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REVIEW ARTICLE

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Roles of the apicoplast across the life cycles of rodent and human malaria parasites

Hayley D. Buchanan[1,2] | **Christopher D. Goodman**[2] | **Geoffrey I. McFadden**[2]

[1]Department of Infectious Diseases, Faculty of Medicine, Dentistry and Health Sciences, Melbourne Medical School, The University of Melbourne, Melbourne, Vic., Australia

[2]Faculty of Science, School of BioSciences, The University of Melbourne, Melbourne, Vic., Australia

Correspondence
Hayley D. Buchanan, Department of Infectious Diseases, Melbourne Medical School, Faculty of Medicine, Dentistry and Health Sciences, The University of Melbourne, Melbourne, Vic. 3010, Australia.
Email: hayley.buchanan@unimelb.edu.au

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Abstract

Malaria parasites are diheteroxenous, requiring two hosts—a vertebrate and a mosquito—to complete their life cycle. Mosquitoes are the definitive host where malaria parasite sex occurs, and vertebrates are the intermediate host, supporting asexual amplification and more significant geographic spread. In this review, we examine the roles of a single malaria parasite compartment, the relict plastid known as the apicoplast, at each life cycle stage. We focus mainly on two malaria parasite species—*Plasmodium falciparum* and *P. berghei*—comparing the changing, yet ever crucial, roles of their apicoplasts.

KEYWORDS
apicoplast, malaria, metabolism, *Plasmodium*, plastid

BROADLY, malaria parasite development can be split into three distinct life cycle phases: blood, mosquito and liver. Each of the phases has a different purpose and involves very different morphological forms, modes of nutrition and significantly different rates of proliferation. In addition, the vertebrate stages are predominantly intracellular, whereas mosquito stages are all extracellular. This variety of form and function is remarkable for a unicellular parasite with a relatively modest number of genes. Within the vertebrate, malaria parasites can achieve huge population numbers (Figure 1)—up to 10^{13} parasites per individual human host in an extreme infection (White & Pongtavornpinyo, 2003). In mosquitoes, parasite numbers are much lower, and a single parasite zygote can achieve transmission from the insect to a new vertebrate if things go well for it.

Our understanding of parasite development through each of the three main stages comes primarily from laboratory-based studies of *P. falciparum* and several rodent malaria species. Both the *P. falciparum* and *P. berghei* life cycles can be recapitulated in their entirety in the laboratory. Transmission of the rodent malaria is easier, but the advent of humanized mice, with human tissue engrafted into the liver and/or blood of mice, now make it practical (albeit expensive) to passage *P. falciparum* through its life cycle (Vaughan et al., 2012). The developmental processes at each stage differ somewhat between species, and the dynamics of this development is further influenced by the laboratory conditions imposed. Whether development occurs in vitro or in vivo, for example, and whether the host–parasite relationship is an organic one, or an artificial one (i.e. the use of laboratory mice as a non-natural host) employed for ease of laboratory experiments (Beier, 1998; Vaughan et al., 1994; Yoeli, 1965). These factors undoubtedly impact the applicability of our findings to the situation in the field. Furthermore, environmental temperature during development also significantly affects parasite numbers and duration of stage-specific development periods (Beier, 1998). Despite these limitations, laboratory study of the parasite progression through its life cycle has yielded powerful insights into the underlying metabolisms and revealed vulnerabilities, a few surprises and some puzzling questions, particularly with respect to apicoplast biology.

