7% parasitemia. At least 3% of this total parasitemia must be ring-stage parasites.

If the number of early-stage gametocytes reached 0.05% prior to plating, the experiment would not proceed. For the CQ experiments, cultures had to be plated at 2% HCT in a total volume of 10 mL. This ensured there was enough parasite quantity for RNA and hemozoin analysis. For each timepoint, 7-8 mL of culture were collected and processed as detailed in Section 3.6.

While the use of RT-qPCR for gene expression analysis presents multiple limitations, most prominent among these is the large culture amount required to obtain adequate levels of RNA for analysis. This method also does not allow for gene expression on the single-cell level, which would be particularly useful for analysis of sexual conversion, as it would allow for identification at the earliest identifiable point in the intraerythrocytic cycle.

In attempts to conduct a deeper and more precise analysis of sexual conversion, we assessed the utility of a RNA-based fluorescent in-situ hybridization (RNA-FISH) assay. The goal of this assay was to be able to identify sexual conversion at the earliest identifiable point, while using less sample quantity. Considering all genes involved early in sexual conversion would be of very low expression, we decided to adapt Thermo Fisher Scientific's PrimeFlow RNA Assay for our use. This assay uses branched-DNA technology, which allows for signal amplification through

pairs of target-specific probes. Using this assay, we also had the ability to combine multiple targets, theoretically allowing us to uniquely identify additional populations.

Custom probes for *ap2-g*, *gexp5*, *actin-1*, and *fp2* were created with the help of ThermoFisher Scientific scientists (Tables 7, 8). To modify the assay for use in erythrocytes, erythrocytes were lysed prior to probe staining, leaving intact parasites that could be visualized through DAPI and Giemsa staining (Figure 26) (specific details in Section 3.14).

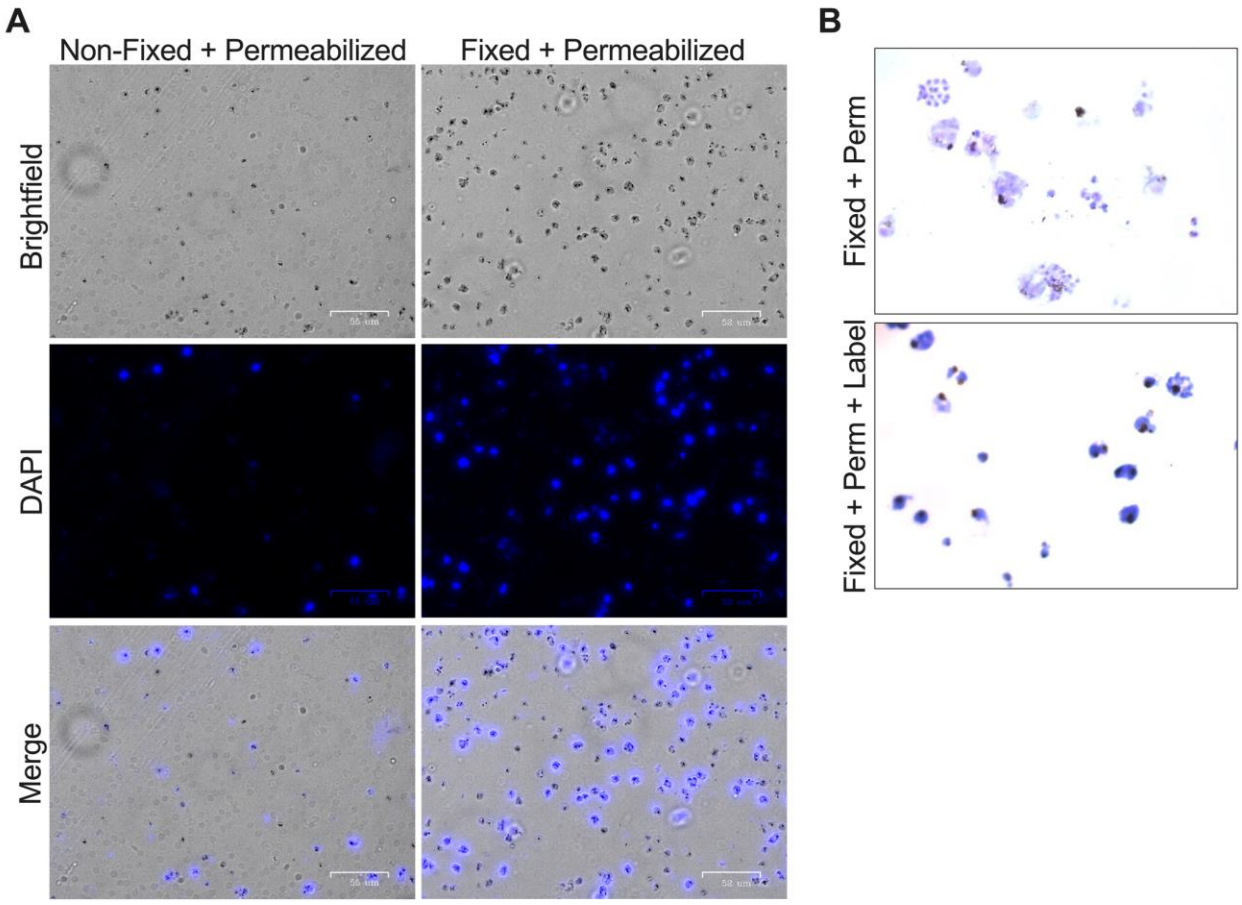

Figure 26. Preparation of RNA-FISH samples.

(A) Brightfield (top), DAPI (middle), and merged (bottom) images of parasites before and after fixing and permeabilization. Fixation increases cell lysis as noted by the increase in DAPI+ cells. **(B)** Giemsa-stained smears fixed + permeabilized and fixed + permeabilized + labelled cells.

Labelling of samples was successful, as apparent through *actin-1* and *fp2* positivity in vehicle controls (Figure 27B). Further gating for the identification of sexually converted cells produced results in slightly higher than expected ranges for the vehicle sample (Figure 27B). Upon investigation of 50 nM CQ-treated samples, we found poor parasite recovery, as indicated by lower *actin-1* expression (Figure 27C).

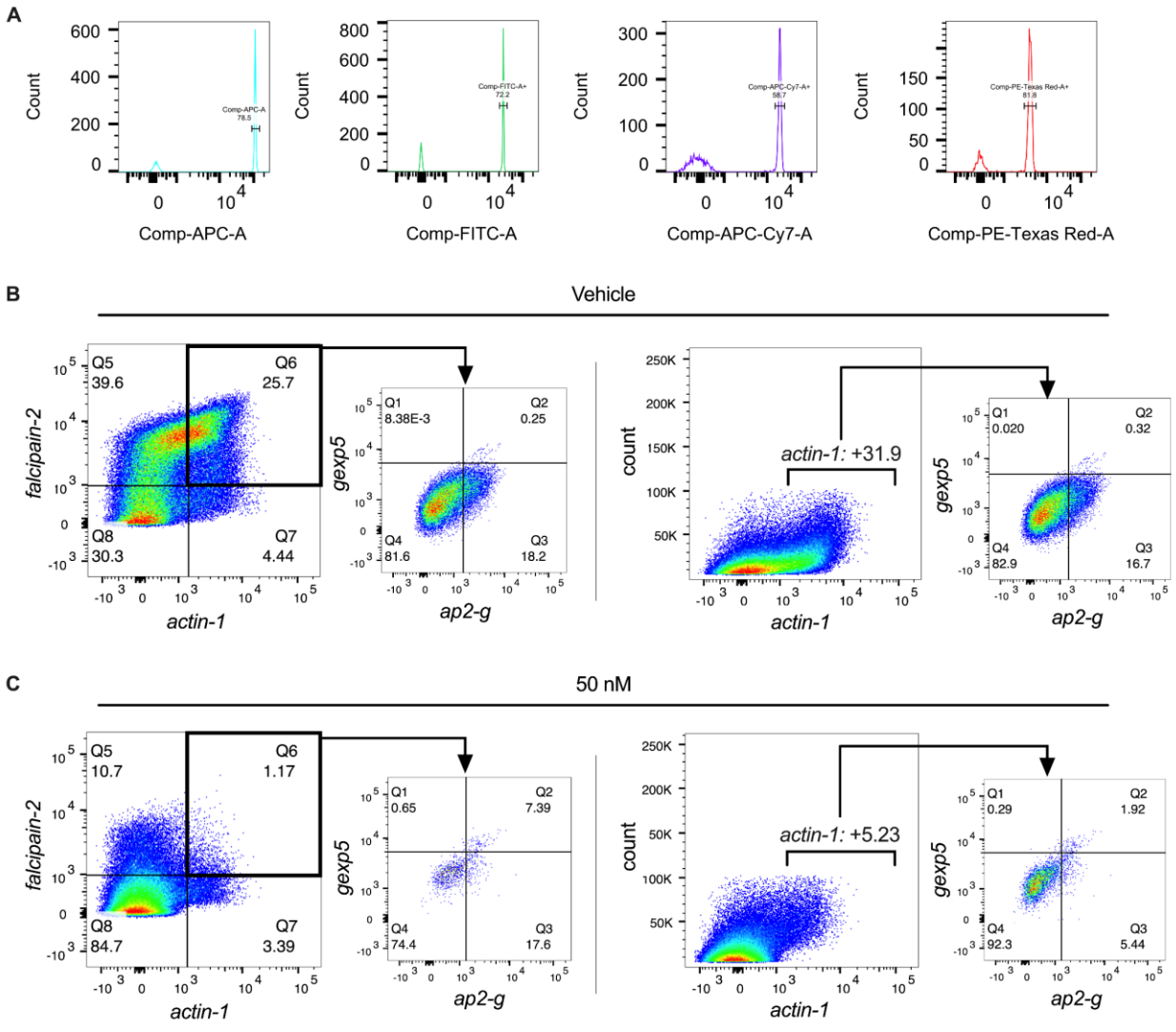

Figure 27. Utility of RNA-FISH assay for *P. falciparum* gene expression analysis.

