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REVIEW

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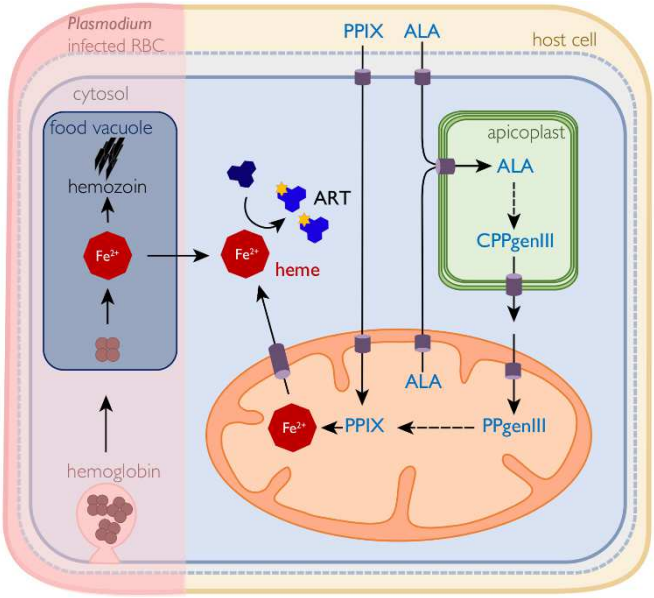

Heme is a ubiquitous cofactor essential for most organisms. The human pathogens *Toxoplasma gondii* and *Plasmodium spp.* can salvage heme or its intermediates from their host, but largely rely on its *de novo* synthesis utilizing an unusual heme synthesis pathway, which spans across three subcellular compartments. This review discusses our understanding of heme acquisition in apicomplexans, its role in metabolism, and in the activation of the antimalarial drug artemisinin (ART).

Supply and demand – heme synthesis, salvage and utilization by Apicomplexa

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The Apicomplexa phylum groups important human and animal pathogens that cause severe diseases, encompassing malaria, toxoplasmosis and cryptosporidiosis. In common with most organisms, apicomplexans rely on heme as co-factor for several enzymes, including cytochromes of the electron transport chain. This heme derives from *de novo* synthesis and/or the development of uptake mechanisms to scavenge heme from their host. Recent studies have revealed that heme synthesis is essential for *Toxoplasma gondii* tachyzoites, as well as for the mosquito and liver stages of *Plasmodium spp*. In contrast, the erythrocytic stages of the malaria parasites rely on scavenging heme from the host red blood cell. The unusual heme synthesis pathway in Apicomplexa spans three cellular compartments and comprises enzymes of distinct ancestral origin, providing promising drug targets. Remarkably given the requirement for heme, *T. gondii* can tolerate the loss of several heme synthesis enzymes at a high fitness cost, while the ferrochelatase is essential for survival. These findings indicate that *T. gondii* is capable of salvaging heme precursors from its host. Furthermore, heme is implicated in the activation of the key antimalarial drug artemisinin. Recent findings established that a reduction in heme availability corresponds to decreased sensitivity to artemisinin in *T. gondii* and *Plasmodium falciparum*, providing insights into the possible development of combination therapies to tackle apicomplexan parasites. This review describes the microeconomics of heme in Apicomplexa; from supply, either from *de novo* synthesis or scavenging, to demand by metabolic pathways, including the electron transport chain.

Abbreviations

ALA, 5-aminolevulinate; ALAD, 5-aminolevulinate dehydratase; ALAS, 5-aminolevulinate synthase; ART, artemisinin; ATP, adenosine triphosphate; CoA, coenzyme A; COX, cytochrome c oxidase; CPDH, coproporphyrinogen III dehydrogenase (oxygen independent); CPOX, coproporphyrinogen III oxidase; CPPgenIII, coproporphyrinogen III; CRISPR, clustered regularly interspaced short palindromic repeats; ETC, electron transport chain; FECH, ferrochelatase; HMB, hydroxy methylbilane; HRM, heme regulatory motif; OXPHOS, oxidative phosphorylation; PBG, porphobilinogen; PBGD, porphobilinogen deaminase; PPgenIX, protoporphyrinogen IX; PPIX, protoporphyrin IX; PPOX, protoporphyrinogen oxidase; PV, parasitophorous vacuole; RBC, red blood cell; TCA cycle, tricarboxylic acid cycle; UPgenIII, uroporphyrinogen III; UROD, uroporphyrinogen decarboxylase; UROS, uroporphyrinogen III synthase.

Introduction

Heme is a ubiquitous, complex organic molecule, essential to nearly all organisms across the three branches of life (Archaea, Bacteria and Eukarya). It belongs to the class of tetrapyrroles, including porphin, the simplest tetrapyrrole, porphyrins such as heme, chlorins including chlorophylls and corrins such as cobalamin (vitamin B12) (**Figure 1**) [1]. Tetrapyrroles are often chromophores due to their high degree of conjugation, e.g. chlorophyll grants the green color to leaves and heme which colors blood red. Tetrapyrroles typically conjugate a metal ion in their center. In the case of heme, iron is coordinated by four nitrogen atoms of the porphyrin ring and can alternate between a reduced and oxidized state, enabling the redox and electron transfer reactions of heme. This flexibility makes heme an essential cofactor for many enzymes; famously it enables the binding and transport of oxygen in hemoglobin. Additionally, heme is the functional group in the antioxidant function of peroxidases and catalases, as well as the electron transfer reactions of cytochromes in the mitochondrial respiratory chain [2]. Besides its role as a crucial cofactor, heme is also vital for the regulation of iron homeostasis and serves as a signaling molecule [3-5]. These diverse, essential functions imply that most organisms must acquire heme from their environment or synthesize it *de novo*.

The Apicomplexa phylum comprises obligate, intracellular parasites which are highly evolutionary divergent from model eukaryotes. Members of this phylum have developed innovative strategies for metabolite synthesis and uptake, including for heme, across their complex lifecycles. Many apicomplexan parasites are medically relevant pathogens, including *Toxoplasma gondii*, which infects approximately one third of the human population [6], *Plasmodium spp*. which currently cause close to 500,000 deaths per year [7] and *Cryptosporidium*, one of the main causes of deaths in children under 5 years of age [8]. Several recent studies have probed the importance of heme synthesis in *T. gondii* [9-11] and *Plasmodium spp*. [12-15] and have shed light on its role in the activation of artemisinin (ART) [16-18]. Given the importance of heme in the biology and treatment of these pathogens, we aim to provide an overview over of the recent findings in the field, with a focus on the heme synthesis pathway in apicomplexan parasites.

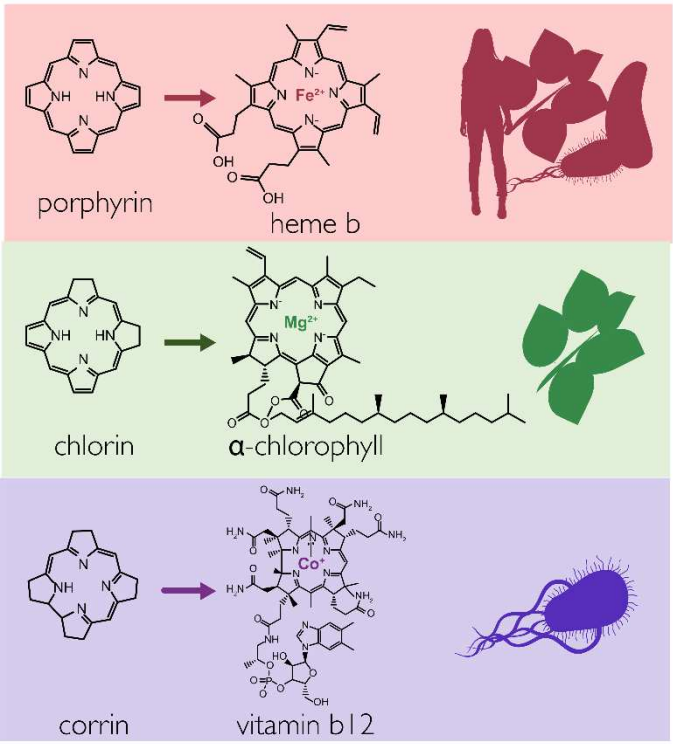

Fig. 1. The structure and origin of porphins and their derivatives. Porphins are a group of molecules consisting of four pyrrole rings, including porphyrins, chlorins and corrins. The former is the precursor of heme, an essential cofactor for nearly all cells and can be synthesized by many different organisms, including bacteria, archaea and eukaryotes (including plants, fungi, animals, protists). While most mammalian cells can produce heme, synthesis largely occurs in developing erythroid cells in the bone marrow and in the liver. Chlorophyll, a derivative of chlorins, is synthesized in the plastids of photosynthetic organism including plants and shares much of its synthesis pathway with that of heme. Corrins are the precursor of cobalamin or vitamin B12, an essential vitamin for humans. Cobalamin is synthesized by some bacteria and archaea, including *Pseudomonas dentrificans* and *Salmonella typhimurium* via an aerobic or anaerobic synthesis pathway.