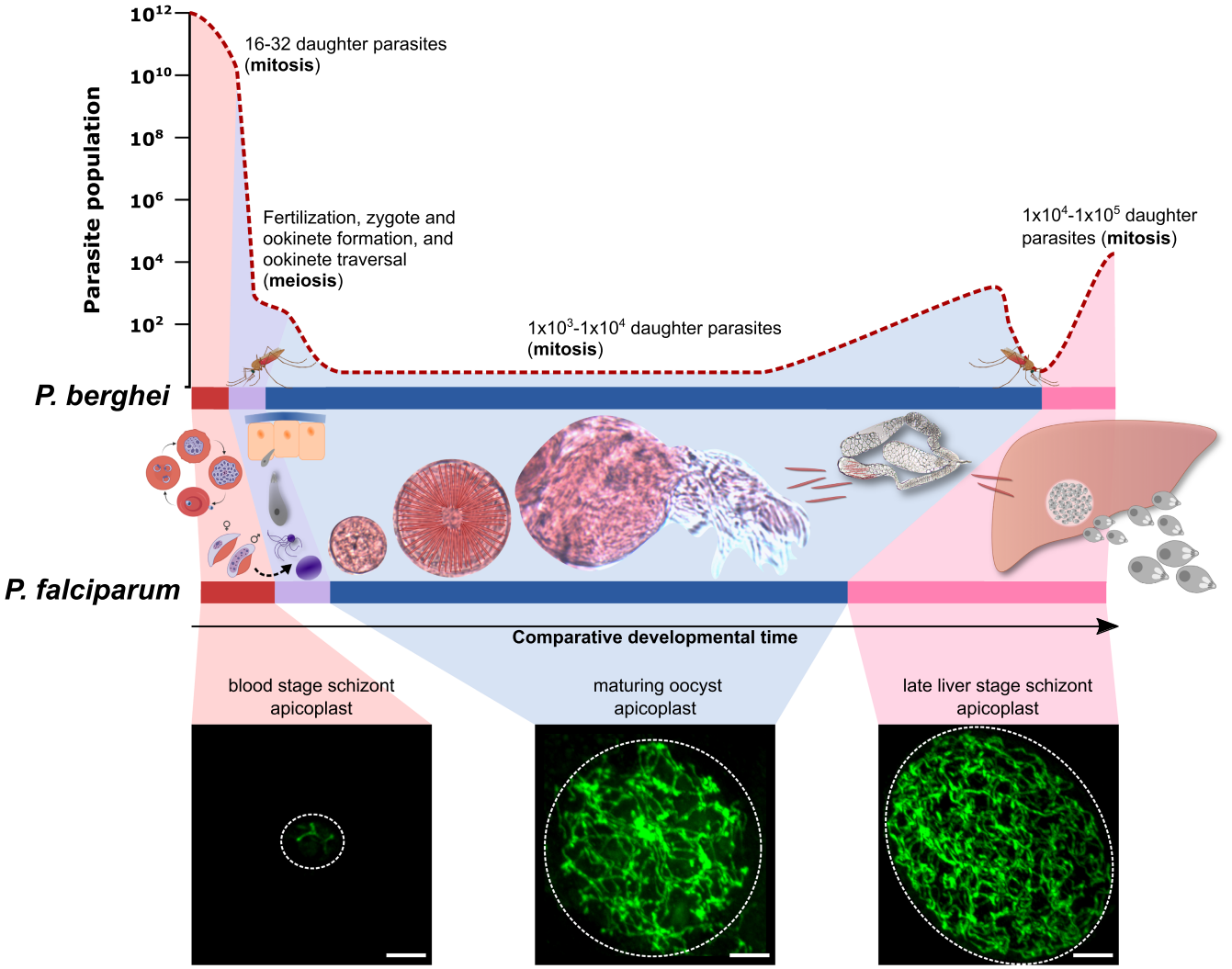

FIGURE 1 An overview of malaria parasite development and approximate parasite population number (red dotted line) and approximate number of daughter cells produced by each mother cell during each developmental stage. A comparison of the duration of each life cycle stage between *P. berghei* and *P. falciparum* (colored bars) is shown, with a representative immunofluorescence image depicting apicoplast morphology underneath. Asexual blood stage development (red bar) takes approximately 24-h for *P. berghei*, whereas *P. falciparum* takes 48-h. Parasite population numbers during the asexual blood stage can reach upward of 1×10^{12} in a high parasitaemia infection, before drastically reducing upon transmission to mosquitoes. Maturation of gametocytes takes around 26-h post blood stage patency for *P. berghei*, but between 9–12 days for *P. falciparum* (time not indicated). Once an *Anopheline* female takes a blood meal from an infected host, gamete activation, fusion, zygote formation, ookinete formation, and traversal across the mosquito midgut wall all happen within 24-h for *P. berghei* and within 36-h for *P. faciparum* (mauve bar). This represents a clear bottleneck for the parasites, as population numbers dip towards their lowest when an oocyst finally forms under the basal lamina of the mosquito midgut. Oocyst development, sporogony, and migration of infection-ready salivary gland sporozoites takes up to three weeks for *P. berghei* yet only takes up to two weeks for *P. falciparum* (blue bar). After the bite of an infected *Anopheline* female (another population bottleneck), liver stage development in a naïve host takes a little over two days for *P. berghei* (about 56-h), yet takes between 6–7 days for *P. falciparum* (pink bar). The disparity in developmental time, particularly between mosquito and liver stages between the two species, highlights potential differing metabolic needs during these developmental stages. However for both species, both mosquito and liver stage development are more onerous for the parasites metabolically, which is reflected in apicoplast size and morphology during these stages (bottom three images, apicoplast labeled in green, white dotted line represents the parasite boundary: scale bar = 5 μm). Created in part with BioRender.com