(A) Gating of compensation controls for PrimeFlow probes specific for *actin-1* (APC-Cy7), *falcipain-2 (fp2)* (503), *ap2-g* (APC), and *gexp5* (PE-Texas Red). **(B, C)** Vehicle **(B)**, and 50 nM CQ-treated **(C)** samples stained with PrimeFlow probes specific for *actin-1, fp2, ap2-g, and gexp5*. After defining populations based on fsc versus ssc and

doublets exclusion, the *actin-1+*,*fp2+* positive populations were selected and analyzed for the gametocyte specific genes *ap2-g* and *gexp5* (left panels, B-C). Alternatively, after defining populations based on fsc versus ssc and doublets exclusion, the *actin-1+* population was selected and analyzed for gametocyte specific genes *ap2-g* and *gexp5* (right panels, B-C).

To observe gametocyte-positive populations, we looked at both the *actin-1+*/*fp2+* population, as well as the *actin-1+* -only population (Figure 27, B-C, left and right panels respectively). As these samples were taken at 30- hours post CQ exposure at the early ring phase, Hb digestion should actively be taking place, and therefore FP2 should be highly expressed. While this was the finding among the vehicle samples, we found the total contribution of *fp2* to be much less among the 50 nM treated group. This may indicate that of the cells within the sample population, few were actively digesting Hb (Figure 27C). This finding is supported by culture images at 30- hours displaying delayed parasite morphology (Figure 24H). Interestingly, though there was little change among the *ap2-g+*/*gexp5+* expression results among the vehicle for either method, the 50 nM treatment group displayed large differences, particularly among *ap2-g*. Specifically, *ap2-g* was much more highly expressed among the *actin-1+*/*fp2+* population, than the *actin-1+* -only population (Figure 27C). Though the *ap2-g+* population was comparable between that of the vehicle and 50 nM treated group, *ap2-g+*/*gexp5+* double positivity was notably higher among the 50 nM group (Figure 27B,C). As *gexp5* has been suggested to act as a co-regulator with *ap2-g* for the expression of other gametocyte-specific genes, these results likely indicate a higher degree of sexual conversion among the treated culture (502, 504, 505).

Though initial experiments produced interesting results, the utility of the assay ultimately did not circumvent the need for large culture volumes. Moreover, as there is a high degree of parasite loss throughout the two-day staining process, this method is both technically challenging and more time consuming than qPCR, with the additive requirement of additional controls.

However, future advances which result to streamline the assay or reduce the cost of reagents, may make its feasible for future investigation.

4.3.3 Summary

The increased rates of gametocyte conversion observed among the CQ-treated groups are in line with other reports which have found an increase in sexual conversion with antimalarial exposure (319, 501). It is known intracellular heme levels are increased during CQ treatment, which in these experiments is supported in the 30 nM treatment group by the change in hemozoin content in the absence of a change in total parasitemia or parasite morphology (Figure 24) (499). Therefore, the increase in sexual conversion observed here supports a relationship between heme concentration and gametocyte conversion.

4.4 Heme treatment increases gametocyte conversion in *P. falciparum* independent of a change in asexual proliferation

To determine whether directly altering extracellular hemin concentrations has a similar influence on sexual conversion, we treated cultures with hemin for 48 hours as performed in the previous experiment (Figure 28A). Though no changes in total parasitemia were observed at the time of NAG addition between all treatment groups, interestingly, we found a non-significant increase in gametocytemia among cultures exposed to hemin concentrations between 10-50 μM (Figure 28B, C). Among these treatment groups, GCRs were also found to be significantly increased (Figure 28D).

Considering heme is a powerful oxidant, contributing to parasite stress which may, as in the case of Hb S-Ery and during CQ treatment, result in limiting parasite proliferation, we next assessed total parasitemia through six days of culture. Interestingly, we found the 50 μM treatment group experienced delayed proliferation at day four (Figure 28E-F). However, all groups reached the same peak parasitemia at day 6, indicating there was no significant impairment to parasite growth (Figure 28G). We further assessed culture appearance on day 10 and found mature gametocytes in all treatment groups (Figure 28H). Image quantification supported the finding of increased gametocyte conversion among the higher treatment groups, as total gametocytemia was found to increase with increasing doses of hemin (Figure 28I).

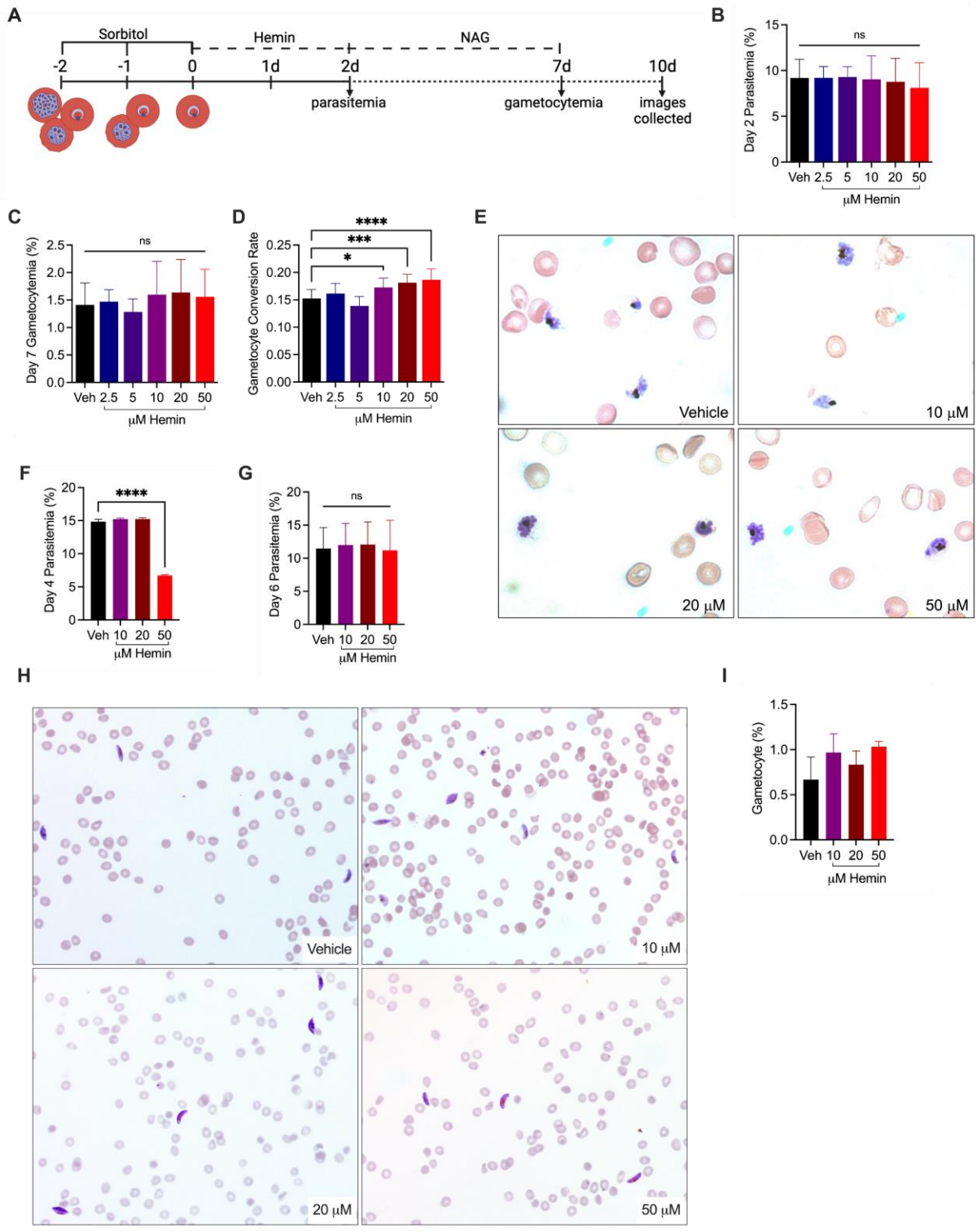

Figure 28. Hemin exposure increases rate of sexual conversion.

(A) Schematic of experimental design. Hb A-Ery cultures were synchronized to a four- hour window and plated during the early ring stage (0-4 hrs post infection). Media was changed daily, with NAG addition at 48- hours. Created with BioRender.com **(B)** Day two parasitemia and **(C)** and day seven gametocytemia measured by flow cytometry. **(D)** Sexual conversion rate calculated from flow cytometry measurements of gametocytemia at day seven divided by the

peak parasitemia at day two (n=12), *p<0.05, ***p<0.001, ****p<0.0001, one-way ANOVA**. (E)** Representative images of hemin-treated and vehicle cultures on day four, showing no change parasite morphology. Images captured using Olympus Provis microscope (100x) and obtained from blood smears stained with Giemsa. **(F)** Day four and **(G)** and day six parasitemia measured by flow cytometry. **(H)** Representative images on day 10 of culture. Images captured using Olympus Provis microscope (40x) and obtained from blood smears stained with Giemsa. **(F)** Quantification of gametocytemia from representative images (n=3). Representative images of culture development taken at 24-, 30-, and 40- hours post CQ exposure. Images captured using Olympus Provis microscope (100x) and obtained from blood smears stained with Giemsa.