Heme demand in protozoan parasites

Among the Apicomplexa, the requirement for heme varies drastically between species. These differences likely reflect the various niches the parasites occupy throughout their lifecycles. Apicomplexans frequently infect multiple host species and undergo both sexual and asexual replication. In order to survive in these often dramatically different environments, parasites must have the flexibility to metabolically adapt [19-21]. Underlining this, *T. gondii* encodes 13 cytochromes, as well as a catalase, that depend on heme as a cofactor, indicating a relatively high demand for heme [22, 23]. *Plasmodium falciparum* expresses 8

cytochromes and no heme-dependent catalase [22, 23]. While in contrast, *Cryptosporidium parvum* lacks a conventional mitochondrion and only expresses a single heme-containing enzyme of unknown function, likely indicating a very low requirement for heme in these parasites [22-25]. As proteins of the electron transport chain (ETC) are the largest destination for heme within these organisms [22, 23], changes to the mechanisms of energy generation are expected to have large impacts on the heme requirements for the Apicomplexa.

The coccididan parasite *T. gondii* undergoes asexual replication in its intermediate hosts in two distinct stages: the fast growing tachyzoites which are responsible for severe symptomatic disease in susceptible hosts [26-28], and the slow growing, encysted bradyzoites which persist for the lifetime of their host [29]. Tachyzoites and bradyzoites differ not only in their growth rate, but also in their metabolism [30-32]. The former rely on the mitochondrial respiratory chain for over 90% of their adenosine triphosphate (ATP) production through oxidative phosphorylation (OXPHOS) [33]. In bradyzoites however, OXPHOS is largely inactive and ATP is instead predominantly generated through glycolysis, which does not require heme-containing enzymes [34-36]. In fact, reduced activity of the respiratory chain has been suggested to be one of the factors involved in tachyzoite-to-bradyzoite conversion [37]. Beyond ATP production through OXPHOS, conversion of the saturated fatty acid stearate (C18:0) to the monounsaturated oleate (C18:1) is also heme dependent. This desaturation reaction relies on the stearoyl-coenzyme A (CoA) desaturase, cytochrome b5 and the nicotinamide adenine dinucleotide phosphate (NADPH) cytochrome b5-reductase. Interestingly, all 3 genes are expected to be fitness-conferring based on a genome-wide Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) fitness screen [38], although a recent study suggested dispensability of stearoyl-CoA desaturase in *T. gondii* tachyzoites *in vitro* and in mice [39]. While the fatty acid elongation pathway is presumably essential in tachyzoites [40], *T. gondii* has shown remarkable capacity to compensate for defects in fatty acid synthesis through increased salvage [10, 40]. A recent study revealed that *T. gondii* bradyzoites efficiently salvage oleate and store excess of it in lipid droplets [41], suggesting that stearoyl-CoA desaturase might be dispensable at this stage. Given the reduced replication rate, and reduced activity of the heme-dependent oleate synthesis and OXPHOS, it is likely that bradyzoites have a reduced requirement for heme compared to tachyzoites. *T. gondii* undergoes sexual reproduction inside felids. Due to the very limited accessibility of these life cycle stages, little is known about their metabolism. Demand for heme in sexual stages will depend on how these stages generate energy as well as on the availability of heme in the cat gut environment. Recent, elegant studies suggest that these stages may become more tractable in the future [42, 43], opening the possibility for these questions to be addressed.

The requirement for heme also varies across the life cycle of *Plasmodium spp*. Asexual, blood-stage *Plasmodium* have unlimited access to glucose and were shown to rely mainly on glycolysis to generate ATP, while secreting excess lactate through a formate/nitrite transporter as a partially oxidized end product [44-46]. This dependence on glycolysis for energy production may cause a reduced demand for heme in blood stage parasites. Nevertheless, the mitochondrial ETC, including heme-containing cytochromes, is essential for *Plasmodium* blood stage parasites [47]. The dihydroorotate dehydrogenase (DHODH), an enzyme of the essential pyrimidine biosynthesis pathway, relies on ubiquinone-mediated re-oxidation in the inner mitochondrial membrane [48-50]. Furthermore, chemical inhibition of heme-dependent stearoyl-CoA desaturase arrested the growth of intraerythrocytic *P. falciparum*, indicating that heme-dependent oleate synthesis is likely active and essential in these stages [51]. Optimal efficacy was observed in schizonts which exhibit a high demand for membrane synthesis [51], while the role of stearoyl-CoA desaturase in liver and mosquito stages remains unclear. Blood stage parasites differentiate into gametocytes which are also dependent on glycolysis for energy production, suggesting a minor role for mitochondrial respiration and possibly a similarly reduced requirement for heme [45]. Once inside the mosquito, transcriptomic and proteomic analyses have revealed upregulation of tricarboxylic acid (TCA) cycle enzymes and the ETC, indicating an increased reliance on mitochondrial metabolism, likely accompanied by a higher demand for heme [52].

protein	function	*P. falciparum*		*P. berghei*			*T. gondii*		
		gene	phenotype Zhang (2018)	gene	phenotype Bushell (2017)	phenotype Stanway (2019)	gene	phenotype Sidik (2016)	phenotype Krishnan (2020)
cytochrome b	ETC	mal_mito_3		PBANKA_MIT01900			TGME49_330000		
cytochrome c1	ETC	PF3D7_1462700	non-mutable	PBANKA_1326300	essential		TGME49_246540	-4.36	-6.33
cytochrome c	ETC	PF3D7_1404100	non-mutable	PBANKA_1038000	essential		TGME49_219750	-3.51	
cytochrome c2	unknown	PF3D7_1311700	non-mutable	PBANKA_1410200	dispensible	dispensible	TGME49_229420	0.61	
CoxI	ETC	mal_mito_2		PBANKA_MIT01800			TGME49_255060		
cytochrome b5-1	Δ9-desaturase partner	PF3D7_1232300	non-mutable	PBANKA_1447000			TGME49_276110	-3.01	-5.34
cytochrome b5-2	unknown	PF3D7_0918100	non-mutable	PBANKA_0819100	slow	dispensible	TGME49_313580	-1.51	-1.51
cytochrome b5-3	unknown	PF3D7_1428700	mutable	PBANKA_1016000			TGME49_254090	-0.68	
cytochrome b5-4	fatty acid hydroxylation	absent		absent			TGME49_240770	-0.08	
cytochrome b5-5	sulfite oxidation	absent		absent			TGME49_295720	-1.46	-3.37
cytochrome b5-6	unknown	absent		absent			TGME49_273530	-0.02	
cytochrome b5-7	unknown	absent		absent			TGME49_276990	0.47	
cytochrome P450	unknown	absent		absent			TGME49_315770	0.8	
catalase	antioxidant defense	absent		absent			TGME49_232250	2.08	-0.44

Fig. 2. The fitness-conferring role of heme containing enzymes in *P. falciparum*, *P. berghei* and *T. gondii*.
This figure, adapted from van Dooren *et al*., lists the heme-containing enzymes in *Plasmodium* and *T. gondii*. For each gene, the fitness score from recent genome wide fitness screens is given where available. Gene annotations in red represent those encoded on the mitochondrial genome, for which no fitness screen data is available to date. Based on the available screens, cytochrome c1, cytochrome c and cytochrome b5-1 are likely fitness-conferring or essential in both *Plasmodium* erythrocyte stages and *T. gondii* tachyzoites. Intriguingly, the sole cytochrome P450 in *T. gondii* is dispensable, while its overexpression is associated with enhanced pathogenicity (see Zhang *et al*., 2017, Oncotarget, Functional characterization of a unique cytochrome in *Toxoplasma gondii*).

This was experimentally confirmed by tracking the development of mutant parasites lacking succinate dehydrogenase (a component of complex II) or a subunit of the mitochondrial ATP synthase (complex V), revealing that mitochondrial respiration is essential for the formation of oocysts and sporozoites within the vector [46, 53]. Similarly, in liver stage *Plasmodium yoelii*, enzymes of the TCA cycle and ETC are overexpressed at the transcript and protein levels compared to blood stage parasites, indicating an increased reliance on OXPHOS for energy production [54]. Indeed, a genome-wide screen revealed that components of the respiratory chain, including a subunit of the mitochondrial ATP synthase and the flavoprotein subunit of complex II, are essential for liver stage metabolism [55].