REPLICATION RATES AND PERIODS

Parasite replication by schizogony during blood, mosquito and liver stages is roughly similar with respect to orders of magnitude between *P. falciparum* and *P. berghei* for each stage, but duration of each stage varies (Figure 1) (Baton & Ranford-Cartwright, 2005; Beier, 1998; Vaughan & Kappe, 2017). Although the asexual cycles differ in duration between the two species—24-h for *P. berghei* and 48-h for *P. falciparum*—this phase of the parasite replicative cycle is relatively short and low demand in comparison to mosquito and liver stages (Figure 1). One disparity not shown, and minimally discussed here, is the maturation of mosquito-ready gametocytes—the sexually committed, blood stage

parasite forms infective to mosquitoes. In *P. berghei* mature gametocytes develop at 26-h post patent blood stage infection, whereas *P. falciparum* gametocytes take up to 12-days to develop (Mons et al., 1985; Ngotho et al., 2019). This long duration to mosquito-ready sexual forms in *P. falciparum* coincides with a distinct morphological difference from other species, with *P. falciparum* gametocytes having an eponymous falciform shape but most other species having round gametocytes (Dixon & Tilley, 2021).

The stages of gamete activation, fusion, ookinete traversal across the mosquito midgut and establishment of oocysts are a genetic bottleneck for both species. As with all eukaryotic sex, the number of individual parasites is halved when gamete fusion occurs. Post fertilization, the life cycle has an extended window of relatively low numbers of individuals. Timewise, gametes activate within minutes of being taken up by the mosquito in her malaria-infected blood meal. Fertilization to form a zygote typically occurs within three hours, and the subsequent ookinete stage forms within a day. The zygote/ookinete is under threat of being digested with the blood meal and must escape the gut. After gliding in a helical path through the blood meal (Kan et al., 2014), the ookinete then traverses the mosquito midgut wall to settle under the basal lamina and become an oocyst; a process that takes ~24-h for *P. berghei* and ~36-h for *P. falciparum* (Figure 1) (Beier, 1998).

Sporogony—the development of an oocyst into thousands of sporozoites—varies considerably between rodent and human malaria species in both the number of sporozoites each oocyst in a single mosquito produces and the time it takes for sporozoites to develop end egress. Sporozoites can be detected egressing from some oocysts as early as 7–10 days after oocyst formation, with all oocysts maturing and releasing their sporozoites over the course of up to 2–3 weeks for *P. berghei* and up to 1–2 weeks for *P. falciparum* (Figure 1). Sporogony is thus the slowest proliferative stage in the life cycle and the only one with significant variation in the synchronicity of the timing of the developmental program. What is more, only around a quarter of total sporozoites ever make it to the salivary glands (Beier, 1998).

When the female mosquito takes another blood meal, fewer than 100 sporozoites are injected into a naïve host by the bite (Beier, 1998; Jin et al., 2007), thus constituting another bottleneck in parasite numbers proceeding to the next stage. Once sporozoites reach and invade a hepatocyte, liver stage development takes ~2 days for *P. berghei* and up to 7 days for *P. falciparum* (Figure 1), with both liver stage schizonts generating tens-of-thousands of daughter merozoites,—a remarkable proliferation rate for any organism (Vaughan & Kappe, 2017).

The disparities between *P. berghei* and *P. falciparum* in duration of development during blood, mosquito, and liver stages suggests differing metabolic requirements. Malaria parasites appear to scavenge most of the necessities during each life cycle stage but de novo synthesis of anabolites is also essential to provide what the host cannot. Genetic studies suggest that this de novo synthesis is relied on more heavily in the insect and liver stages (Stanway et al., 2019). Consequently, the *Plasmodium* spp. apicoplast—a hub of metabolic activity (Ralph et al., 2004)—is larger, and apparently more active during insect and liver stages than it is during blood stage (Figure 1). The differences in apicoplast activity during the three broad developmental phases will be discussed here, as will overarching differences between *P. berghei* and *P. falciparum* that are highlighted by the differing developmental timelines in the insect and liver stages. This review highlights recent advances in our understanding of apicoplast activity during the three key phases of the life cycle. For background details on general apicoplast metabolism see (Kloehn et al., 2021) and (Ralph et al., 2004).