4.4.1 Changes in *ap2-g* expression in response to heme treatment are not reflected in histone occupancy

Aiming to further confirm the observed change in GCR during hemin exposure, we next investigated relevant gene expression and epigenetic marks associated with the process of sexual conversion. Total gene expression of *ap2-g* was increased among treatment groups between 48-64-, and 72-80-hours post exposure, overall supporting the observed increased rates of sexual conversion (Figure 29). Minimal changes were observed among other early gametocyte markers assessed.

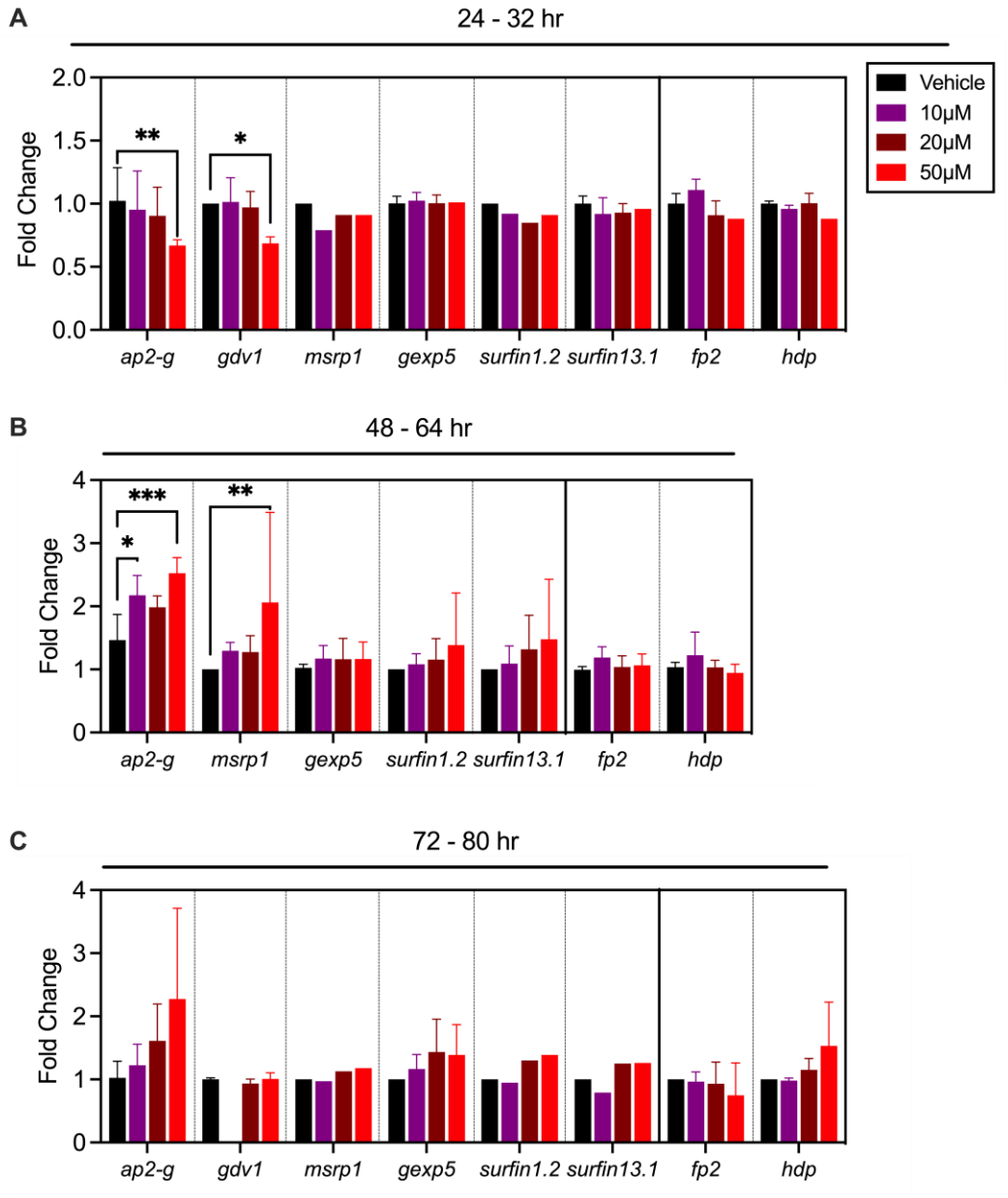

Figure 29. Gene expression after hemin exposure reflects changes in gametocyte conversion.

Gene expression analysis of *ap2-g*, *gdv1*, *msrp1*, *gexp5*, *surfin 1.2*, *surfin 13.1*, *fp2*, and *hdp* between **(A)** 24-32- hours, **(B)** 48-64- hours, and **(C)** 72-80- hours of hemin exposure (n=6). *p<0.05, **p<0.01, ***p<0.001, two-way ANOVA.

Going further, we attempted to identify changes in the epigenetic landscape of *ap2-g*. The *ap2-g* gene locus remains epigenetically repressed by H3K9me3 during asexual replication. De-repression of *ap2-g* is therefore marked by H3K9Ac. In order to reveal potential changes in

H3K9me3 occupancy between treatment groups, we pursued both native (nChIP) and cross-linked ChIP (xChIP) analysis.

Chromatin digestion was initially optimized without fixation (nChIP), wherein it was determined a combination of sonication and micrococcal nuclease (MNase) digestion produced the most reliable digestion results, with bands between 200-800 bp (Figure 30). MNase and exonuclease were used because of the inherent preference MNase has toward AT-rich sequences (458). The subsequent brief sonication served to further release chromatin from nuclear debris. The same digestion protocol was used with xChIP, with the only modification being samples were de-crosslinked overnight prior to confirming digestion on an electrophoresis gel.

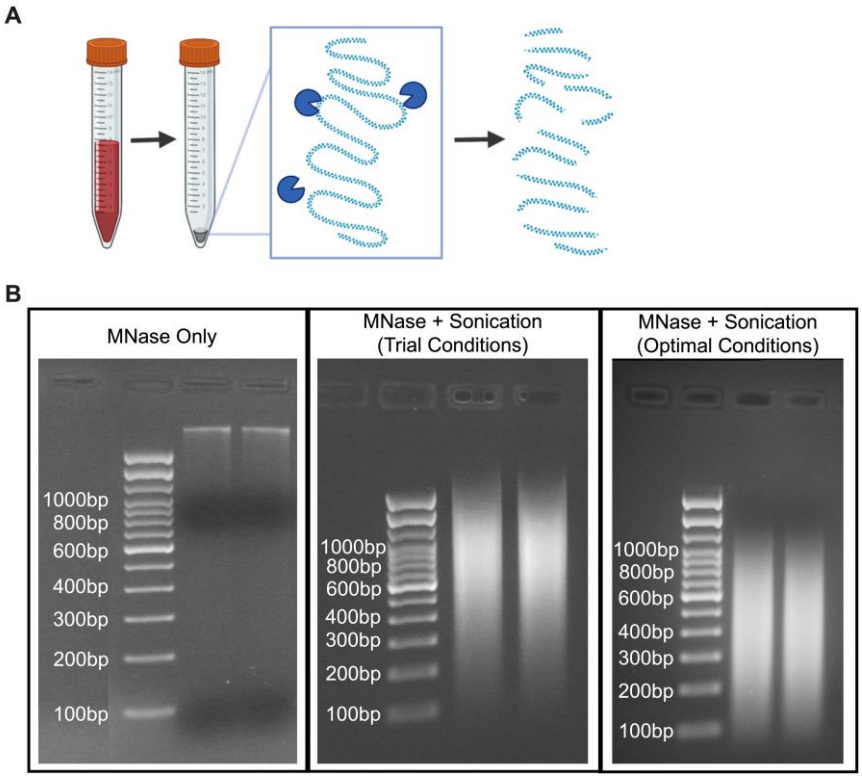

Figure 30. Optimization of chromatin digestion for ChIP analysis.

(A) Schematic representing nuclei isolation and chromatin digestion. **(B)** Gel electrophoresis of digestion conditions, with the right-hand panel displaying most optimal conditions with fragment sizes between 200 – 800 bp.

4.4.1.1 nChIP of vehicle and hemin-treated samples results in low recovery

Native ChIP was performed initially because the epigenetic modification of interest was a histone modification. As these epigenetic modifications are already tightly associated with the DNA, it was assumed cross-linking would not be required for stabilization of the proteins. Upon performing ChIP (as described in section 3.13.1), we consistently found our recovery to be much lower than anticipated (Figure 31). Therefore, in order to potentially increase recovery, we proceeded to xChIP.