In summary, *Plasmodium* blood stages depend on heme for metabolic processes, including pyrimidine and oleate biosynthesis. However, mosquito and liver stage parasites rely increasingly on mitochondrial metabolism, probably leading to a higher demand for heme compared to blood stage *Plasmodium*, which ironically reside in an extremely heme-rich environment. **Figure 2** highlights the fitness score of different heme-containing proteins in *P. falciparum*, *P. berghei* and *T. gondii* based on the data from recent genome wide CRISPR fitness screens [10, 38, 55-57]. The *Plasmodium* screens cover only the erythrocytic stages [56, 57], with the exception of Stanway *et al*., which also covers other life cycle stages, but only provides data on two heme-containing enzymes in *P. berghei* [55]. The available *T. gondii* screens are limited to assessing the fitness cost in tachyzoites [10, 38]. The heme-containing components of the ETC, cytochrome b and cytochrome c oxidase (complex IV) subunit I (COXI), are among the few proteins encoded in the highly reduced mitochondrial genomes of *Plasmodium spp.* and *T. gondii* [58, 59]. While these mitochondrial genes have not been targeted genetically, several lines of evidence indicate that they are essential: cytochrome b, encoded in the mitochondrial genome, is part of the cytochrome bc1 complex, which is likely essential in *Plasmodium* and *T. gondii*, based on the essentiality of its nuclear encoded subunit, cytochrome c1 [10, 38, 56, 57]. Similarly, interactors of mitochondrially encoded COXI, which together form complex IV, are fitness conferring based on genome fitness screens. Furthermore, various antimalarials, including the first line drug atovaquone, target cytochrome b within the cytochrome bc1 complex [60, 61]. Lastly, the mitochondrial ribosomes, which in *Plasmodium* only translate one other heme-independent protein, the cytochrome c oxidase subunit III (COXIII), are essential [62, 63]. Curiously, the *T. gondii* nuclear genome encodes partial copies of the mitochondrial

cytochrome b and COXI [64], although their functional significance is unknown.

Looking beyond Apicomplexa, the demand for heme also differs drastically between other organisms. Cytochromes of the mitochondrial respiratory chain form one of the largest groups of heme-containing enzymes and thus are a major sink for heme in most cells. The exception to this are anaerobic organisms, such as *Entamoeba*, *Trichomonas* and *Giardia*, which have no or reduced respiratory chain activity, likely accompanied by a reduced requirement for heme [22]. This is exemplified by *Entamoeba histolytica* which lacks a conventional mitochondrion and thus expresses no cytochromes or other heme-containing proteins [22, 65, 66]. However, proteins involved in trafficking iron and heme have been identified in *E. histolytica* and it has been proposed that the parasite secretes heme-binding proteins as a mechanism for iron acquisition. More surprisingly, a homologue of ferrochelatase (FECH), the enzyme catalyzing the final step in the heme synthesis pathway, was identified [67, 68]. However, it remains unclear if the putative FECH of *E. histolytica* is functional, and whether these parasites have any requirement for heme, besides serving as an iron source. Another anaerobic parasite, *Giardia intestinalis*, expresses five heme proteins and incorporates heme into a soluble cytosolic form of cytochrome b5 of unknown function [69, 70]. While most organisms require heme to some degree, the kinetoplastid plant parasite *Phytomonas serpens* is highly atypical in that it is able to survive and grow entirely devoid of heme [71]. *P. serpens* does not require heme for its ETC and lacks many of the proteins known to contain heme. While its synthesis of ergosterol relies on the heme-dependent lanosterol 14α-demethylase (CYP51), *P. serpens* can survive by incorporating the ergosterol precursor lanosterol into its membranes, making it the only known aerobic eukaryote able to grow in the absence of heme [71].

Availability and uptake of exogeneous heme in Apicomplexa

The availability of exogeneous heme throughout the development of Apicomplexa also varies. *T. gondii* tachyzoites can replicate within most cell types, however labile, non-protein bound heme levels are low in the host cytosol (~25 nM, [72]) and the vast majority of heme is sequestered by chaperones or incorporated into proteins and may be difficult to access. Bradyzoites face the same challenge within neurons and muscle cells [73], however the surrounding cyst wall has been shown to be largely permeable for compounds <10 kDa and fully permeable for compounds <1 kDa [74], suggesting that any free heme, with a molecular weight of 615 Da, could freely pass into the vacuole. Similarly, heme is likely abundantly present for salvage by schizonts and gametes in the cat intestine, where it is taken up into epithelial cells through the heme carrier protein 1 (HCP1) [75]. Free environmental oocysts, however, likely have no access to exogeneous heme and must rely on *de novo* synthesis or significantly reduced demand during this quiescent life cycle stage.

Particularly striking are the differences in heme availability during the life cycle of *Plasmodium spp*. Throughout the asexual development in erythrocytes, *Plasmodium* relies on the ingestion and proteolytic digestion of hemoglobin to fulfill its amino acid needs and thereby releases large amounts of heme [76, 77]. During replication, parasites take up such large amounts of hemoglobin that heme concentration reaches toxic levels within the food vacuole, and must be detoxified through deposition in a crystalline structure, termed hemozoin [78]. The mechanism of hemozoin formation has been well studied in *Plasmodium,* due to its importance as a target for antimalarial drugs, and will not be discussed in detail here [79-84]. The vast majority of heme released from hemoglobin digestion is confined within the food vacuole as hemozoin [85]. However, parasites deficient of heme synthesis grow and develop unimpaired in red blood cells (RBCs), highlighting that *Plasmodium* utilizes host derived heme for cellular functions [12-15]. In contrast to the heme-rich milieu in the blood, heme availability may be limited in the mosquito gut and in hepatocytes. In the mosquito gut, heme is expected to gradually decline following the blood meal as it is excreted or absorbed into tissues and used for egg development [86], while heme levels in the salivary gland are unknown. *Plasmodium spp*. must therefore be able to carefully meet its varying heme demand, while facing fluctuating environmental conditions ranging from heme limiting to toxic levels.

Apicomplexa and other parasites have evolved expert strategies to exploit the metabolism of their host, salvaging various nutrients including sugars, amino acids, nucleobases, lipids, fatty acids,

vitamins and co-factors [21, 30, 87, 88]. Hence, many parasites have lost the capacity to synthesize certain metabolites and have developed scavenging mechanisms, allowing the pathogens to save energy by reducing their genome and metabolic capabilities [89]. The heme auxotroph trypanosomatid *Leishmania amazonensis* expresses a gene with 45% similarity to a *C. elegans* heme transporter [90, 91]. Remarkably, expression of this divergent *Leishmania* Heme Response-1 (LHR-1) protein in a yeast strain deficient in heme synthesis restored its growth [90]. LHR-1 is essential for *Leishmania* promastigotes and virulence in mice [90, 92] and a related transporter was later identified in *Trypanosoma brucei* [93]. More recently, another plasma membrane transporter, the feline leukemia virus subgroup C receptor-related protein (LmFLVCRb), was shown to be implicated in heme transport in *Leishmania major* and is essential for virulence [94]. Trypanosomatids, which possess more heme containing proteins than *T. gondii* or *Plasmodium spp*. [22], but lack functional heme synthesis pathways and rely on its salvage, can nonetheless survive and propagate in environments in which heme-levels fluctuate and may reach critically low levels, such as the digestive tract of insect vectors [90, 95, 96] (reviewed in [97]). Instead, *Plasmodium* is unable to acquire sufficient heme in the similar environment of the mosquito's digestive tract [12, 13]. This suggests that *Plasmodium spp*. lack specific heme uptake mechanisms which allow the efficient uptake of heme, or that *Plasmodium* has a particularly high requirement for heme during oocyst maturation which cannot be met by uptake. Similarly, growth impaired *T. gondii*, in which a heme synthesis enzyme was deleted, could not be rescued by provision of exogenous heme [9, 10], indicating that *T. gondii* is unable to efficiently salvage heme. Indeed, no heme transporters have been identified in apicomplexans to date but specific transporters or other unidentified uptake mechanisms must exist in various species such as the *Gregarina* and *Theileria* which lack enzymes of the heme biosynthesis pathway but encode heme-containing enzymes.

Heme synthesis enzymes in Apicomplexa: conservation, localization and evolutionary origin

The enzymes encoding the heme synthesis pathway have been lost multiple times in the evolution of parasitic protozoans, including from entire families, such as *Trypanosoma*, *Giardia* and *Trichomonas,* but have typically been maintained in free-living protists such as *Chromera velia* [22]. **Figure 3** summarizes the conservation of heme synthesis enzymes across selected parasitic protozoa. Within the Apicomplexa, the ability to synthesize heme has also been lost several times; *Piroplasmida* including *Babesia* and *Theileria,* as well as *Cryptosporidia* and *Gregarina* are all missing some or all of the enzymes of the heme biosynthetic pathway and are hence unable to synthesize heme [22, 23, 98] (**Figure 3**). From examining available genomes, it is anticipated that *Cryptosporidia* have a low requirement for heme due to the lack of cytochromes. *Babesia* and *Theileria* also lack components of this pathway but encode cytochromes and a heme oxygenase-like protein [23], which was shown in *Plasmodium* to bind heme and its precursor protoporphyrin IX (PPIX), but does not catalyze heme degradation, suggesting an alternative function [99]. Thus, *Babesia* and *Theileria* appear to have some requirement for heme and must scavenge it from their hosts through a currently unknown mechanism [23]. In contrast, *T. gondii* and *Plasmodium spp*. express all of the enzymes necessary to synthesize heme *de novo* [23, 100] (**Figure 3**). Given the loss of many metabolic pathways in parasitic protozoa, the ability to synthesize heme was probably only retained in parasites where salvage of heme or its precursors was not possible throughout the complete life cycle.

