Lipid Synthesis in Protozoan Parasites: a Comparison Between Kinetoplastids and Apicomplexans

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Abstract

Lipid metabolism is of crucial importance for pathogens. Lipids serve as cellular building blocks, signalling molecules, energy stores, posttranslational modifiers, and pathogenesis factors. Parasites rely on a complex system of uptake and synthesis mechanisms to satisfy their lipid needs. The parameters of this system change dramatically as the parasite transits through the various stages of its life cycle. Here we discuss the tremendous recent advances that have been made in the understanding of the synthesis and uptake pathways for fatty acids and phospholipids in apicomplexan and kinetoplastid parasites, including *Plasmodium*, *Toxoplasma*, *Cryptosporidium*, *Trypanosoma* and *Leishmania*. Lipid synthesis differs in significant ways between parasites from both phyla and the human host. Parasites have acquired novel pathways through endosymbiosis, as in the case of the apicoplast, have dramatically reshaped substrate and product profiles, and have evolved specialized lipids to interact with or manipulate the host. These differences potentially provide opportunities for drug development. We outline the lipid pathways for key species in detail as they progress through the developmental cycle and highlight those that are of particular importance to the biology of the pathogens and/or are the most promising targets for parasite-specific treatment.

Keywords

Apicomplexa; Kinetoplastida; fatty acid synthesis; phospholipid synthesis; drugs; protozoa

1. Introduction

Diseases caused by protozoan parasites are among the most pressing concerns on the global health agenda. Within their ranks are not only some of the most widespread and important

infectious diseases, but also many of the most sorely neglected [1]. In this review we will focus on two protozoan phyla in particular, Apicomplexa and Kinetoplastida, which stand out in their overall public health impact with respect to incidence and severity of the diseases they cause [2]. Not only are these the most important groups of parasites, they also are the best studied. Facile experimental model systems have been established for several members of both phyla. Driven by powerful genetics these models have yielded considerable insight into parasite metabolism. There are numerous significant metabolic differences between these parasites and their human host, and we will highlight these differences, as they afford opportunities for drug development. We will provide a brief introduction to the parasite models that are the focus of this article for readers unfamiliar with the organisms. We will describe the particular importance of lipid metabolism for pathogens to introduce the subsequent systematic and comprehensive discussion of the biosynthesis pathways of major lipid classes for both phyla.

1.1. Apicomplexan Parasites

Apicomplexans are eukaryotic pathogens named after the complex of secretory and cytoskeletal organelles located at the apical end of the parasite cell. Apicomplexa are intracellular parasites and the organelles of the apical complex are required for host cell invasion. Three genera represent a particular threat for human health and therefore will be the main focus of this review: *Plasmodium*, *Toxoplasma* and *Cryptosporidium*. Note, however, that there are numerous additional apicomplexans that are important veterinary pathogens.

Five species of *Plasmodium* collectively are the causative agents of malaria, a disease that claims the lives of about a million individuals every year. Most of these deaths are due to *Plasmodium falciparum* and occur in small children in sub-Saharan Africa. *Plasmodium* has a complex life cycle, and as we will see, profound metabolic and biochemical changes are associated with each life cycle transition. Infection of a human occurs through the bite of a mosquito, whereby a small number of sporozoites are injected with the saliva. The sporozoites travel to the liver and invade hepatocytes, where they massively proliferate. Merozoites are then released into the bloodstream, where they invade and replicate in red blood cells. It is this intraerythrocytic phase that is responsible for the disease. Control of malaria rests on the prevention of transmission (e.g. through treated bed nets) and drug treatment. The genetic malleability of the parasite and its propensity to evolve drug resistance [3] has long haunted malaria control. This famously includes the loss of chloroquine as an effective therapeutic. There is grave concern about the future of the current artemisinin-based treatment regimen with first signs of treatment failure emerging in South East Asia [4, 5]. A constantly evolving portfolio of new anti-malarials is required to keep up with the ever-changing parasites. Understanding parasite metabolism is an important cornerstone of this agenda.