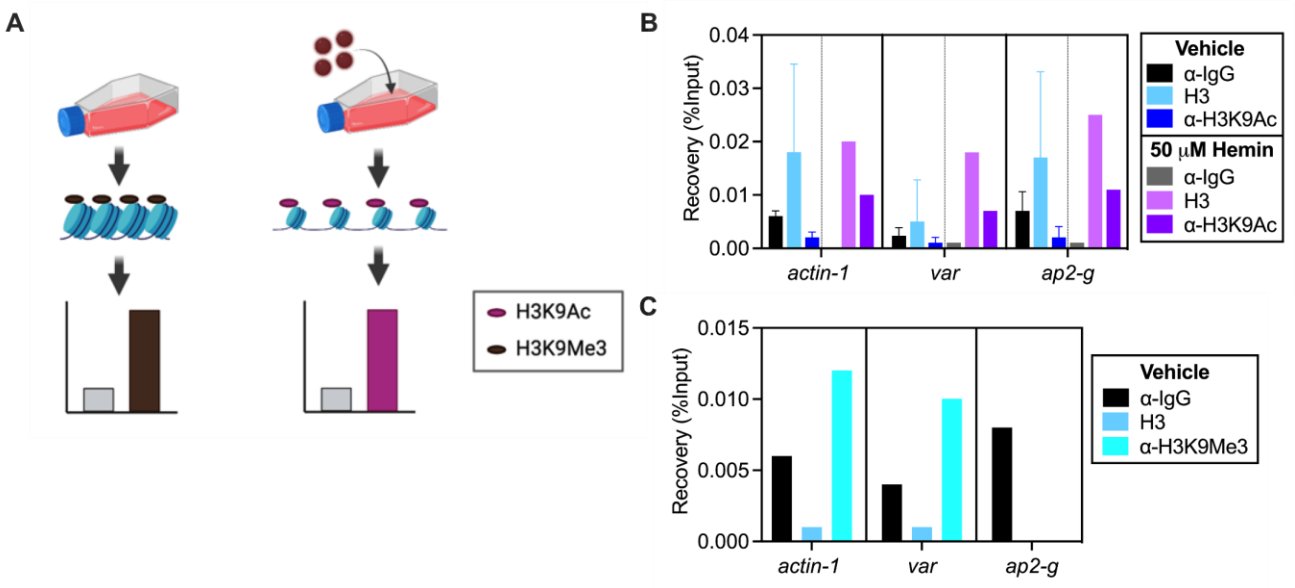

Figure 31. nChIP of vehicle and hemin-treated samples.

(A) Schematic of ChIP analysis and expected results with the corresponding methylation/acetylation status. **(B-C)** ChIP-qPCR represented as recovery of % input. **(B)** H3K9Ac and **(C)** H3K9Me3 of nChIP. Tested loci include *actin-1*, used as a euchromatic control, and *var* used as a heterochromatic control. Anti-rabbit IgG was used as a negative control. Results are obtained from biological replicates.

4.4.2 No differences in histone occupancy are observed with xChIP

In xChIP, formaldehyde is used to first crosslink proteins prior to erythrocyte lysis with saponin. For this procedure, methods were followed as described by Hoeijmakers and Bartfai

(458). In performing the ChIP, we received much better recovery compared to nChIP. Unfortunately, however, we were ultimately unable to reveal any differences in histone occupancy between the heme treated and untreated conditions (Figure 32).

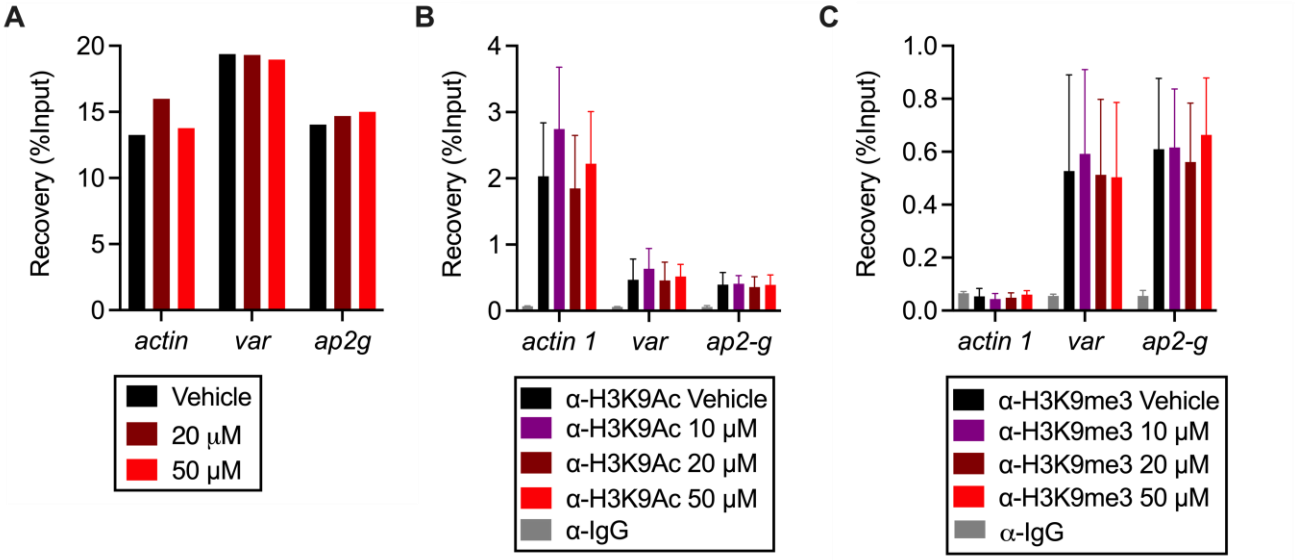

Figure 32. xChIP diplays no change in histone occupancy between treatment groups.

ChIP-qPCR represented as recovery of % input. Recovery of **(A)** H3, **(B)** H3K9Me3, and **(C)** H3K9Ac occupancy at selected loci in vehicle and hemin treated Hb A-Ery cultures at 48-64 hrs of hemin treatment. Results display much greater rates among all groups (n=6). Tested loci include *actin-1*, used as a euchromatic control, and *var* used as a heterochromatic control. Anti-rabbit IgG was used as a negative control. Results are obtained from biological replicates.

4.4.3 Summary

These results show exposure to increasing concentrations of extracellular hemin increase the rate of sexual conversion in Hb A-Ery cultures. Notably, the rate of increase in the culture exposed to 50 μM was similar to those that were obtained from day four and eight in the Hb S-Ery cultures. Though we found increased *ap2-g* expression during hemin treatment, supporting the previously observed increase in sexual conversion, we were unable to determine a difference in

the epigenetic landscape of *ap2-g*. However, considering the overall rate of asexual replication remained >80%, a modest difference, if any at all, was anticipated.

5.0 Discussion

Sexual conversion dynamics of *P. falciparum* are known to be influenced by different sources of environmental pressure, and the ability of *Plasmodium spp.* to alter its reproductive investment is an instrumental survival strategy in line with many evolutionary biology theories (360, 365, 366). As specific sources of sexual stage initiation remain elusive, a growing understanding of how *Plasmodium spp*. balance intra-host survival with inter-host transmission is critical to understanding the trends and fluctuations in pathogen virulence (362, 363).

This dissertation research demonstrates that gametocyte conversion is positively influenced by variation of the Hb A protein. As the efficiency of Plasmodial enzyme mediated hydrolysis of Hb have previously been shown to vary between Hb types, and Hb degradation is integral to the intraerythrocytic cycle, we focus on this process as a potential factor for the observed change in reproductive effort. After revealing FP2-induced hydrolysis of Hb S is significantly more efficient than Hb A, and demonstrating the effect CQ has in increasing both sexual conversion and intracellular heme levels, we implicate heme, as a product of Hb hydrolysis, to be a potentially relevant component for gametocyte conversion. We go on to expose Hb A-Ery cultures to increasing levels of hemin and discover increased rates of sexual conversion with increasing exposure concentrations. Ultimately, this establishes heme as a causative factor for sexual conversion.

5.1 The rate of gametocyte conversion in *P. falciparum* is influenced by variation of the Hb protein

To our knowledge, this is the only study directly comparing the generation and maturation of gametocytes *in vitro* between multiple Hb types. Though it has been well established that sexual conversion rates fluctuate *in vivo*, the average commitment rate per asexual cycle has been estimated to be <1% (506). Therefore, a naturally occurring environmental condition which we found to result in an average conversion rate of 30% *in vitro* is striking, and incredibly relevant for both the development of public health-based methods to combat malaria transmission, and the identification of targets to hinder sexual conversion.

To understand variances in the rate of sexual conversion and maturation, we used two methods of calculation. The first method measured mature gametocytes entirely through flow cytometry after NAG elimination of asexual stages and is based off the induction of gametocytogenesis within Hb A-Ery cultures. We chose to use this method to induce gametocytogenesis because it is known to induce sexual conversion in Hb A-Ery cultures as a result of terminal investment by promoting a high culture parasitemia (337, 338, 340, 364, 443, 507). However, because parasitemia was not increasing at a comparable rate between all cultures, we can conclude gametocytogenesis was not being induced in the same manner. Therefore, the obtained values cannot not be directly compared without recognizing a few important insights.

Primarily, the differences in overall parasitemia on day seven of culture is an important distinction relevant to the calculation of sexual conversion rate. While in the Hb A-Ery cultures, parasitemia is continuously increased to a high degree over the seven-day period, parasitemia within the Hb AS- and Hb S-Ery cultures does not increase. If we were to assume the rate of sexual conversion was consistent between all three cultures, in the absence of an increase in total

parasitemia, the overall ratio of gametocytes to asexual stages on day seven would be higher among the cultures with the lower level of proliferation than the cultures with the higher level of proliferation. Therefore, though we can assume that any gametocytes produced over the first three replication cycles were effectively diluted out of the conversion calculation because of the high level of parasitemia that was reached among the Hb A-Ery cultures, the same cannot be concluded for the Hb AS- and Hb S-Ery cultures. Ultimately, this indicates the calculated sexual conversion rate among the Hb AS- and Hb S-Ery, the latter of which was found to average around 50%, is likely an exaggerated quantification.

In examining the change in total parasitemia throughout the three cycles of asexual replication which occurred from day one to day seven, we have established that among the Hb AS- and Hb S-Ery cultures, parasitemia was not increasing after the first replication cycle. This difference in total parasitemia was expected and was likely contributed to by decreased invasion due to altered remodeling of the Hb AS- and Hb S-Ery cytoskeletons, and impaired parasite growth due to diminished protein export and the limited availability of required nutrients. Interestingly, over the first replication cycle, morphological analysis and parasitemia by flow cytometry show that each culture appears to be developing synchronously, with no significant changes observed in overall parasite development. Based on parasitemia acquired from day one however, we do know invasion was slightly less efficient within the Hb AS and Hb S-Ery cultures. If this pattern of invasion continued, even despite potential equivalence in development, schizogony, and egress, the parasitemia among the Hb AS- and Hb S-Ery cultures would still consistently be below that of the Hb A-Ery culture. However, because total parasitemia does not increase in the Hb AS- and Hb S-Ery cultures, we cannot conclude development, schizogony, and egress are happening equally. Though it is therefore tempting to conclude impaired development, schizogony, and egress are the

likely cause for the observed deficiency in parasitemia compared to the Hb A-Ery culture, this does not explain the parasitemia plateau reached between days three and seven.