Heme can be synthesized *de novo* from simple amino acids and organic acids in 8 or 9 enzymatic reactions [101]. While animals and fungi synthesize heme from succinyl-CoA and glycine (C4-pathway) in the mitochondria and cytosol, plants generate heme from glutamyl-tRNA (C5-pathway) in the plastid and mitochondria. Both, the C4- and C5-pathway occur in bacteria, although the C5-pathway is more common [102]. In animals, the initial step of heme biosynthesis is catalyzed by 5-aminolevulinate synthase (ALAS) and takes place in mitochondria, generating 5-aminolevulinate (ALA) from glycine and succinyl-CoA [103].

ALA is transported out of the mitochondria and the subsequent four steps occur in the cytosol, producing coproporphyrinogen III (CPPgenIII). CPPgenIII is trafficked back across the outer mitochondrial membrane and is converted to PPIX which is transported across the inner mitochondrial membrane and converted to heme, through the incorporation of iron by FECH [103].

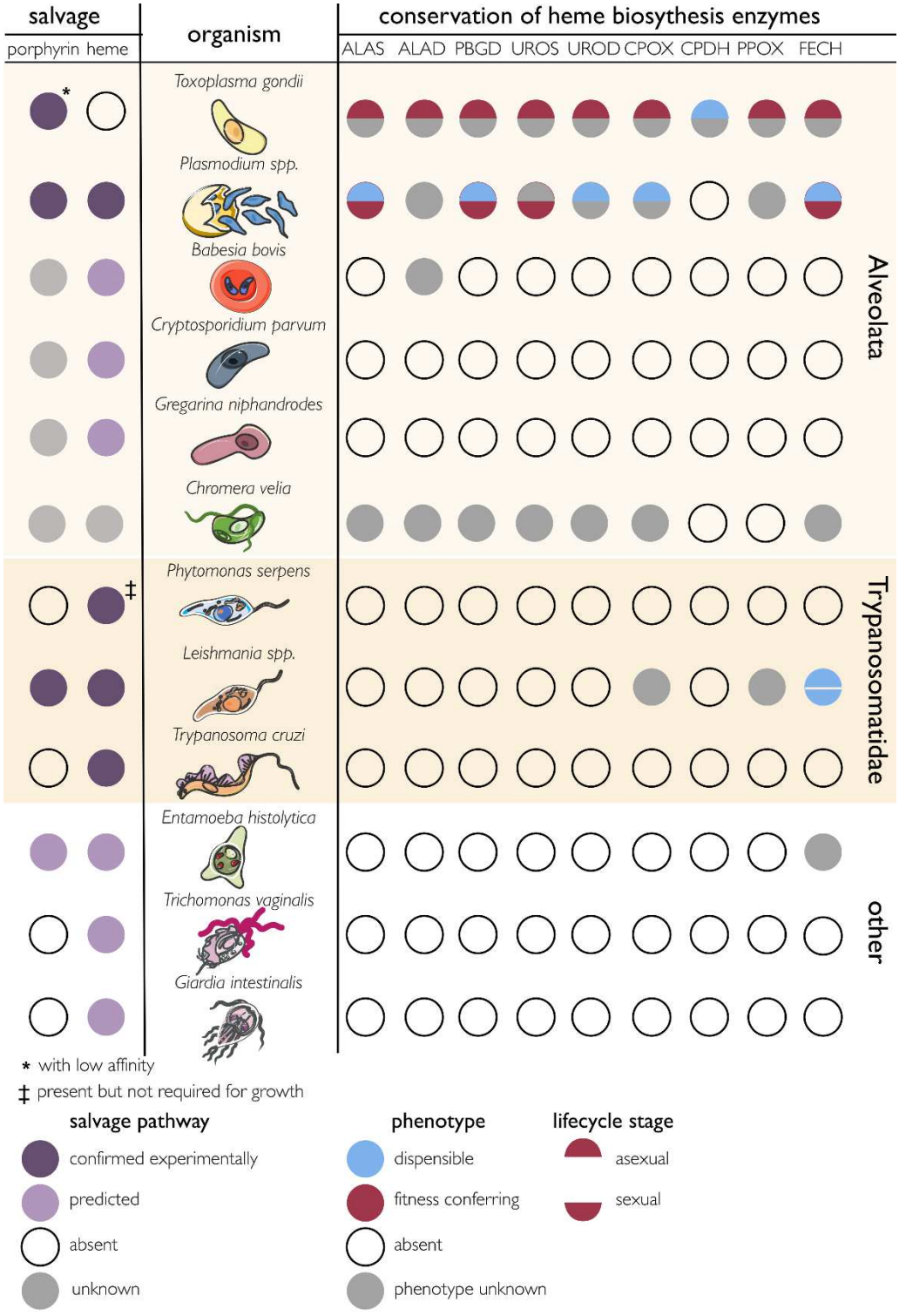

Fig. 3. Conservation and fitness-conferring roles of heme biosynthesis enzymes across selected protists. Enzymes required for heme synthesis have been lost independently in a number of parasitic protists. Where studied, the importance for heme synthesis across the sexual (lower half circle) and asexual (upper half circle) life cycle phases of these parasites is indicated (references given in the text). In addition to heme synthesis, some parasites are able or predicted to scavenge heme or its porphyrin precursor from their environment, although in most cases the transporters have not been identified or characterized. Abbreviations*: ALAS*, 5-aminolevulinate synthase; *ALAD,* 5-aminolevulinate dehydratase; *PBGD*, porphobilinogen deaminase; *UROS*, uroporphyrinogen III synthase; *UROD*, uroporphyrinogen decarboxylase; *CPOX*, coproporphyrinogen III oxidase; *CPDH*, (oxygen independent) coproporphyrinogen III dehydrogenase; *PPOX*, protoporphyrinogen oxidase; *FECH*, ferrochelatase. Cartoons of parasites are adapted from Servier Medical ART available under smart.servier.com

While most enzymes in this pathway originated from the eukaryotic nucleus, ALAS and the uropophyrinogen synthase (UROS) have been proposed to be of α-proteobacterial origin [100, 104]. In contrast to the mammalian pathway, heme synthesis in plants occurs in the plastid. However, the final two enzymes dually localize to the plastid and to the mitochondria, thus, heme can be synthesized in the plastid, or in the mitochondria following import of PPIX. PPIX is also an intermediate of chlorophyll, which is generated exclusively in plastids. Hence, PPIX is either processed by the FECH to generate heme or by the magnesium chelatase, committing to chlorophyll synthesis. The flux through these two pathways is regulated at multiple levels to balance heme and chlorophyll abundance [105]. Unsurprisingly, most enzymes in the heme synthesis pathway of plants are of cyanobacterial origin, however, the porphobilinogen deaminase (PBGD) has been described to be of α-proteobacterial origin and the coproporphyrinogen oxidase (CPOX) may be derived from the primary host nucleus [100, 104]. Some heme synthesis enzymes have been proposed to interact with each other, forming supramolecular protein complexes which facilitate the shuttling of intermediates and increase the efficiency of the pathway [106].

Within the apicomplexans, phylogenetic analyses revealed a hybrid heme synthesis pathway, reflecting the unique evolutionary history of these parasites [22, 100, 107-109]. Generation of ALA from succinyl-CoA and glycine is catalyzed by ALAS in the mitochondrion, as in animals and yeast. In *T. gondii*, the succinyl-CoA is expected to be derived from the TCA cycle, as inhibiting the pathway resulted in a decrease in heme production [16], while glycine can be derived from serine in the cytoplasm or directly salvaged from the host and transported to the mitochondria [110]. The subsequent four steps occur in the apicoplast, a relict plastid organelle derived from secondary endosymbiosis of a red alga [108, 111, 112]. CPPgenIII is then transported to the cytosol where CPOX, which catalyzes the conversion of CPPgenIII to PPgenIX, is localized [113]. CPOX is cytosolic in fungi and yeast, however, it localizes to the mitochondria in animals [103]. Curiously, coccidians and chromerids express a putative oxygen-independent bacterial-type coproporphyrinogen III dehydrogenase (CPDH) which is also predicted to catalyze the conversion of CPPgenIII to PPgenIX, via a radical s-adeonsyl-methionine (SAM) mechanism [88, 101, 102, 110]. The final two enzymes of the pathway, PPOX and FECH, localize to the mitochondrion in *Plasmodium spp*. and *T. gondii* [114, 115]. **Figure 4** highlights the structures of heme intermediates, the enzymes and their respective localization in animals, plants and apicomplexans.

The initial apicomplexan heme synthesis enzyme, ALAS, is, as in animals and yeast, of α-proteobacterial origin. The four subsequent apicoplast-localized enzymes have been proposed to be of varying origin but are at least partially derived from cyanobacterial origin [22, 100]. Curiously, although the localization of PPOX resembles that in animals, it has been proposed to be of cyanobacterial origin [22, 100, 107]. The FECH, in contrast, is of proteobacterial origin [116]. This peculiar hybrid pathway including enzymes from various ancestors dispersed over three cellular compartments is so far unique to apicomplexans and closely related organisms [98, 117, 118]. It has been speculated that the mitochondrial/cytosolic- and the plastid-localized heme synthesis pathway probably co-existed following the endosymbiotic acquisition of the plastid [23]. Over time, transporters enabling the redistribution of intermediates between organelles were acquired or developed from other existing transporters and redundant enzymes were lost.