THE *PLASMODIUM* spp. APICOPLAST

The sequencing of the '35-kb circular DNA' (Williamson et al., 1994; Wilson et al., 1996) and subsequent localisation of some plant-like genes in a membrane bound compartment (now called the apicoplast) in malaria parasites (Gardner et al., 1991; Howe, 1992; Williamson et al., 1994), marked a significant change in our understanding of *Plasmodium* spp. Localizing several of these plant-like genes to an organelle of apparent endosymbiotic origin (McFadden et al., 1996) suggested that an early common ancestor of *Plasmodium* spp. parasites, and indeed the entire Phylum Apicomplexa, was photosynthetic. There was initial disagreement as to the exact evolutionary origins of the apicomplexan plastid (Funes et al., 2002; Köhler et al., 1997; Waller et al., 2003; Williamson et al., 1994), but it is now widely accepted to derive from the secondary endosymbiosis of a red alga (Burki et al., 2020; Fast et al., 2001). Owing to this ancient endosymbiotic event, the apicoplast is surrounded by four membranes and contains its own genome (the 35 kb circular element) and metabolic pathways of bacterial origin (Ralph et al., 2004). Given that these metabolic pathways are evolutionarily distinct from those of the vertebrate host, the apicoplast has long been perceived as an attractive drug target (Fichera & Roos, 1997; Ralph et al., 2001; Wiesner & Jomaa, 2007). Indeed, many drugs are known to target apicoplast-resident pathways (Biddau & Sheiner, 2019). Disruption of the *Plasmodium* spp. apicoplast with inhibitors leads to parasite death (Dahl et al., 2006; Dahl & Rosenthal, 2007; Goodman et al., 2007; Uddin et al., 2018; Wiesner et al., 2003), demonstrating the essentiality of the organelle during symptomatic blood stages of malaria. The apicoplast is essential for all life cycle stages, although its metabolic activity varies both between species and between

developmental stages, and it seems to be more heavily relied upon during the highly replicative mosquito and liver stages (Figure 1).

THE APICOPLAST DURING BLOOD STAGES—SOME UPDATES

Whole genome mutagenesis screens in *P. falciparum* (Zhang et al., 2018) and *P. berghei* (Bushell et al., 2017) have been critical in giving us an understanding of metabolic functionality of the malaria parasite during the genetically tractable blood stages. These studies revealed that a substantial proportion of genes involved in apicoplast function are essential for both species in blood stage (Bushell et al., 2017; Zhang et al., 2018), confirming what we knew from chemical inhibition and gene-by-gene investigations of the organelle. Despite the wide variety of biogenesis and maintenance pathways these genes service, it seems that they are only essential during blood stage to maintain the apicoplast so that it can sustain largely one main pathway, the MEP/DOXP/non-mevalonate pathway for synthesis of isoprenoid precursors, such as isopentenyl pyrophosphate (IPP) (Yeh & DeRisi, 2011). Indeed, when provided with exogenous IPP in the growth medium, *P. falciparum* parasites are able to grow and replicate during blood stages without an intact apicoplast (Yeh & DeRisi, 2011). However, the emphasis here should be on 'intact' apicoplast because another apicoplast activity—crucial synthesis of coenzyme A (CoA)—has recently been shown to occur in what is apparently an echo of the apicoplast in apicoplast minus parasites (Swift et al., 2021). When the apicoplast is ablated, an echo of apicoplast-destined vesicles apparently have nowhere to go and accumulate in the cytosol (Yeh & DeRisi, 2011). Swift et al. (Swift et al., 2021) provide evidence that these vesicles function to convert dephospho-CoA into CoA using a dephospho-CoA kinase originally destined for the apicoplast. This step in CoA synthesis is an essential process, even in apicoplast 'minus' parasites. Thus, at least two apicoplast activities—IPP generation and CoA synthesis—are indispensable in blood stage *P. falciparum* parasites. Of course, all other activities crucial to the growth, maintenance and biogenesis of the apicoplast underpin these two activities and are, therefore, also essential. IPP and CoA must be exported from the apicoplast, and even from apicoplast-destined vesicles in the case of CoA, but how this export is achieved remains totally unknown (Kloehn et al., 2021; Sayers et al., 2017).