When looking specifically at the Hb S-Ery cultures, we see total parasitemia decline by approximately 25% between day one and three. If this pattern were to continue with GCRs comparable to that of the Hb A-Ery cultures, the total parasitemia at day seven would be less than the observed value. Though with the available dataset it is impossible to decipher what contributed more to the decline in parasitemia, a potential solution to the observed discrepancy would be an increased rate of sexual conversion. In this case, more parasites would be carried over through the time period, thus contributing to the total parasitemia measurement.

This explanation is additionally supported by the observed decline in total parasitemia after the addition of NAG at the seven-day point. A decline indicates some degree of asexual replication was taking place. As discussed previously, if we consider the ratio of gametocytes to asexual stages among the Hb AS- and Hb S-Ery cultures to be skewed, the sexual conversion rate calculation is likely inflated. However, an alternative interpretation of these results indicates that 50% of the culture at day seven were already gametocytes. For 50% of the entire culture to be gametocytes at day seven, and the parasitemia trend to align with what was observed, a 10% rate of sexual conversion does not mathematically align. Instead, a rate of at least 25% would need to be applied.

The likelihood of a skewed gametocyte to asexual stage ratio between experimental groups was an oversight in the initial experimental plan. In order to correct for this calculation error, a second method for determining sexual conversion rate was developed. The second method relied entirely on microscopic quantification of blood smears and determined sexual conversion after one single replication cycle after inoculation. Though sexual conversion rate determination through microscopy presents sources of variability, to validate this method, we present in parallel total

parasitemia obtained from flow cytometry to show little variation between the two methods. Additionally, there are differences in invasion between the Hb A-, Hb AS-, and Hb S-Ery cultures, which is apparent from the parasitemia on day one. This difference was not found to be statistically significant, and the lack of morphological differences on day two indicate the rate of sexual conversion determined from the first cycle was a point of valid comparison.

We determined sexual conversion using both day four and day seven blood smear analysis to provide additional rigor and give insight to any differences uncovered during gametocyte maturity. The day-four analysis gives more accurate insight to the actual rate of sexual conversion as the measurement is taken at the closest point in which sexual development can be visually distinguished. As shown, the day four results reveal a conversion rate of approximately 30% for the Hb S-Ery cultures, compared to 10% among the Hb A-Ery cultures.

As *P. falciparum* gametocytes mature over the course of 7-10 days, an important distinction arises in defining sexual commitment versus sexual differentiation. While the former term is meant to describe the number of parasites undergoing sexual conversion, or the point at which an asexual parasite becomes a sexual stage parasite, the latter describes the process of gametocyte maturation. By analyzing sexual conversion again on day seven, we are offered unique insight into potential differences between gametocyte maturation, and sexual commitment.

The day seven results indeed reveal a lower rate of sexual conversion compared to those observed on day four. This is most apparent among the Hb S-Ery cultures, which dropped from 30% on day four, to 20% on day seven. The Hb A-Ery culture also experienced a decreased rate, though slightly less dramatic (approximately 8% from 10%). Importantly, between all experimental groups, no day seven rates were found to be significantly decreased from the day four rates, ultimately indicating Hb type does not play a significant role in the success of

gametocyte maturation. It is vital to note that gametocytes are extremely sensitive to temperature changes, and thus can be easily lost during *in vitro* culture. Though extreme care was taken with these cultures to mitigate potential loss, it is impossible to completely disregard handling error that may have contributed to changes between day four and day seven.

Comparing the resulting rates obtained from the two methods of sexual conversion calculation, we observed the second method to result in a lower sexual conversion rate, most obviously among the Hb S-Ery cultures. A difference in rate is also observed among the Hb A-Ery culture, however, this was expected as the first method for quantification effectively induces gametocytogenesis. Therefore, the higher rate observed in method one compared to method two, indicates that induction was successful. Interestingly, the rate of conversion in the Hb AS-Ery cultures was found to be consistent between the two methods. Potential explanations for this finding could be in relation to a balanced rate of sexual conversion through day seven equaling the percent lost after NAG addition.

To further validate these findings, we completed a set of experiments to determine whether total parasitemia was a factor for sexual conversion. Though it is known the condition of high parasitemia contributes to gametocytogenesis induction, this experiment sought to investigate whether the condition of low parasitemia plays a similar role. These experiments were necessary because total parasitemia on day seven significantly differed between experimental groups. Therefore, if the condition of low parasitemia is a factor for gametocytogenesis induction, we could not conclude Hb variation was the sole cause for the observed phenomenon. These experiments were conducted in Hb A-Ery cultures. Cultures were started with varying levels of parasitemia, which corresponded to the total parasitemia conditions in the Hb variant cultures. While the low parasitemia group had a comparable total parasitemia to the Hb S- and Hb AS-Ery

cultures, we were unable to identify any gametocytes. This establishes the calculated sexual conversion rates were not biased by differences in total parasitemia.

Furthermore, the results of these validation experiments together with the experiments set in Hb variant cells demonstrate two defined strategies of reproductive investment by *P. falciparum:* terminal investment and reproductive restraint. The lack of proliferation coupled with the increased rate of sexual conversion in the Hb S- and Hb AS- Ery cultures indicate terminal investment, while the lack of sexual conversion during the same level of total parasitemia in the Hb A-Ery culture indicates reproductive restraint.

Unfortunately, we were unable to adequately examine potential differences in microgametocyte exflagellation among Hb S-Ery. Examination of Hb AS- and Hb A-Ery exflagellation however, reveals that microgametocytes generated in the Hb AS-Ery cultures exhibit equal exflagellation competencies. As this dataset was not able to be normalized to total gametocytemia, it may be reasonably hypothesized that Hb AS-Ery produce microgametocytes that are actually more competent than Hb A-Ery, considering the observed differences in total gametocytemia from other datasets. This is an interesting avenue for future investigation.

5.2 Dysregulated Hb digestion is a potential factor in the promotion of gametocyte conversion

In considering possible explanations for the observed adjustment of reproductive investment, we were first drawn to investigating the Hb protein. Looking at the rates of sexual conversion obtained from the Hb AS-Ery cultures, we see that they land almost perfectly in between those of the Hb A- and Hb S-Ery cultures. Though perhaps unsurprising at first, in viewing

it from the perspective of the Hb protein, these results give the appearance of a dosing effect on gametocyte conversion by Hb S. As Hb digestion is an integral role for parasite development within the erythrocyte, we sought to analyze potential differences in Hb digestion efficiency between the experimental Hb groups.

Our analysis of Hb digestibility by *P. falciparum* FP2 revealed Hb S to be more readily hydrolyzed compared to Hb A (Figure 22). Degradation of Hb was further validated by measuring total Hb in the remaining sample at the 24-hour timepoint which overall reflected the hydrolysis results. The different trends in hydrolysis were consistent over four different concentrations. Though we were unable to make these concentrations physiologically relevant, we did increase the Hb concentration as much as the assay conditions would allow prior to Hb precipitation.

Though FP2 is only one of several *P. falciparum* proteases known to be active in the Hb degradation process, it has been shown to be active during the early phases of Hb digestion and is an essential protease in the process, with disruption resulting in the accumulation of undigested Hb within the digestive vacuole (453). We acknowledge this assay is a simplified version of Hb digestion, but it is worth noting knock-out studies of other hemoglobinases have suggested they play more of a cooperative function to the overall process (453, 508). This redundance in function highlights the importance of Hb digestion to the parasite, as in the potential absence or dysfunction of one hemoglobinase, there is a backup in place that can, but perhaps not as effectively, hydrolyze Hb for the parasite's development (508).

While direct *in vivo* translation using this assay may be outside its scope, we did adjust the assay from other studies to make it as physiologically relevant as possible. These adjustments include the alteration of the pH to mimic that of the food vacuole, and the reduction of the concentration of the reducing agent to what is considered physiological (107, 124, 509). In making

these adjustments, the real strength of this assay is that it provides a clean examination of Hb digestion. Iterations of this assay have been used in prior studies to draw similar conclusions relevant to Hb digestion efficiency between variants of the Hb protein, as well as to investigate the role of other proteins in the Hb to hemozoin formation process (455, 490). Therefore, we conclude the increased degradation of Hb S by FP2 in these experiments provides a reasonable reference point for *in vivo* hydrolysis.

Significance between hydrolysis is noted as early as six-hours post exposure, and notably, these differences are confirmed through no-enzyme containing controls. Relating to the intra-erythrocytic cycle, the process of Hb digestion begins around 24-hours post invasion and reaches its peak around 30-hours post invasion. Though it is impractical to translate these findings by time, it is interesting to note we see the greatest changes in hydrolysis at earlier timepoints as the concentration of Hb increases. Considering the physiological concentration of the erythrocytes is much higher than what we were able to include in this assay, it's interesting to consider whether this trend continues under physiological concentrations, particularly when we think about what is happening during Hb hydrolysis. When Hb is digested within the parasite, free heme is released, which the parasite must actively convert to hemozoin. Our finding of an increased rate of Hb S hydrolysis by FP2 therefore indicates heme is being released more rapidly compared to Hb A (427). We found Hb AS to fall in between that of Hb A and Hb S, somewhat mimicking what was observed among sexual conversion. Though it is unclear whether hydrolysis of Hb takes place solely in the DV, it is assumed a degree of hydrolysis occurs during Hb transit from the erythrocyte cytosol to the DV. In both scenarios, an increased rate of heme release would have consequences to the overall PV environment.