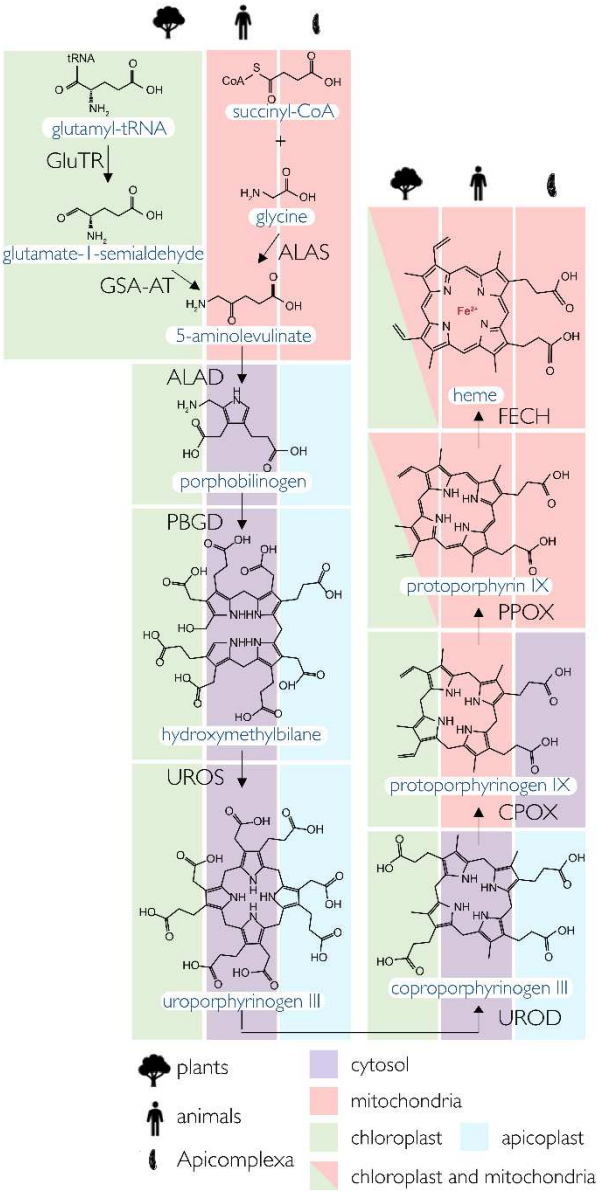

Fig. 4. Heme synthesis in plants, animals and apicomplexans.
Plants, animals and humans synthesize heme using a similar set of enzymes. In apicomplexans, as well as in animals, 5-aminolevulinate (ALA) is generated from glycine and succinyl-CoA, catalyzed by the 5-aminolevulinate synthase (ALAS). In contrast, plants generate ALA from glutamyl-tRNA (GluT) using GluT reductase (GluTR) and glutamate 1-semialdehyde aminotransferase (GSA-AT). The subsequent enzyme reactions are identical, but the localization of the enzymes differ between organisms. Plants synthesize heme in the chloroplast, while the last two enzymes dually localize to the chloroplast and mitochondria. In animals, heme synthesis occurs in the mitochondria and cytosol. Uniquely, heme synthesis enzymes are dispersed across three cellular compartments in the apicomplexans: the mitochondrion, apicoplast and cytosol. Abbreviations*: ALAS*, 5-aminolevulinate synthase; *ALAD,* 5-aminolevulinate dehydratase; *PBGD*, porphobilinogen deaminase; *UROS*, uroporphyrinogen III synthase; *UROD*, uroporphyrinogen decarboxylase; *CPOX*, coproporphyrinogen III oxidase; *PPOX*, protoporphyrinogen oxidase; *FECH*, ferrochelatase.

The role of heme synthesis in *Plasmodium*

Heme synthesis in *Plasmodium* spp. follows the enzymatic reactions outlined above. However, the importance of *de novo* synthesis changes throughout *Plasmodium's* complex lifecycle where it faces vastly different environments in terms of heme abundance and must adapt its metabolism accordingly [45, 55, 57]. Early studies proposed that, despite the uptake of large amounts of host derived heme, *Plasmodium de novo* heme synthesis is active in RBCs and provides a promising drug target [119]. However, later on, many of the enzymes of the pathway have been directly knocked-out, including ALAS, PBGD, CPOX, uroporphyrinogen decarboxylase (UROD) and FECH with no defects in intraerythrocytic parasite replication or gametocyte conversion [12-15, 120, 121]. Two studies used ^{13}C-labelled ALA and mass spectrometry to specifically probe *de novo* heme biosynthesis in *Plasmodium* [12, 15]. While both studies showed that labelled atoms derived from ^{13}C-ALA are incorporated into parasite derived heme in intraerythrocytic wildtype parasites, Sigala *et al*. showed that this activity is dependent on residual heme synthesis enzymes in the RBC upon non-physiological provision of exogeneous ALA, as neither saponin-lysed asexual parasites nor gametocytes utilized ^{13}C-ALA for heme synthesis [15].

In contrast to the high levels of heme present in erythrocytes, parasites likely have limited access to exogeneous heme in the mosquitos. Deletion of both ALAS and FECH resulted in a defect in male gamete formation, reduced oocyst numbers and a lack of sporozoites in the salivary gland, indicating a block in transmission of both *P. berghei* and *P. falciparum* [12, 13]. Two subsequent studies confirmed a defect in oocyst maturation in *P. berghei* upon obstruction of either mitochondrial ALAS and FECH or the apicoplast resident heme synthesis enzymes, PBGD and UROD [14, 121]. While Rizopoulos *et al*. reported that liver stage development, although retarded, could be completed by parasites lacking ALAS, Nagaraj *et al*. proposed that heme synthesis is absolutely essential for liver stage development [13, 14]. Both studies highlighted that supplementation with exogeneous ALA in mice or mosquitos could rescue the phenotypes of parasites lacking ALAS [13, 14], seemingly consistent with the observation that *Plasmodium spp*. can salvage exogeneous ALA for heme synthesis [12]. Rathnapala *et al*. later used a novel approach to

demonstrate that FECH deficient sporozoites, distinguishable by fluorescence from FECH expressing sporozoites, were unable to complete liver stage development [121]. In summary, heme synthesis is inactive and dispensable for intraerythrocytic stages but becomes essential during development in the mosquito vector and in the liver stages. The fitness-conferring roles of heme synthesis enzymes are summarized in **Figure 3**.

The role of heme synthesis in *Toxoplasma gondii*

In *T. gondii* the first demonstration of the importance of heme production came from chemical inhibition of ALAD using succinylacetone which led to a block in parasite replication [122]. The relevance of heme synthesis was further supported by a genome-wide CRISPR fitness screen, where all heme synthesis genes were assigned a fitness score of less than -2, indicating a fitness-conferring role in *in vitro* growth [38]. Recently, several enzymes of the pathway have been examined in detail [9-11], confirming that several heme synthesis enzymes are highly fitness-conferring in *T. gondii* (ALAS, ALAD, PPOX, CPOX), although interestingly, so far only FECH was found to be essential in tachyzoites under standard culture conditions [9-11] (**Figure 3**).

Deletion of the first enzyme in the *T. gondii* heme pathway, ALAS, caused a significant reduction in parasite replication [9, 10]. Krishnan *et al*., reported full restoration of heme levels and rescue of parasite growth following supplementation with ALA, while Bergmann *et al*., only reported partial restoration of intracellular heme levels and a relatively poor restoration of fitness. This may reflect variations in techniques or possibly the level of adaptation which occurred during the generation and selection of these mutant strains. The subsequent enzymes operating in the apicoplast: ALAD, PBGD and UROS have not been disrupted individually to date and it remains unclear whether these are essential. A fitness-conferring role as for the other enzymes in the pathway is anticipated given the data from a genome-wide fitness screen [38]. *T. gondii* ALAD (also known as porphobilinogen synthase, PBGS) has been characterized biochemically and structurally [122, 123]. These studies revealed that an extended C-terminus of the *T. gondii* enzyme is responsible for an unusual octamer formation, which does not require metal ions for its stability [122, 123]. The functional significance of these alterations remains to be determined and may provide a possible novel drug target. Furthermore, pharmaceutical inhibition of ALAD using succinylacetone indicated essentiality of the enzyme and the heme synthesis pathway [122]. However, it remains unclear how inhibition of the host cell ALAD affected parasite growth in these experiments and off-target effects cannot be excluded and have been suggested [120].