Chemically rescuing *P. falciparum* apicoplast-minus parasites with addition of IPP to the growth medium has enabled validation of apicoplast-targeting drugs, allowing clear identification of those drugs with a primary apicoplast target from a host of other parasiticidal agents (e.g. fatty acid biosynthesis inhibitors) that are apparently off-target and have no impact on the apicoplast (Uddin et al., 2018; Wu et al., 2015). IPP rescue has also turned out to be a convenient tool to confirm the indispensability of blood stage apicoplast-targeted genes, pathways, and membrane transporters where knockouts would otherwise have been lethal (Meister et al., 2020; Sayers et al., 2017; Tan et al., 2021; Tang et al., 2019; Walczak et al., 2018). IPP supplementation has also delivered the answer to one of the most enduring mysteries since the discovery of the apicoplast, namely why do malaria parasites take two blood stage cycles to die when their apicoplasts are compromised? Dubbed 'delayed death', this mysterious phenomenon whereby malaria parasites treated with apicoplast housekeeping inhibitors die in the second intraerythrocytic developmental cycle (IDC) post drug treatment has puzzled apicoplast afficionados for 22 years (Dahl & Rosenthal, 2007; Goodman et al., 2007). Kennedy and colleagues (Kennedy et al., 2019) showed, through chemical rescue with IPP and downstream isoprenoid products, that parasite death in the second IDC post drug treatment is caused by loss of protein prenylation and consequent disruption of vesicle trafficking essential for endocytotic feeding on hemoglobin (Kennedy et al., 2019). Perturbation of vesicle trafficking affects myriad parasite processes, and parasite death is delayed since IPP and downstream products are gradually exhausted, essentially resulting in death by parasite fatigue (Kennedy et al., 2019). By isolating protein prenylation from the numerous other downstream isoprenoid-requiring pathways as the proximate cause of parasite death in the second IDC following drug treatment (Kennedy et al., 2019), we can now understand the action of apicoplast housekeeping inhibitors. This insight is not only intellectually satisfying but it also has profound consequences for how we deploy apicoplast inhibiting drugs. For instance, the key antimalarial artemisinin is potentiated by parasite haem digestion (Pandey et al., 1999), but Kennedy et al. showed that haem digestion is perturbed by common apicoplast inhibitors (Kennedy et al., 2019), which begs the question of whether apicoplast inhibitors might be antagonistic to artemisinin, and indeed any derivatives of artemisinin requiring activation by haem digestion. Early indications are that doxycycline—a widely used, apicoplast targeting antimalarial (Dahl et al., 2006; Goodman et al., 2007)—is indeed antagonistic to artemisinin because it impedes haem digestion and thereby blunts the impact of artemisinin (Crisafulli et al., 2021).

Supplementing in vitro cultures of *P. falciparum* with IPP is expensive. As a workaround, a parasite line (*Pf*Mev) that can make IPP without recourse to the apicoplast pathway was recently engineered (Swift et al., 2020). *Pf*Mev parasites generate IPP in the cytosol via heterologous expression of the so-called mevalonate pathway (Swift et al., 2020), which is common to organisms without a plastid. *Pf*Mev requires exogenous

supply of mevalonate, which is considerably more affordable than IPP and offers numerous options for exploring apicoplast essentiality, the first example being the above-mentioned apicoplast phosphorylation of dephospho-CoA. It was using this parasite line that CoA was identified as essential during asexual blood stage growth in *P. falciparum* (Swift et al., 2021), and it was shown that the synthesis pathway remained active after apicoplast disruption, continuing to function in vesicles that would normally be trafficked to the organelle (Swift et al., 2021). Both of these insights highlight that apicoplast activity during blood stages is more complex than has been held over the past decade. What other apicoplast functions can be supported in such a dispersed manner after organelle disruption in these biochemically active apicoplast-like vesicles?