We observed the lowest rate of hydrolysis among the sample received from day 0-2 Towne's pups, which express Hb A, Hb S, and Hb F (Hb ASF). This result was anticipated, as Hb F has previously been shown to be resistant to other *P. falciparum* hemoglobinases and is resistant to acidic conditions as the γ-subunits contain positively charged amino acids which interact with bicarbonate ions (379, 490).

Though *Plasmodium spp.* have developed mechanisms to detoxify heme and complete their lifecycle within the most heme-rich cell within the human body, interestingly they are also able to synthesize heme de novo (510, 511). Though this de novo synthesis has been shown not be required for the intraerythrocytic phase of infection, heme does serve as an important cofactor for metabolic processes necessary for survival and growth, and it is widely assumed this heme acquisition comes from the Hb digestion, as well as uptake from the host cell (512-516).

Heme is involved in the synthesis of essential biomolecules, including proteins, nucleic acids, and lipids, that are required for parasite replication and proliferation (110, 111, 137, 453-455). One particularly important pathway is the biosynthesis of heme-containing proteins, such as the cytochromes, which are involved in the electron transport chain and other redox reactions within the parasite's mitochondria (513-515, 517). Interestingly, heme seems to play a role in other processes, as it has been shown to interact with genes involved in merozoite invasion (518). For these reasons, it is unsurprising that heme is a major factor for many antimalarials, both in its increasing concentration leading to overwhelming toxicity to the parasite, as in the case of CQ, and in using its concentration for the mechanism of action for the therapeutic, as in the case of artemisinin (107).

Though the results of the hydrolysis assay were in contradiction to our initial hypothesis of Hb variants being resistant to parasite-mediated digestion, considering the interesting and

convoluting relationship *P. falciparum* has with heme, we returned to our initial question of Hb variation and sexual conversion. As referenced previously, recent studies have concluded that CQ can promote sexual conversion (319, 501). Though the mechanism of this finding is not understood, it is known CQ increases the intracellular concentration of heme by blocking the formation of hemozoin (232, 497-499, 519). We found this to be an interesting parallel to the results received from the hydrolysis experiments, ultimately leading us to the question of whether increased heme concentration may be associated with gametocyte conversion.

Initially exploring this question, we wanted to better understand the relationship between CQ, hemozoin formation, and sexual conversion. Using sub-optimal doses of CQ, which effectively limited the production of hemozoin without necessarily causing developmental changes to the parasite, we indeed found sexual conversion to increase, most significantly among the 30 nM treated group. Interestingly, this treatment group also showed the least amount of total hemozoin production, but we observed little change in parasite morphology at the time of hemozoin sampling. This is an important distinction, because lack of hemozoin formation may also indicate a lack of parasite development, which is exemplified in the 50 nM treatment group, where we did observe a change in parasite morphology through 40- hours. If Hb digestion is still taking place but hemozoin is not being produced, this indicates an increase in heme. Overall, we found a negative correlation between the rates of sexual conversion to the amount of hemozoin formed. Though we were unable to quantify heme specifically, previous studies have shown CQ concentrations of 40 nM to increase labile heme 2-fold compared to vehicle treated controls (499). This observation is additionally relevant to the first replication cycle of the Hb S-Ery cultures, as though we know asexual proliferation is impacted, within the first asexual cycle, we show little change in parasite morphology (Figure 16E).

The increased rates of gametocyte conversion are reflected in gene expression analysis, as differences in early-gametocyte genes are apparent, particularly at 30- hours post exposure. Though non-significant, both *gdv1* and *ap2-g*, are increased among all treatment groups, which as noted previously, are two genes thought to be essential for the sexual commitment (Figure 25). Also of note is the change in *fp2* expression at 30- and 40- hours post CQ exposure. Most Hb digestion occurs around 30- hours post invasion. The observed increase in *fp2* expression indicates a degree of Hb digestion dysregulation, though interestingly, as may be expected, we do not see increased levels of *hdp*.

In attempts to visualize expression on the single-cell level, we developed a second method for determining sexual conversion rate with a RNA-FISH assay. The rationale for this assay was to identify specific populations of early gametocyte gene expression under different treatment conditions and relate that to potential changes in Hb digestion and heme release. We had also intended to use the assay to re-investigate sexual conversion among Hb-variants, as it would provide a more rapid and, ideally, less reagent-consuming method. Unfortunately, we found the total sample volume needed to complete the assay was not feasible for these purposes.

5.3 Hemin exposure promotes sexual conversion in *P. falciparum in vitro*

Collectively, these results indicate heme may be a causative factor for promoting sexual conversion in *P. falciparum*. Therefore, continuing along this line of investigation, we asked the question of whether altering levels of hemin *in vitro* would have the same effect on sexual conversion rates. Upon directly manipulating levels of extracellular hemin, we found sexual conversion to increase (Figure 28). This increase in sexual conversion was reflected to a small

degree in gene expression analysis, which revealed significant increases among *ap2-g* during the second replication cycle (Figure 29).

Concentrations of hemin that are physiologically relevant during both malaria and SCD were used, as both diseases are known to cause elevated extracellular heme levels which contribute to pathology (520-522). Interestingly, the observed increase in sexual conversion was comparable to what we observed in Hb S-Ery cultures and occurred in the absence of a significant change in parasite proliferation. Though we do see day four parasitemia to be hindered among the 50 μM treatment group, all cultures reached the same peak in total parasitemia by day six (Figure 28G). This finding differentiates from what was observed in the Hb AS- and Hb S-Ery cultures, as well as those treated with CQ, as in all previous cases we observed a definitive limitation in parasite proliferation. Among any of these former conditions, cultures were incapable of reaching the level of total parasitemia that was reached among the accompanying vehicles. This observed limitation on proliferation corresponded with an increase in sexual conversion, which models the terminal investment hypothesis of reproductive investment formerly discussed (159, 360, 366).

However, the same cannot be determined for the hemin- treated cultures, as all cultures were ultimately able to reach the same peak in total parasitemia. Though growth may have been limited to a certain extent, for example potentially by an increase in intraerythrocytic cycle time, total proliferation was not limited. To our knowledge, this is the only example of a treatment causing an increase in sexual conversion independent of a change in total asexual proliferation. As other studies have investigated, and in some cases identified, other stimulants of sexual conversion, with increasing and/or decreasing concentrations of the stimulant, proliferation is affected (318, 343, 360, 363, 366). This pattern also does not align with the reproductive restraint hypothesis, as we still see an increase in sexual conversion rate. Rationalizing this finding, we may conclude

heme acts a direct inducer of sexual conversion, that is, it does not act by affecting overall parasite "state", as has been observed. Instead, it appears heme acts through specifically enhancing sexual commitment, independent of an effect on proliferation.

Notably, erythrocytes have been shown to contain at least one transporter capable of importing heme from the extracellular environment. The ATP-binding cassette sub-family B member 6 (ABCB6), is expressed on the plasma membrane of erythrocytes (523, 524). Therefore, though we were unable to directly deliver heme into erythrocytes or quantify intracellular heme after exposure, in seeing a response to the hemin exposure, we conclude hemin was delivered to infected cells. More interesting to note is that Hb S-Ery contain increased levels of heme even while uninfected (427, 495, 496).

Considering the effect on gametocyte gene expression in response to heme, we next sought to capture changes among the epigenetic landscape relevant to sexual commitment during heme treatment. Though we had determined both sexual conversion and *ap2-g* expression to be significantly increased between 48-64- hours post hemin exposure, the degree of this change on a whole-culture level was subtle. In other words, more investment was still being put into asexual replication, and found to be equal among all treatment groups as we found no change in asexual proliferation. Therefore, though we were unable to show changes in histone occupancy during ChIP analysis, this was not entirely surprising considering at least 80% of each culture was still asexually replicating, and thus would largely be expected to have *ap2-g* silenced by H3K9me3.