Toxoplasma and *Cryptosporidium* were initially recognized as opportunistic infections in immunocompromised individuals and received particular attention as late stage manifestations of AIDS [6, 7]. The sexual phase of the *Toxoplasma* life cycle occurs in the intestinal tract of cats and results in the shedding of spore-like oocysts, which then become highly infectious to other animals and humans upon consumption of contaminated food or water. Within intermediate hosts, tachyzoites cause rapid systemic infection. The onset of immune control eliminates tachyzoites, but bradyzoites persist within tissue cysts for the life of the host. In immunocompromised adults, chronic infection is reactivated leading to *Toxoplasma* encephalitis. In addition, congenital toxoplasmosis is a significant concern during pregnancy. While anti-folate treatment is effective in controlling tachyzoites and acute disease, there are no drugs available to eliminate chronic infection, which is problematic in a variety of clinical settings [8]. Cryptosporidiosis is an acute enteric disease

that typically is self-limiting. However, recent studies show that in particular in malnourished children the disease can be severe, protracted, and life threatening [9]. *Cryptosporidium* has a single host life cycle restricted to the intestinal epithelium, and oocysts are the only mode of transmission. Nitazoxanide was approved by the FDA for treatment but only shows moderate efficiency in immunocompetent children and produces no benefit in immunocompromised patients. Finding a reliable drug remains an important goal [10].

1.2. Kinetoplastid parasites

Kinetoplastids are flagellated protists that share a mitochondrial genome of unique organization and localization called the kinetoplast. There are numerous human pathogens among these early-branching eukaryotes, including African and American trypanosomes and many different species of *Leishmania*. All of these pathogens have insect and mammalian hosts. The different life cycle forms can be morphologically distinguished based on the position and length of the flagellum. More importantly, they also show remarkable metabolic differences. Stages from insect and mammalian hosts can be cultured axenically, which has greatly facilitated biochemical and genetic studies. *Trypanosoma cruzi* is the causative agent of Chagas disease, which is endemic in large parts of South America (note that although both the parasite and the vector are present in North America, transmission is rare). *T. cruzi* is transmitted by reduvid, or kissing bugs. Promastigote stages replicate within the bug's midgut and infective metacyclic trypomastigotes are deposited with the feces onto the skin of the mammalian host. In the mammalian host, the parasite cycles between replicative intracellular amastigotes and trypomastigotes. Importantly, replicating amastigotes are free in the host cell cytoplasm providing intimate access to host metabolites. Chagas disease is characterized by chronic and progressive inflammatory tissue damage, in particular of the heart muscle. Treatment is available but is not consistently effective and suffers from significant adverse effects [11].

Trypanosoma vivax, *T. congolense* and *T. brucei brucei* cause Nagana, an important cattle disease that severely limits ranching and dairy production in many parts of Africa. *T. brucei gambiense and rhodesiense* are morphologically indistinguishable from the bovine subspecies and are the causative agents of Human African Trypanosomiasis, also known as sleeping sickness. All African trypanosomes are vectored by tsetse flies, where they are present as procyclic trypomastigotes, epimastigotes and metacyclic trypomastigotes. In the mammalian host, trypomastigotes replicate extracellularly in the bloodstream causing anemia and cachexia. In humans, the blood phase is ultimately followed by invasion of the central nervous system. Untreated infection is invariably fatal. Several drugs are available for treatment of Human African Trypanosomiasis and Nagana, but there are severe limitations. Some of the drugs have grave and even life threatening adverse effects, are limited in their potency once the parasites have entered the brain, or are only active against certain species or subspecies. Lastly, there is treatment failure and overt resistance to several drugs, in particular in veterinary practice [12–14].

Parasites of the genus *Leishmania* are transmitted by a variety of sand flies, where promastigotes replicate in the midgut and are mechanically introduced into the bite site. In the mammalian host, *Leishmania* parasites proliferate as intracellular amastigote in macrophages, and they do so within a fully matured and acidified phagolysosome [15]. A large number of different *Leishmania* species are responsible for a broad spectrum of disease manifestations, with currently 12 million people being infected worldwide. In its most benign form, leishmaniasis presents as a self-limiting skin ulcer at the bite site (cutaneous leishmaniasis). In contrast, progressive non-healing erosion of mucosal tissue in the vicinity of the bite site is characteristic for mucocutaneous forms. In the most severe visceral disease, parasites spread systemically causing hepatosplenomegaly, cachexia and

immunosuppression. The etiology of these various forms appears to be closely linked to an unbalanced immune response to the chronic infection resulting in too much or too little inflammation and parasite control. Visceral leishmaniasis is fatal if not treated. Several drugs are available [16], but treatment of leishmaniasis remains challenging due to adverse effects, the requirement of lengthy regimens, limited drug availability and expenditure [17].