Teasing out the essential functions of the apicoplast during blood stages of *P. berghei* has not been so easy, mainly because blood stages cannot be effectively cultured, and IPP supplementation in vivo is impractical. Nevertheless, an excellent suite of tools available in the rodent malaria model *P. berghei* have allowed us to drill into metabolic functions of the apicoplast during blood stages in a targeted way (Gomes et al., 2015; Schwach et al., 2015) and also at the whole genome level (Bushell et al., 2017). Thus, like *P. falciparum*, many apicoplast resident metabolic pathways, such as fatty acid biosynthesis and heme synthesis, are not essential for blood stage growth in rodent malaria (Nagaraj et al., 2013; Pei et al., 2010; Rathnapala et al., 2017; van Schaijk et al., 2014; Yu et al., 2008).

Given the enormous numbers of parasites that develop in mammalian hosts, it is perhaps counterintuitive that the replicative demands of malaria parasites during blood stages are relatively low (Figure 1), but such is the power of exponential growth, and may reflect grerater availability and ease of access to scavenged resources. From the above discussions, it is apparent that the apicoplast does only two things for blood stage parasites: build IPP and phosphorylate dephospho-CoA. Indeed, these minimal activities likely reflect that the apicoplast is not relied upon during the blood phase nearly as heavily as elsewhere in the life cycle, and the rest of this review examines the apparently increased roles of the organelle in the other two major phases of the life cycle.

THE APICOPLAST DURING MOSQUITO STAGES—SOME UPDATES

During mosquito stage development, the oocyst mother cells of both *P. falciparum* and *P. berghei* generate thousands of daughter cells known as sporozoites (Figure 1). The large, unicellular sporoblast mother cell (~50 μm diameter) within an oocyst has a relatively small surface area to volume ratio, but when we consider thousands of thin, elongate sporozoites built within the confines of the oocyst, it is obvious that a tremendous amount of bounding membrane must be generated during this schizogony. Nevertheless, apicoplast localized fatty acid biosynthesis enzymes are not essential for sporogony in *P. berghei* (Yu et al., 2008) nor in another rodent malaria model *P. yoelii* (Vaughan et al., 2009; Pei et al., 2010). This is in stark contrast to *P. falciparum*, where parasites lacking key apicoplast localized fatty acid biosynthesis enzymes, FabI and FabB/F, fail to produce sporozoites, implying that apicoplast fatty acid biosynthesis is essential for oocyst maturation in *P. falciparum* (van Schaijk et al., 2014). This disparity is somewhat difficult to reconcile considering the parasites are generating similar numbers of sporozoites per oocyst in similarly nourished mosquitoes (Figure 1). Perhaps the more rapid maturation of oocysts in *P. falciparum* mandates apicoplast production of fatty acids, or perhaps boutique fatty acids, only able to be made by the apicoplast are required by *P. falciparum* sporozoites. Alternatively, it may simply reflect differences between the *Plasmodium* species in their ability to scavenge resources during the differing sporogony periods. For instance, a recent study where both *P. berghei* and *P. falciparum* were starved of vector-derived lipids during mosquito stage development found that both species responded with slowed metabolism and subsequent reduced oocyst growth (Habtewold et al., 2021). These effects were reversed when infected mosquitoes were given additional blood meals during early-mid stage oocyst development, and the rescue was more pronounced for *P. falciparum* parasites, where the supplementary blood meal was needed earlier (Habtewold et al., 2021). Thus, the reduced developmental time for *P. falciparum* during mosquito stages may result in an earlier and more rapid need for metabolites such as fatty acids, that cannot be supplied from the mosquito host in time, therefore necessitating de novo synthesis. Indeed, a recent publication shows that multiple blood feeds for mosquitoes infected with *P. falciparum* speeds up parasite development (Shaw et al., 2020). Attempting post infection blood feeds for mosquitoes infected with *P. berghei* and *P. falciparum* possessing fatty acid biosynthesis gene disruptions may help to dissect these differences.