6.0 Conclusions and Future Directions

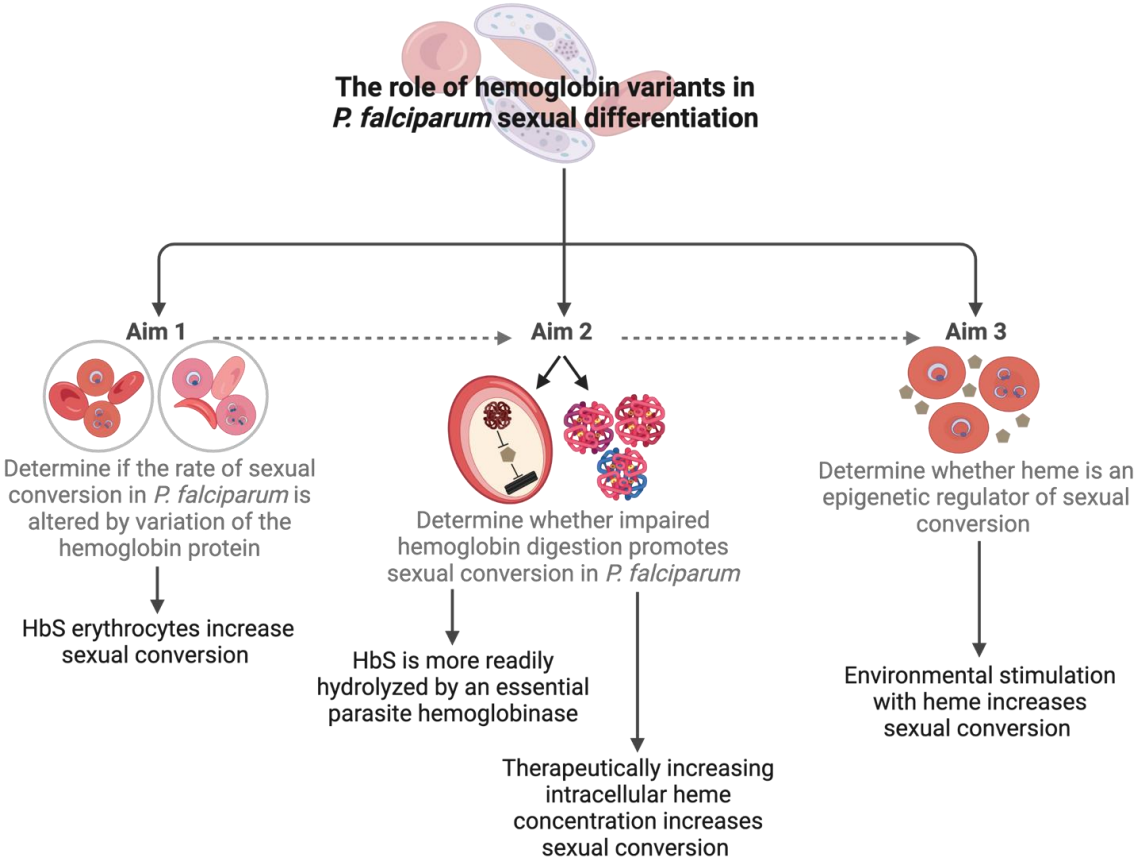

Figure 33. Summary of specific aims and conclusions.

Created with Biorender.

Collectively, these results provide experimental support for previous epidemiologic studies which have observed differences in gametocyte carriage among individuals with Hb variants (Figure 33) (332-334, 386, 441). Furthermore, our findings which indicate heme concentration is a causative factor for the initiation of sexual conversion, are highly relevant to the biology of Hb S- Ery, as they are known, even in uninfected states, to have higher concentrations of intracellular heme, as well as higher concentrations of extracellular heme (427, 495, 496). Therefore, we

propose increased heme concentration, resulting from the increased digestion of Hb S as well as underlying differences in total heme concentration, as a causative factor for the promotion of sexual conversion among Hb S- carrying individuals.

The finding of an increased rate of sexual conversion among Hb S-Ery cultures and during CQ exposure satisfies a survival purpose which can be explained by life history theory. As little intra-host survival is achieved during these conditions, parasites seem to "abandon ship" and divert effort into ensuring the success of inter-host transmission in hopes of acquiring a more suitable host for asexual proliferation (360, 366, 525). Intriguingly, though direct exposure to hemin did not significantly alter intra-host survival, we still observed an increase in sexual conversion rate, decoupling the idea of an induction of sexual conversion because of a decreased rate in asexual proliferation. This suggests that heme, instead of causing a population-based effect, acts more specifically in the induction of sexual conversion.

So then, how can this conclusion be rationalized between the three experiments (Hb S-Ery vs CQ exposure vs hemin exposure)? Under the Hb- S Ery and CQ experimental conditions, it is obvious there is a distinct effect on proliferation. However, we learn from the hemin exposure experiments, this effect on proliferation is not solely caused by increasing concentrations of heme. In thinking about the natural course of blood-stage malaria, as parasitemia, and thus intra-host success, increases, so does extracellular heme (327). Therefore, not only is it a requirement of the parasite to survive in the most heme-containing cell in the human body, throughout its survival success, it further contributes to the creation of a heme-rich environment. It is well known Hb S-Ery are resistant to *P. falciparum* infection, which results from a culmination of factors relating to invasion, efficiency of protein export, and early immune clearance (412, 417-419, 424, 425, 526). Though we know CQ increases intracellular heme concentration, we also know it causes a

dysregulation of the Hb digestive process, ultimately affecting the parasite's ability to acquire the essential amino acids otherwise gained from Hb digestion, and likely therefore resulting in parasite death (232, 498, 527). In the case of heme exposure, however, the only thing affected is heme concentration. Though this exposure certainly alters the external and internal environment for the parasite, these environmental changes *in vivo* are changes ultimately indicative of intra-host success. Therefore, though we don't necessarily see an improvement in "state" during heme treatment, heme could essentially be acting as a signal of improved state. From this perspective, we can apply the life history theory of improved state, wherein when conditions are improving for the parasite, investment into sexual conversion increases (Figure 34).

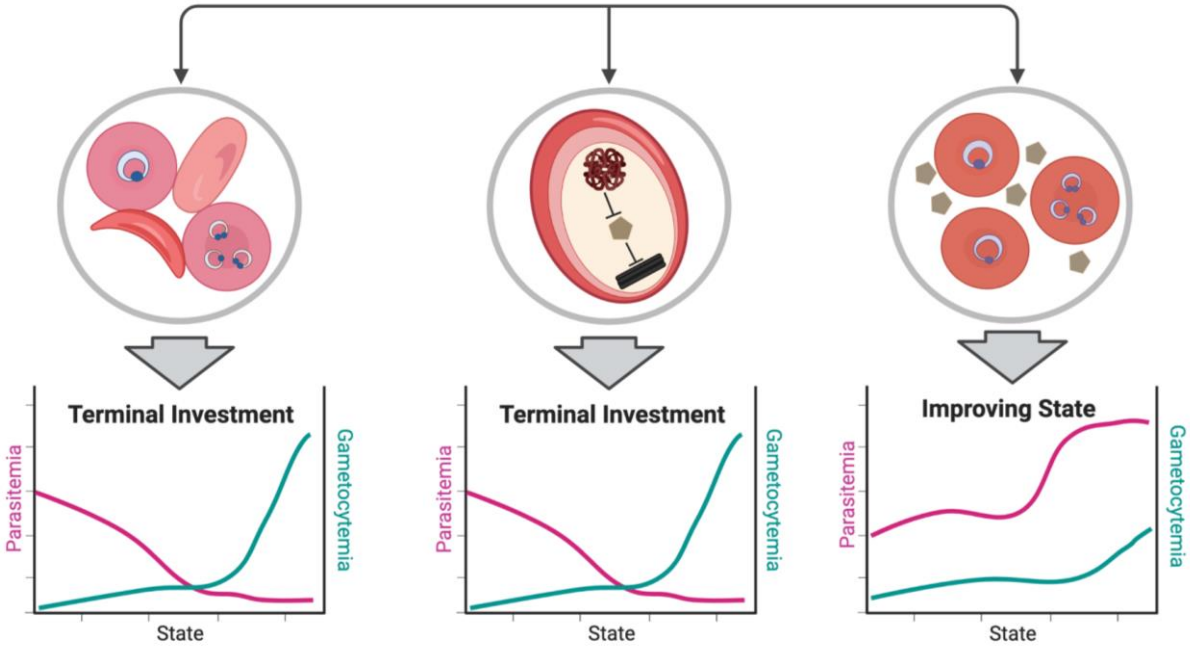

Figure 34. The relationship between heme exposure and reproductive investment in *P. falciparum*.

6.1 Future directions

The exact mechanism of how heme contributes to the initiation of sexual conversion is an area for future investigation. Though we can conclude sexual conversion occurred as a result of enhanced *ap2-g* expression resulting from heme exposure, we cannot conclude heme acts directly on *ap2-g* expression. Potential avenues of further investigation may include a deeper analysis of the role of heme causing oxidative stress, heme's function in transcription factor regulation, or heme's role in modifying chromatin structure leading to alterations in gene expression. The rationale for these avenues is discussed in the following sections.

6.1.1 Oxidative stress

Excess heme is known to cause oxidative stress, and oxidative stress is known to promote sexual conversion (161, 363, 528, 529). However, it remains unknown if oxidative stress as a result of increased heme acts directly on the expression of genes which promote sexual conversion, or if the affected redox state of the PV affects the activity and efficiency of signaling molecules which then promote gametocyte conversion. Current findings in the field relevant to both oxidative stress and generalized stress, provide more support for the latter hypothesis. For example, a more recent analysis of the effects of antimalarials on sexual conversion determined the effect of antimalarials is more likely a response to stress, rather than a specific component of the antimalarial itself (530). This included those antimalarial therapeutics which are known to cause oxidative stress, namely chloroquine and artemisinin. The same is hypothesized about the role of LysoPC depletion in the promotion of sexual conversion. The depletion of phosphatidylcholine is thought to prompt sexual conversion in lieu of asexual replication, as otherwise, merozoite formation would be negatively

impacted (343). Therefore, in both scenarios of oxidative stress and generalized or metabolic stress, sexual conversion has been found to be a response to state, rather than a directive from a specific molecule.

However, this distinction circles us back to heme's effect on parasite proliferation. If sexual conversion is a reactive response to oxidative stress, we would expect to see a clear change in proliferation or morphology. Therefore, investigation of sexual conversion as a direct response to heme-initiated oxidative stress should aim to address [1] heme's effect on the asexual proliferative capacity of *P. falciparum*, and [2] distinctions between the stress responses of *P. falciparum* which are known to results in sexual conversion (i.e. oxidative and nutrient). Though we determined the proliferative capacity of *P. falciparum* was not overall affected during heme exposure, we did observe changes in asexual proliferation. Therefore, a better understanding of whether and how heme impacts asexual proliferation should be investigated in order to fully understand the effects on sexual conversion. Moreover, as discussed, it is widely accepted that stress promotes sexual conversion. Therefore, in further proving or understanding the direct role of heme and distinguish between heme-initiated oxidative stress and other stress responses, it would be necessary to fully characterize these responses.