The enzyme catalyzing the last step of heme synthesis in the apicoplast, UROD, has been disrupted genetically [11]. Down-regulation of UROD is associated with a reduction in free heme levels, as well as a dramatic reduction in parasite growth and fitness. In this study, heme intermediate levels were not quantified, and it is unknown if these parasites could be rescued through addition of ALA. The CPPgenIII synthesized by UROD is exported across the four membranes of the apicoplast through an unidentified transporter or transporters to the cytosol. Once there, CPOX converts CPPgenIII into PPgenIX, the only reaction which occurs in the cytosol [9, 113]. In addition to CPOX, *T. gondii* encodes another oxygen-independent enzyme (CPDH) potentially catalyzing the same reaction. CPDH is only found in the coccidians but is predicted to be dispensable in tachyzoites [38] which was recently confirmed experimentally [10]. Parasites lacking CPDH had no fitness defect, while those lacking either CPOX or both enzymes showed equally reduced intracellular heme levels and a similar dramatic reduction in growth and fitness, excluding a redundant function in tachyzoites. CPDH expression is upregulated in bradyzoites during chronic infection, when parasites might have to cope with reduced oxygen levels in large cysts. However, Krishnan *et al*. identified no defect in cyst size or number in parasites lacking CPDH [10]. Hence, the function of the conserved CPDH in the coccidian subgroup of Apicomplexa remains unclear. It may have a role in the sexual life cycle stages, where it is upregulated, and future studies may be able to investigate this intriguing question [124].

CPOX generates PPgenIX in the cytosol which is then transported into the mitochondrion and converted to PPIX by PPOX. Deletion of PPOX also resulted in reduced heme levels and a loss of parasite fitness [9]. However, compared to other knockouts (KOs) in the pathway, deletion of PPOX has a milder phenotype [9], in a similar manner to

Saccharomyces cerevisiae where PPOX is the only non-essential heme synthesis enzyme [125]. It is possible that oxidation of PPgenIX to PPIX may occur inefficiently without enzymatic activity of PPOX, which would explain why loss of PPOX is tolerated at a significant but less severe fitness cost compared to other enzymes in the same pathway [9]. Spontaneous oxidation of PPgenIX has been previously suggested [126] but it remains unclear to what extent it occurs *in vivo*. Interestingly, in apicomplexans, PPgenIX is generated in the cytosol, while in animals it is likely produced in the mitochondria. In apicomplexans, the cytosolic milieu may thus facilitate the spontaneous oxidation due to its higher oxygen concentration and lower reducing redox potential compared to mitochondria [127-129]. For PPIX, derived from spontaneous oxidation of PPgenIX, to contribute to heme synthesis, it must be transported into the mitochondrion, which presupposes that the mitochondrial transporter does not discriminate between PPIX and PPgenIX. The existence and importance of spontaneous oxidation in the cytosol remains to be determined and may rely on the identification of the putative transporter. Despite their fitness-conferring role, several targeted genes upstream of FECH (ALAS, CPOX, PPOX) could be deleted successfully. While their loss severely affected growth, viable parasites could be maintained in culture [9, 10]. The final step in the construction of heme is the incorporation of iron (Fe^{2+}) into the porphyrin ring by FECH. Strikingly, despite multiple attempts, a KO of FECH could not be generated [9, 10] and parasites conditionally depleted of FECH failed to propagate in culture [10]. Taken together, the results of these recent independent studies showed that enzymes of the *T. gondii* heme synthesis pathway are highly fitness-conferring and their loss is associated with a dramatic reduction, but not a complete loss of heme in cultured tachyzoites. Thus far, only the final enzyme, FECH, was identified to be essential in cultured tachyzoites. While the rich culture medium *in vitro* may allow survival of parasites lacking ALAS, CPOX or PPOX, all mutant strains were found to be avirulent in mice, highlighting that heme synthesis is essential for virulence *in vivo* [9].

Beyond tachyzoites, little is known about the role of heme synthesis in other life-cycle stages of *T. gondii* and can only be inferred from RNA sequencing data [124]. Based on RNA expression data, it appears that heme synthesis may be least active in bradyzoites, as all enzymes of the pathway are repressed, except for the putative oxygen independent CPDH [124]. Perhaps most relevant is the expression level of ALAS, a rate-limiting enzyme in other organisms [130]. ALAS expression is reduced in all life cycle stages compared to tachyzoites, possibly indicating reduced activity of the heme synthesis pathway [124]. In yeast, ALAD and PBGD have also been proposed to have rate-limiting functions [131]. The former is down-regulated in all life-cycle stages compared to tachyzoites. PBGD, on the other hand, is only down-regulated dramatically in bradyzoites [124]. Slow growing bradyzoites likely have generally reduced metabolic demands compared to the rapidly replicating tachyzoites. Additionally, as discussed above, bradyzoites are likely less dependent on ATP generation through OXPHOS, which may further contribute to reduced heme requirements and thus reduced synthesis [34, 35]. However, it remains unclear if bradyzoites truly have reduced heme requirements, and if these are met through depressed *de novo* synthesis or if bradyzoites increasingly rely on scavenging.

Regulation of heme synthesis in Apicomplexa

In model organisms, heme synthesis is highly regulated at multiple levels based on the state of the cell and availability of precursors [103, 131, 132], e.g. ALAS activity is inhibited by binding to heme, allowing negative regulation of heme production [133]. In extracellular parasites, addition of ALA leads to an excess of PPIX but no or only a modest increase in heme, indicating that ALA stimulates PPIX synthesis in *T. gondii*, while FECH is rate limiting for its conversion to heme [16]. The same effect of ALA supplementation is well known for mammalian cells and has been exploited for photodynamic therapy of cancer cells which become light sensitive upon PPIX accumulation [134-136]. In Apicomplexa, ALAS likely has a rate-limiting function [137], consistent with the presence of a cysteine-proline dipeptide (CP-motif) in the N-termini of *T. gondii* and *P. falciparum* ALAS [23, 100]. These CP-motives enable the transient binding of heme and are the most prominent heme-regulatory motif (HRM) [138]. While binding of heme to these HRMs typically modifies the stability and/or catalytic activity, Varadharajan *et al*., provide evidence that excess heme disables the translocation of ALAS into the mitochondrion of *P. falciparum*, thus regulating its activity [137]. Another study proposed that, not

only ALAS, but also ALAD (PBGS) is inhibited by heme in a concentration-dependent manner in *Plasmodium knowlesi* [139]. The absence of a CP-motif in the N-terminus of the *P. knowlesi* enzyme suggests a different mode of regulation. In contrast to the *Plasmodium* and human ALAD, the *T. gondii* ALAD has a ~200 amino acid extended N-terminus which also includes a CP-motif, possibly involved in regulating its activity. Using experimental or computational approaches to identify heme-regulatory motives and determine unknown protein-heme interactions could be one way of identifying regulatory mechanisms of heme synthesis in Apicomplexa [138].

Other elegant studies have helped to reveal complex putative regulatory mechanisms: it was shown that ablation of the function of the protease DegP2 leads to a reduction in heme levels in both *T. gondii* and *P. falciparum* [16]. DegP2 is outside of the canonical heme synthesis pathway and its targets within the cell remain unknown [16, 17]. However, in both yeast and mammalian cells, the protease ClpX partially unfolds ALAS and thereby aids in the incorporation of its cofactor, pyridoxal 5-phosphate, enabling its activity [140]. A related regulatory role could be carried out by DegP2 in the Apicomplexa. If, or how DegP2 senses heme levels is unclear, but it does include a CP-motif in its N-terminus. Further research into potential regulation of this pathway in the parasites would be valuable in determining the diversity of regulatory approaches utilized by eukaryotes.

Metabolic adaptations in heme-depleted *Toxoplasma gondii* tachyzoites

Heme acts a key prosthetic group within several enzymes. In *T. gondii*, 14 proteins are predicted to contain heme (**Figure 2**), 4 of which are required for the ETC [22, 23]. It is likely that depletion of heme-bound cytochromes in the ETC is largely responsible for the fitness defect seen upon depletion of heme. Tachyzoites acquire the majority of their ATP through OXPHOS [33] and indeed, depletion of heme by down-regulation of UROD or straight KO of CPOX leads to a dramatic decrease in mitochondrial respiration [10, 11] and a drop in ATP levels [11]. Upon deletion of CPOX, parasites respond to the drop in mitochondrial respiration by markedly increasing the rate of glycolysis, although it is likely that this cannot fully compensate for the hampered ATP production in these parasites [10]. Interestingly, upregulation of glycolysis was not seen in parasites conditionally depleted of UROD. However, it is possible that over the relatively long process of generating a ΔCPOX parasite line, adaptation occurred, leading to selection of parasites with markedly increased rates of glycolysis. In contrast, the sudden loss of enzyme function upon conditional depletion was likely too rapid for the parasite to compensate in this way. This potential adaptation highlights the possible metabolic flexibility of *T. gondii* and underlines the caution required in interpreting metabolic phenotypes after genetic perturbations. These findings have implications for potential drug treatment. Parasites appear unable to adapt to a sudden drop in heme synthesis but may be able to adapt over longer periods to move away from OXPHOS and instead rely on glycolysis for energy generation. Regardless of the partial adaptation of these parasites, the dramatic fitness defect of heme-deficient parasites *in vitro* and particularly *in vivo* suggests that heme synthesis enzymes may be promising drug targets to treat toxoplasmosis [9-11].