Similarly, just as *P. berghei* and *P. falciparum* differ in their requirements for de novo synthesized fatty acids, they also rely differently on apicoplast localized lipoate synthesis during mosquito stage development. Lipoic acid synthesis in the *Plasmodium* spp. apicoplast serves to provide substrates to function in the fatty acid biosynthesis pathway, and vice versa (Kloehn et al., 2021). A key apicoplast localized enzyme in this pathway, LipB, is dispensable during mosquito stage development for *P. berghei* (Falkard et al., 2013), but knockouts of the orthologue in *P. falciparum* were recently shown to be essential for sporogony (Biddau et al., 2021). This disparity in lipoic acid synthesis requirements between *P. berghei* and *P. falciparum* mirrors the fatty acid synthesis

needs, and the two pathways are interdependent (Kloehn et al., 2021). Given that these two pathways go on to become essential during liver stages of *P. berghei*, the duration of parasite development (Figure 1) may again be crucial for fatty acid/lipoic acid synthesis requirements, in addition to magnitude of replicative need.

There is a conspicuous gap in our knowledge of apicoplast localized membrane transporters for *Plasmodium* spp. (Kloehn et al., 2021). Given that the apicoplast is bound by four membranes, substrates required for apicoplast resident metabolic pathways and energy sources for housekeeping must be imported, and their products exported for proper parasite function. Although the inner and outer triose phosphate transporters (iTPT and oTPT, respectively) in *P. falciparum* were identified and characterized some time ago (Lim et al., 2010; Mullin et al., 2006), and several other apicoplast localized transporters have since been identified in *P. berghei* (Sayers et al., 2017), very little is known about the shuttling of metabolites into and out of the apicoplast, especially in mosquito and liver stages.

A recent study identified an apicoplast localized folate transporter essential for normal sporozoite development in *P. berghei* (Korbmacher et al., 2020). This putative folate transporter, FT2, is one of two likely folate transporters identified in the *Plasmodium* spp. genome (Salcedo-Sora et al., 2011), with the other, FT1, localized to the parasite periphery and predicted to be involved in folate salvage (Salcedo-Sora et al., 2011). Folate intermediates likely need to be transported into the *Plasmodium* spp. apicoplast for protein translation, as the organelle requires a formylated methionine to initiate translation (Habib et al., 2016; Jackson et al., 2011). This has so far remained experimentally unvalidated, but the identification of the apicoplast localized FT2 in *P. berghei* indicates that folate products are required in the apicoplast for normal parasite development in the mosquito (Korbmacher et al., 2020). It is then tempting to speculate that the underlying reason for lack of sporulation would be the lack of properly regulated protein translation in the apicoplast, perhaps leading to an imbalance in metabolism during oocyst development in *P. berghei*. Recall that even though de novo fatty acid biosynthesis is not required during oocyst development in *P. berghei*, apicoplast resident heme, and iron–sulfur cluster biosynthesis certainly are (Charan et al., 2017; Rizopoulos et al., 2016).

These studies together highlight both the indispensable role that apicoplast metabolism has during sporogony for *P. berghei* and *P. falciparum*, and that there is an intriguing interdependence between apicoplast resident metabolic and housekeeping pathways, the optimal functioning of the latter being essential to the former. Importantly, our lack of knowledge of metabolite movement into and out of the apicoplast makes teasing apart apicoplast requirements during mosquito stage development difficult and underlines that need to dissect this further during mosquito and liver stages to understand the full picture of malaria parasite transmission.

THE APICOPLAST DURING LIVER STAGES—SOME UPDATES

Much of what we know about liver stages of malaria has come through the study of rodent malaria models, largely because this system provides the simplest source of sporozoites to infect livers or cultured liver cells (Vaughan & Kappe, 2017). However, the advent of humanized-liver mouse models in malariology (Vaughan et al., 2012) to permit in vivo studies of *P. falciparum* liver stage infections in chimeric mice is a game changer (Button-Simons et al., 2021; Foquet et al., 2018; Yang et al., 2017a; Yang et al., 2017b). Apicoplast biology during liver stage of *P. falciparum* however, remains hardly studied, primarily because gene perturbations must be done during blood stage and genes essential in mosquito stages are awkward to ablate during liver stages (Biddau et al., 2021; van Schaijk et al., 2014). It would be interesting to perform mixed infections with these parasite lines in an attempt to overcome the mosquito stage block and study apicoplast metabolic requirements during liver stage, much like what has been done with other gene knockouts with mosquito stage blocks in *P. berghei* (Rathnapala et al., 2017). These mixed infections result in genetically blended coenocytic oocysts (Simon et al., 2021) with a mixed cytoplasm, and protein carryover to the haploid sporozoites allows knockouts that would normally arrest during mosquito stages to be carried through to liver stage (Rathnapala et al., 2017). This may assist in reconciling what we know of apicoplast biology during liver stages in rodent malaria with *P. falciparum* and aid progression of liver stage attenuated parasite vaccine strategies (Mo & McGugan, 2018; Sahu et al., 2021; Vaughan et al., 2018) and selection of prophylactic antimalarials.