6.1.2 Gene regulation

It is known heme is able to regulate gene expression in humans and other organisms (531-534). For example, heme has been shown to play a role in transcription factor regulation in *Saccharomyces cerevisiae* through the binding of heme-responsive motifs in the transcription factor Hap1 (535). In humans, heme plays a well-established role in the control of Bach1, a transcription factor which regulates the expression of genes involved in the response to oxidative

stress. Heme binding of Bach1 promotes its degradation, and allows the expression of genes such as *Hmox1,* which function in heme degradation (536-540). Though in both of these examples heme's role in transcriptional or translational control occur in responses relevant to heme metabolism, because of heme's critical role in *Plasmodium* development, a wider understanding of heme's impact on gene regulation could not only answer questions relevant to sexual conversion, but open new avenues for the control of asexual replication. Therefore, this avenue of investigation should seek to determine [1] heme's role in the control of *ap2-g* and other regulatory elements currently understood as relevant to *ap2-g* control.

6.1.3 Chromatin structure and organization

While the previous two avenues of investigation are more broad examinations of the potential effects of heme on either pathways or gene regulation, this last route aims to investigate a more specific target. As discussed in section 1.3.4, the organization and structure of chromatin can have profound effects on overall gene expression (541). Heme has been shown to affect chromatin structure, specifically by altering the acetylation and methylation status of histone proteins (531). Recently in humans it was found that heme is a strong activator of histone lysine demethylase 4 (KDM4), which contains a heme-binding PAS domain. The majority of KDMs contain JmjC domains, which seemingly confer the demethylase activity (542). Interestingly, *P. falciparum* encodes three proteins which contain JmjC domains, and inhibitors of these domains were found to effectively prevent gametocyte development (543-545). In all organisms, KDMs containing Jmj domains are the only family of histone demethylases enzymatically capable of removing trimethyl marks (545-547). Therefore, it is possible heme has a role in the regulation of *P. falciparum* Jmj- containing proteins, which may have a role in the demethylation of H3K9me3

sites and the de-repression of *ap2-g*. Therefore, this route of investigation should aim to determine [1] the role of heme in the regulation or control of *PfJmjC1, PfJmjC2,* and *PfJmj3,* and [2] whether heme directly associates with *PfJmjC1, PfJmjC2,* and *PfJmj3.* These primary determinations may open additional avenues for transmission inhibition.

6.2 Final remarks

Heme is a fundamental molecule needed for the survival of both human and Plasmodial species. Balancing its toxic effects with the concentrations needed for life, require a complex relationship among many signaling pathways, molecules, and metabolic processes. Here, we conclude that heme acts as a direct inducer of gametocyte conversion in *P. falciparum*. With Hb S-Ery known to contain increased concentrations of heme, and increased action of parasite-mediated hydrolysis of Hb S leading to higher concentrations of heme within infected erythrocytes, we also conclude heme is a contributing factor to increased sexual conversion observed among Hb S-Ery and other Hb variants.

7.0 Public Health Implications

With over 40% of the global population considered at-risk of malaria disease at any time of the year, malaria is amongst the leading concerns of global health today. The significant economic burden malaria inflicts has been estimated to incur around $12 billion USD annually in direct and indirect costs (548). These costs result primarily from losses in human capital, as individuals are often forced to miss school and work, as well as long-term health complications resulting from chronic infection. Reactive responses to the disease ultimately increase healthcare costs, therefore further limiting the economic growth potential of the most affected countries. Moreover, with the most significant mortality effects impacting pregnant women and children under the age of five, maternal and child health, which are two of the most important indicators of a country's overall health and well-being, continuously suffer.

Though effective prevention and treatment options are available, malaria remains to be a significant contributor to global morbidity and mortality, particularly within sub-Saharan Africa. Over the past two years, deaths attributed to *P. falciparum* have actually increased, following a long-standing plateau of cases and deaths which was reached in 2015. Though this is largely due to obstacles following the COVID-19 pandemic, the continued emergence of resistant strains to all currently available antimalarials is a major challenge for ongoing control and elimination efforts (8, 51, 52). To circumvent this surge and regain control in the effort to reduce cases and deaths, new methods to control parasite transmission have long been needed.

Though there has been increased excitement after the European Medicines Agency (EMA) approved the use of the RTS,S vaccine (Mosquirix) for use in moderate-to-high malaria transmission areas, since its implementation, it has been shown to only provide approximately

30% protection against hospital admission. While a momentous stride for both vaccine development and malaria control, the vaccine requires four doses to reach its peak efficacy, thus further adding logistical complications to its application among various populations (549, 550). Therefore, though an important step in the effort to control malaria, the vaccine is far from being the end-all, be-all for malaria elimination.

Fundamentally speaking, in order to effectively eliminate any infectious agent, it's lifecycle must be disrupted. Though *P. falciparum* have long and complex lifecycles which occur over a minimum of 21 days between mosquito vector and human host, the development of specific methods which interrupt these phases have been challenging to create and effectively deploy. This is in part due to the massive efficiency of the lifecycle bottlenecks present throughout the cycle. These bottlenecks effectively alternate between parasite densities so small that they are able to hide from elimination strategies to densities so high, they effectively overwhelm elimination strategies. While most treatment efforts aim to target the intraerythrocytic cycle, which causes human disease, this phase incurs one of the highest parasite densities of all phases throughout the cycle. Therefore, often by the time treatment is introduced, the parasite has already been able to initiate an exit strategy, and thus has sealed the success of their next lifecycle phase. Accordingly, focus has more recently switched to the development of methods which target the pre-erythrocytic cycle (sporozoites), and transmission cycle (gametocytes) within the human host. As total parasite numbers among these stages are greatly reduced compared to that of the asexual erythrocytic cycle, they theoretically provide a much easier target for parasite elimination.

The relationship between malaria and hemoglobinopathies is complex and multifaceted. Particularly that of the Hb S-mutation, which was the major focus in this dissertation research. Though Hb S, when acquired on a heterozygous level, is known to provide protection against the

most severe forms of the disease, the protection is not absolute, and individuals are still susceptible to infection and complications resulting from severe disease. The high prevalence of the various Hb variants which now exist, thought in large part, due to malaria have significant public health implications. These include the apparent under detection and misdiagnosis of malaria amongst individuals with hemoglobinopathies, as well as increased morbidity and mortality from severe malaria which may be affected by trends in hemoglobinopathy carriage. Therefore, the protective effect Hb variation provides against malaria is ultimately balanced against its increased risk of severe disease.

Here, we investigated the potential of another facet to the malaria-hemoglobinopathy relationship; transmission. We found Hb S-Erys promote the formation of sexual stage parasites in the most prominent disease-causes species of *Plasmodium, P. falciparum*, which are essential for host to vector transmission. Though total gametocyte numbers were overall lower in the Hb S-Ery cultures, considering the population trends among malaria-endemic areas and the non-significant increase observed among the Hb AS-Ery cultures, this contribution to transmission may present an untapped target for malaria elimination strategies.

Though it has long been recognized that heme is a therapeutic target for malaria elimination, its action in the promotion of sexual conversion has not been investigated. Here, we show that heme promotes the formation of gametocytes. Therefore, while most methods relating to heme have focused on increasing its intracellular concentration as a way to kill the parasite, it is possible the opposite may also be effective, as a way to either decrease, or entirely inhibit sexual conversion and thus transmission entirely.

As methods of controlling asexual replication are increasingly becoming limited, deciphering new ways to control transmission, such as through the prevention of gametocyte

transfer from human host to mosquito vector, provide new opportunities for the goal of ending malaria (363, 551).

Appendix A

Appendix A.1 List of Abbreviations

AMA-1	apical membrane antigen 1
CM	cerebral malaria
CPM	complete parasite media
CSP	circumsporozoite protein
CQ	chloroquine diphosphate
DBL	Duffy binding-like
EBL	erythrocyte binding-like
FMO	fluorescent minus one
FP2	falcipain-2
GCR	gametocyte conversion rate
GDV1	gametocyte development protein 1
GTS	Global Technical Strategy
Hb	hemoglobin
Hb A-Ery	hemoglobin A erythrocyte
Hb AS-Ery	hemoglobin AS erythrocyte
Hb S-Ery	hemoglobin S erythrocyte
Hda2	histone deacetylase protein 2
HP1	heterchromatin protein 1
HSPG	heparan sulphate proteoglycans
H3K9Ac	histone 3 lysine 9 acetylation
H3K9me3	histone 3 lysine 9 tri-methylated
IFN	interferon
IPM	incomplete parasite media
KAHRP	knob-associated histidine-rich protein
LysoPC	lysophosphatidylcholine
MA-ARDS	malaria-associated acute respiratory distress syndrome
Mnase	micrococcal nuclease
MR4	Malaria Research and Reference Reagent Resource Center
MSP	merozoite surface protein
NAG	N-acetylglucosamine
Ni-NTA	nickel- nitrilotriacetic acid

NK	natural killer
NPP	new permeability pathways
PAMP	pathogen associated molecular patterns
PC	phosphatidylcholine
PfEMP1	*P. falciparum* erythrocyte membrane protein 1
PPM	parasite plasma membrane
PV	parasitophorous vacuole
PVM	parasitophorous vacuolar membrane
RBL	reticulocyte binding-like
RIFIN	repetitive interspersed families of polypeptides
RNA-FISH	RNA- fluorescent in situ hybridization
RON-2	rhoptry neck protein 2
ROS	reactive oxygen species
RT	room temperature
SCA	sickle cell anemia
SCD	sickle cell disease
SCR	sexual conversion rate
SCT	sickle cell trait
SMA	severe malarial anemia
STEVOR	subtelomeric variable open reading frame
TBST	tris-buffered saline with tween 20
VSA	variant surface antigens
WHO	World Health Organization

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