Whether other metabolic adaptations are necessary to survive under a markedly reduced heme level remains unclear. As discussed above, the desaturation of stearate is also dependent on heme but likely dispensable for *T. gondii* [39]. Oleate is one of the most abundant fatty acids in mammalian tissues and cells [141]. Thus, it may be salvaged by *T. gondii* and can be further elongated to generate the *T. gondii*-specific, long chain mono-unsaturated fatty acids which are essential [40]. However, Ramakrishnan *et al*. have proposed that elongases preferentially act on *de novo* synthesized oleate [40] and the role of lipid metabolism in the growth phenotypes in the absence of heme has not yet been examined.

Scavenging and intracellular trafficking of heme intermediates in Apicomplexa

As obligate intracellular parasites, apicomplexans have the option to scavenge metabolites from their hosts. The results above strongly suggest that *T. gondii* is unable to take up exogenous heme, however metabolic intermediates in the heme synthesis pathway do appear to be taken up and utilized. Treatment of extracellular *T. gondii* with ALA leads to an increase in PPIX levels [16], demonstrating that the parasites can take up and utilize this first committed metabolite. Furthermore,

ALA supplementation can rescue heme level and growth in mutant parasites lacking CPOX, although CPOX is downstream of ALA, suggesting that parasites can take up the excess PPIX generated by the host cells. This points towards a potential PPIX salvage pathway in the parasite, which is then converted to heme by *T. gondii* FECH [10]. Crucially, this salvage mechanism appears inefficient and parasites are severely impaired under physiological conditions. In order to be salvaged, intermediates must make their way to the correct destination within the cell. The membrane of the parasitophorous vacuole (PV) has been described as a molecular sieve, through which small metabolites like ALA and PPIX (<3 kDa) [142] can pass through passive diffusion. The parasite plasma membrane and mitochondrial membrane provide more substantial barriers. As for *T. gondii*, uptake of host derived PPIX and its conversion to heme through the parasite FECH was also demonstrated in intraerythrocytic *P. falciparum*. While mature reticulocytes lack mitochondria and thus lack ALAS as well as FECH activity, Sigala *et al*. elegantly demonstrated that exogeneous ALA was incorporated into parasite derived heme, including in parasites which lack CPOX or PBGD. These observations revealed that PPIX synthesis surprisingly occurs in mature RBCs provided with exogeneous ALA and that host derived PPIX is taken up by *P. falciparum* and converted to heme inside the parasite's mitochondrion [15]. The findings by Krishnan *et al*. and Sigala *et al*. strongly support the existence of PPIX transporters at the plasma membrane as well as in the inner mitochondrial membrane in *T. gondii* and *P. falciparum*, which have not been identified to date.

Uptake of PPIX through an unidentified mechanism has also been described in *Leishmania major*, where salvaged PPIX is converted to heme through the parasite's FECH [95]. However, the role of this salvage pathway *in vivo* is unclear, as the *Leishmania* FECH is not required for development in the sandfly or for the formation of skin lesions in mice [95].

Intriguingly, while ALA supplementation has been widely used in studies probing heme synthesis in *T. gondii* and *Plasmodium* and was shown to rescue the phenotype of parasites lacking ALAS [9, 10, 12-15], it remains unclear if intracellular parasites actually take up exogeneous ALA or largely acquire late intermediates benefitting from the ALA induced increase in host PPIX [10, 15]. The latter is strongly supported by the observations by Krishnan *et al*. and Sigala *et al*., who showed that *T. gondii* lacking the enzyme CPOX downstream of ALAS can also be rescued by ALA supplementation and similarly that ALA-induced heme synthesis was occurring in *P. falciparum* which lack PBGD or CPOX [10, 15]. However, ALA is likely salvaged given that it is a small metabolite which easily passes the PV and may be transported across the plasma membrane using a specific ALA transporter or unspecific small polar metabolite transporters. Indeed, Harding *et al*. demonstrated that extracellular *T. gondii* tachyzoites take up exogeneous ALA resulting in increased PPIX levels [16], so that both mechanisms, salvage of ALA as well as PPIX, are likely utilized by parasites with a defect in heme synthesis.

Besides the acquisition of heme intermediates across the plasma membrane, apicomplexans must rely on several transporters to traffic heme synthesis intermediates between organelles. Due to the patchwork nature of the heme biosynthesis pathway, multiple transporters are predicted to be required which are not present in other eukaryotes, including an apicoplast ALA-importer and an apicoplast CPPgenIII exporter (**Figure 5**). Other transporters are likely functionally homologous to mammalian transporters. In mammalian cells, the transporter ABCB6 has been proposed to be a porphyrin transporter in the outer mitochondrial membrane, possibly transporting CPPgenIII into the intermembrane space of mitochondria [143-145]. However, the role and localization of ABCB6 have been a matter of debate [146, 147] and it has been proposed that small metabolites cross the outer mitochondrial membrane through relatively unspecific porin channels [106]. Another mammalian mitochondrial ATP-binding cassette transporter, ABCB7, likely functions in iron-sulfur cluster synthesis in the inner mitochondrial membrane [148] and has been proposed to interact with ABCB10 and FECH to form a complex which is required for heme synthesis [149]. We have identified a putative transporter in *P. berghei* (PBANKA_1364800) and *T. gondii* (TGME49_269000), which shares ~40% identity with ABCB7 and ABCB6. Both apicomplexan genes are reported to be essential based on genome-wide disruption approaches [38, 56] and the *T. gondii* protein TGME49_269000 localizes to the mitochondrial membrane according to the spatial proteomics method hyperplexed Localization of

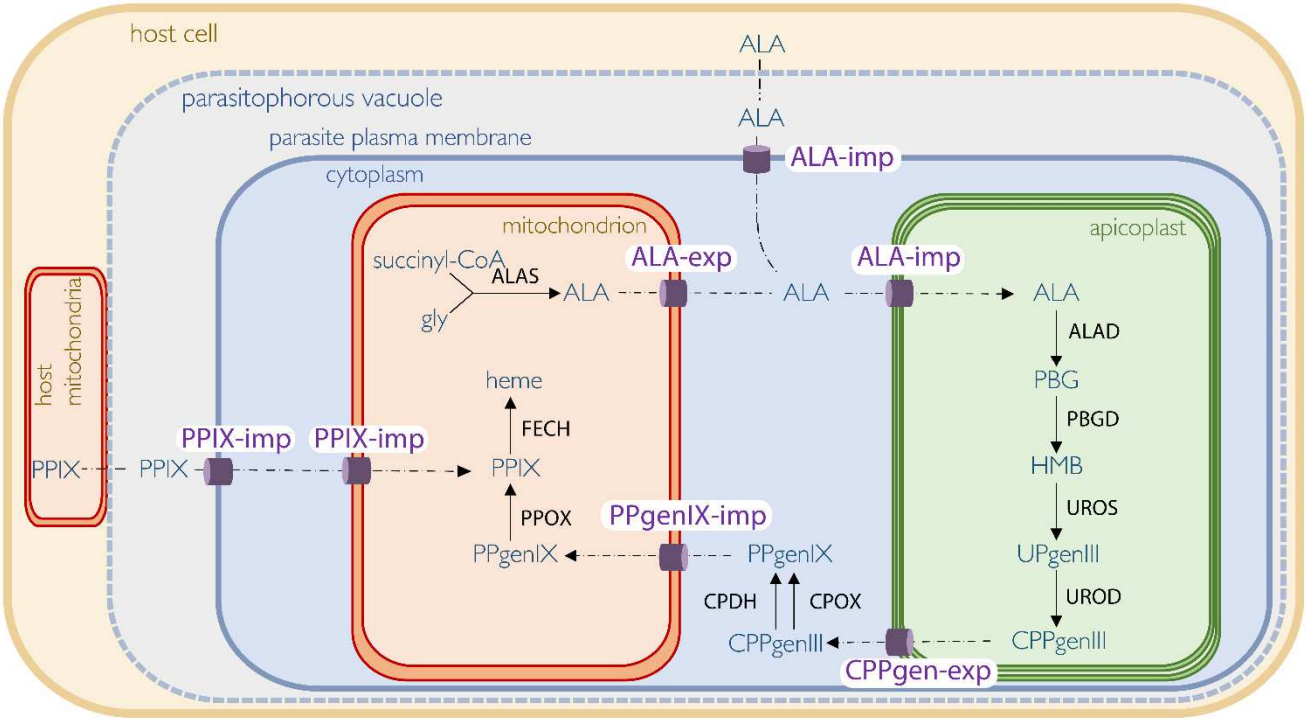

Fig. 5. Several unidentified transporters must enable uptake and intracellular trafficking of heme intermediates in Apicomplexa.
T. gondii **and *Plasmodium*** can take up protoporphyrin IX (PPIX) and 5-aminolevulinate (ALA), necessitating transporters in the plasma membrane of the parasite. Metabolites of this size are expected to freely diffuse through the parasitophorous vacuole. Besides these plasma membrane transporters, *T. gondii* and *Plasmodium spp.* must also express transporters in the mitochondrial and apicoplast membranes to allow the trafficking of heme intermediates between the synthesis enzymes which are dispersed across 3 cellular compartments. These parasite-specific transporters have not been identified to date but may be promising drug targets. Metabolites are depicted in blue, enzymes in black and transporters in purple. Abbreviations*: gly,* glycine; *ALAS*, 5-aminolevulinate synthase; *ALA,* 5-aminolevulinate; *ALA-Exp,* unidentified 5-aminolevulinate exporter in the inner mitochondrial membrane; *ALA-Imp,* unidentified 5-aminolevulinate importer in the plasma membrane and apicoplast membranes; *ALAD,* 5-aminolevulinate dehydratase; *PBG*, porphobilinogen; *PBGD*, porphobilinogen deaminase; *HMB*, hydroxy methylbilane; *UROS*, uroporphyrinogen III synthase; *UPgenIII* uroporphyrinogen III; *UROD*, uroporphyrinogen decarboxylase; *CPPgenIII*, coproporphyrinogen III; *CPPgenIII-Exp*, unidentified exporter of coproporphyrinogen III in the apicoplast membranes; *CPOX*, coproporphyrinogen III oxidase; *CPDH*, (oxygen independent) coproporphyrinogen III dehydrogenase; *PPgenIX*, protoporphyrinogen IX; *PPgenIX-Imp,* unidentified protoporphyrinogen IX importer in the plasma membrane and inner mitochondrial membrane*; PPOX*, protoporphyrinogen oxidase; *PPIX*, protoporphyrin IX; *FECH*, ferrochelatase.