The liver stage represents another mass replication stage for *P. berghei* and *P. falciparum* (Figure 1), and like mosquito stages, it seems that apicoplast activity is more heavily relied upon, at least from what we know in rodent malaria models. Contrary to mosquito stages, apicoplast fatty acid biosynthesis (Pei et al., 2010; Vaughan et al., 2009; Yu et al., 2008) and lipoate biosynthesis (Falkard et al., 2013) are required for normal liver stage development in rodent malaria. Furthermore, from a study involving genetic disruption of SufS in *P. berghei*, there appears to be a liver stage requirement for apicoplast localized iron–sulfur cluster biosynthesis (Charan et al., 2017). *P. berghei* sporozoites with a SufS deletion were able to infect naïve mice, but showed a significant patency delay, indicative of a liver stage developmental defect (Charan et al., 2017). These studies were largely corroborated by a pivotal study by Stanway et al., who performed a novel, high throughput screen

of genes required for parasite liver stage development in *P. berghei* (Stanway et al., 2019). They further dissected necessity of parasite metabolic pathways during liver stage by constructing an integrated phenotype model of their data, allowing for predictions of the physiological mechanisms behind the observed phenotypes (Stanway et al., 2019). Indeed, they showed that all enzymes in the apicoplast localized fatty acid biosynthesis and lipoate synthesis pathways are required for *P. berghei* liver stage development, and these requirements are likely tied to a high need for fatty acids at this stage (Stanway et al., 2019). This was corroborated by their predictive model (Stanway et al., 2019). The screen, however, did not identify iron–sulfur cluster biosynthesis as a necessary pathway for *P. berghei* liver stage development, although, in agreement with the above study (Charan et al., 2017), their integrated predictive model suggested it was required (Stanway et al., 2019). Thus, we may need to resort to gene-by-gene studies of iron–sulfur cluster biosynthesis during liver stage development to reconcile and confirm essentiality during liver stages.

The difference in liver stage development duration between *P. berghei* (around 56-hours) and *P. falciparum* (6–7 days) (Figure 1) prompts speculation as to why there is such a time disparity and whether this has anything to do with apicoplast metabolic activity. The replication magnitude is similar between species, just as it is during mosquito stage, but the requisite time frames are reversed, being longer in *P. berghei* mosquito stages but much shorter in *P. berghei* liver stages (Figure 1). The mosquito stage differences in development time might perhaps be related to temperature—*P. berghei* infects mosquitoes at 21°C and *P. falciparum* at 27°C, but liver stages develop at 37°C. The mosquito's reproductive cycle might also be a factor (Shaw et al., 2021). Whatever the case, it is clear that accelerated development for *P. berghei* during liver stages is coupled with an increased reliance on apicoplast metabolism, particularly fatty acid biosynthesis (Stanway et al., 2019). It would be interesting to find out if the reverse is true for *P. falciparum*, as is seemingly the case during mosquito stage development, and if this is somehow tied to duration of parasite development.

CONCLUDING REMARKS

The apicoplast plays an integral role in *Plasmodium* spp. development through the life cycle, particularly when the replicative demands of the parasite are highest. As outlined here, several recent studies have brought focus on apicoplast activity at each of the three broad life cycle stages and highlighted where gaps in our knowledge exist. Although we at least have a broad map of which pathways are essential in which stage for a rodent malaria parasite and a human malaria parasite, we have a paucity of knowledge of how apicoplast resident metabolic pathway intermediates are transported into and out of the organelle for any *Plasmodium* species, outlined and reviewed in (Kloehn et al., 2021). Furthermore, our understandings of apicoplast biology during liver stages in *P. falciparum* are still rudimentary, and it would be particularly helpful to compare the human parasite liver stage with our growing knowledge of the apicoplast during liver stage development of *P. berghei* (Stanway et al., 2019). Clinically, understanding the apicoplast's role in the liver stage is vital in informing the development and selection of prophylactic antimalarials. Humanized mouse models for malaria should help in addressing this gap.

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ORCID

Hayley D. Buchanan https://orcid.org/0000-0002-2764-1428

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