Organelle Proteins by Isotopic Tagging (hyperLOPIT-) data set [150]. The localization and fitness-conferring role of this ABC transporter are both consistent with a function in porphyrin transport, although its function remains to be characterized. Another putative transporter, transmembrane protein 14C (TMEM14C), has been implicated in the transport of PPIX from the mitochondrial intermembrane space into the mitochondrial lumen in mammalian cells [151]. Harding *et al.* identified a structural homologue of TMEM14C in *T. gondii* (TGME49_228110).

Although its function is currently unknown, deletion of TMEM14C is associated with increased sensitivity to the antimalarial ART, suggesting a possible role in heme metabolism [16]. While mammalian cells transport CPPgenIII across the outer mitochondrial membrane, Apicomplexa must transport PPgenIX based on the different localization of CPOX [9, 103, 113]. Transport of heme intermediates across membranes may be facilitated by the known close physical association of the mitochondrion and apicoplast [152], however protein transporters are probably required to assist in the movement of molecules across the four membranes of the apicoplast and the double membrane of the

mitochondrion. Identification of porphyrin transporters has proven to be challenging, even in well studied model organisms [106, 153-155] and novel techniques will likely be required to identify proposed transport mechanisms. **Figure 5** provides an overview over the many unidentified transporters involved in the uptake of heme intermediates and their intracellular trafficking.

Considering the disparate localization and diverse evolutionary origins, as well as the presence of unique intermediate transporters, the heme synthesis pathway is expected to provide several promising drug targets to tackle toxoplasmosis [9-11]. An intriguing possibility is also to treat *Plasmodium* infection by provoking the accumulation of PPIX, which produces reactive oxygen species upon light exposure, leading to death of the parasite [15, 120].

The role of heme in activation of artemisinin

The unusual heme metabolism of *Plasmodium* is also thought to be a key facet in the activity of the antimalarial drug ART. ART and its derivatives, usually in combination with partner drugs, are frontline malaria treatments and play a vital role in malaria control across the world [156]. ART is a sesquiterpene lactone, derived from the Chinese medicinal herb *Artemisia annua,* which contains an endoperoxidase bridge. The activity of the drug depends on the cleavage of this bridge [157], which a number of *in vitro* and *in vivo* studies have demonstrated requires the iron moiety found within heme [18, 158, 159]. Activation of the drug leads to alkylation of heme as well as hundreds of proteins [18, 160] and lipids [161] within the cell. This widespread alkylation is key to the drug's rapid activity [162] as ARTs are quickly cleared *in vivo* [163].

Heme is required for ART activation, and as heme levels are expected to vary across the Apicomplexa, the sensitivity to ART is also be expected to differ. Indeed, while *Plasmodium spp.* are exquisitely sensitive to ART, *C. parvum* (which has no heme biosynthesis and a low requirement for heme) is comparatively insensitive to ART [164]. *Babesia spp.,* which are resident within erythrocytes, but do not take up large quantities of hemoglobin and do not form hemozoin [165] have an IC_{50} of around 100 times greater than that of *Plasmodium* [166]. *T. gondii*, which relies on its endogenous heme biosynthesis pathway, displays an intermediate sensitivity to ART [16, 17]. Levels of heme also differ significantly throughout the cell cycle of *Plasmodium* [167] and this is reflected in changes in sensitivity to ART treatment. Early after invasion (ring stage), prior to ingestion of significant amount of host hemoglobin, parasites are less sensitive to ART treatment, compared to later in the cell cycle [168, 169]. Together these data across the Apicomplexa hint that heme levels are related to ART-mediated toxicity, providing an unusual role for a metabolite in the mechanism of drug action. Interestingly, both *Plasmodium* and *T. gondii* demonstrate a higher sensitivity to ARTs than mammalian cells [170] despite the presence of heme synthesis in mammalian cells. Although there may be multiple reasons for this, including more robust cellular repair mechanisms in mammalian cells, metabolomic analysis has demonstrated a pool of labile heme in apicomplexan parasites [16], which is not detectable in mammalian cells [171, 172]. This soluble heme may have greater accessibility to the drug, allowing for greater or more rapid activation.

Resistance of *Plasmodium* to ARTs is an important and developing clinical problem [173-175]. Treatment failure in the field is associated with point mutations in the *kelch13* gene [176, 177], however the mechanism underlying this decreased sensitivity remains under debate [178-181]. Given the non-specific nature of ART toxicity, it is likely that multiple pathways have roles in protecting or mitigating drug-induced damage which will not be discussed here. However, given the key role heme has in drug activation, recent studies have suggested that uptake and degradation of host hemoglobin, and its concomitant release of labile heme has an important role in drug sensitivity [182, 183]. Indeed, recently it was shown that mutations in *kelch13* directly impact on hemoglobin uptake and digestion, through alterations in a clathrin-independent endocytic mechanism [180, 182, 184]. These recent studies help to explain the mechanism of action of a number of other resistance-conferring mutations that have been found *in vitro* or from clinical samples [184-187]. These studies show that *kelch13* has a role in endocytosis, and that endocytosis and digestion of host hemoglobin is required for ART-mediated killing [182, 183, 188].

To complement the work from *Plasmodium*, the genetically more tractable *T. gondii* has been

exploited to examine ART in a less heme-rich environment. Using both chemical mutagenesis and genome-wide CRISPR screens [16], mutants with decreased sensitivity to ARTs have been identified. Gratifyingly, both techniques identified the protease DegP2, which appears to modulate heme levels within the parasite through a currently unknown mechanism [16, 17]. Interestingly, detailed analysis of mutants recovered from chemical mutagenesis also uncovered changes in the copy number of mitochondrial DNA [17]. The mitochondrial DNA of apicomplexans is highly reduced but is known to encode two essential heme-containing cytochromes [58, 59], however the mechanism by which amplification of the mitochondrial genome decreases ART sensitivity is unknown [17]. CRISPR-based screening also allowed the identification of enzymes of both the TCA cycle and heme biosynthesis as mediating decreased ART susceptibility [16]. Chemical inhibition of either pathway reduced levels of labile heme within the parasites, in concert with decreased ART-susceptibility. The finding in *T. gondii* that heme levels appear to be the major determinant of ART susceptibility, supports work from *Plasmodium*, as well as confirming the TCA cycle as the source of precursors for heme synthesis, and demonstrated the power of CRISPR screens as tools to determine relevant pathways involved in the mechanism of drug action.

Summary

The synthesis of heme is highly conserved across the kingdoms of life [189]. Within the parasitic Apicomplexa phylum, heme production is differentially required across species and even life cycle stages. The changes in production are influenced by both the supply of exogenous heme to scavenge and the demand for heme, which is closely linked to the metabolic demands of the parasite. The ability of *T. gondii* to metabolically adapt to a heme-deficient lifestyle, albeit limited, highlights the metabolic flexibility exhibited by these parasites which is echoed in the decreased heme levels found in the drug resistant *Plasmodium kelch13* mutants. Heme, either produced *de novo* or scavenged from the host, is key to the susceptibility of apicomplexans to artemisinins. Modulating heme levels within the parasite, possibly through partner drugs, presents an intriguing opportunity for synergistic anti-malarial treatments.

Several questions concerning the synthesis and intracellular roles of heme remain. Transporters of porphyrin intermediates must be identified and characterized to better understand flux through the pathway. In many other organisms, heme production is regulated at multiple levels as excess heme is toxic. Regulation of metabolic processes has been understudied in the Apicomplexa; however, the relatively simple, unidirectional heme biosynthesis pathway may be amenable to such investigation. The role of heme itself as a signaling molecule has been examined in other organisms [3-5] and the abundance of labile heme in Apicomplexa may make this an interesting avenue for further research. Identification of uncharacterized transporters and discovery of divergent regulatory mechanisms in Apicomplexa is expected to reveal novel candidate targets for intervention and help unravel the mode of action of existing drugs.

Author contribution statement

JK, CRH and DSF wrote the manuscript

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