1.3. Lipids and Pathogens

Lipids are critical to the biology of all cells and organisms. They are the main structural elements of all biological membranes, they anchor glycoconjugates and many proteins to membranes, they serve as signaling molecules within and between cells, and they represent a highly efficient store and source of energy and reduction power. Lipids are of particular importance for pathogens, and some pathogens deliberately seek out lipid-rich host niches [18], or enhance the availability of lipids by manipulating the host [19, 20]. Intracellular pathogens have evolved sophisticated mechanisms to manipulate and tap into the lipid metabolism of their host cells. These include interference with vesicular and non-vesicular cellular lipid trafficking in viral [21], bacterial [22] and protozoal [23] pathogens. Within host cells, intracellular pathogens often develop in specialized vacuoles and the flow of lipids between host and pathogen-controlled membranous compartments is key to the pathogen's ultimate success [15, 24–26].

Lipids are not only used by pathogens as food or structural building blocks, but are also important pathogenesis factors that allow the pathogen to evade immune responses, manipulate host processes, and cause disease. In many cases these are specialized lipids synthesized by the pathogen [27]. The best-characterized example in protozoan parasites is a class of specialized glycosylphosphatidylinositol (GPI) lipids. They include GPI-anchored lipophosphoglycans of *Leishmania* species, which are crucial for host specificity and survival of the parasite in the sand fly vector [28, 29], and are also thought to modulate the initial interaction with the mammalian host [30]. In African trypanosomes, GPI-anchored variant surface glycoproteins are at the heart of the antigenic variation mechanism used to enable chronic infection. In *Plasmodium* and *Toxoplasma*, precursor GPI lipids are believed to play important roles as toxins and immune modulators [31–33].

Due to the importance of specialized lipids in mycobacteria, lipid synthesis has been a major target of drug development for tuberculosis [34]. Such examples highlighting the important roles of lipids are also present in protozoan parasites. As we will describe in detail, kinetoplastids and apicomplexans rely on a number of mechanisms for lipid synthesis that are not found, or different from those used in the mammalian host. These include fatty acid synthesis in the mitochondrion and the plastid, specialized elongation and desaturation pathways, and differences in downstream pathways of phospholipid synthesis. The success of miltefosine as an orally available *Leishmania* drug is one important validation of lipid metabolism as a drug target. Lipid turnover in pathogens is complex and involves numerous mechanisms of uptake and synthesis. In the following we will systematically review fatty acid and phospholipid synthesis and uptake pathways in both apicomplexans and kinetoplastids.

2. Fatty acid synthesis in protozoan parasites

The genomes of protozoan parasites encode the enzymes for three distinct biochemical pathways involved in fatty acid synthesis. While there are significant differences between these pathways, the underlying chemistry and sequence of enzymatic reactions is highly conserved (Fig. 1). All pathways synthesize fatty acids by successive addition of two carbon units to a growing carboxylic acid chain that is held via the pantothenyl group of acyl carrier protein (ACP; see Table 1 for names of enzymes of fatty acid synthesis and modification

and their corresponding genes in selected parasites) or coenzyme A (CoA). The length of this starter chain may vary. Fatty acid synthesis type I and II (FASI and FASII) typically produce fatty acids *de novo*, while the fatty acid elongation (FAE) pathway adds two carbon units to a typically much longer starter molecule. As detailed below, FASI and FASII differ in their architecture, FASI is expressed as a single very large polypeptide whereas FASII has multiple individual components (Fig. 1 B,C). The source of carbon to be added is malonyl-CoA, the activated form of a three carbon dicarboxylic acid. Malonyl-CoA is generated by acetyl-CoA carboxylase (ACC) from two molecules of acetyl-CoA (this step typically regulates the flux of fatty acid synthesis). Decarboxylative condensation of the malonyl substrate with the starter yields a chain elongated by two carbon units. The carbons are then fully reduced by the successive action of ketoacyl reductase, dehydratase and enoyl reductase. At this point the fatty acid can be elongated through condensation with another malonyl-CoA, or released by cleaving the thioester bond holding the carboxyl end.

Not all fatty acid synthesis pathways are present in all apicomplexans. The FASII pathway has been thoroughly characterized in *Toxoplasma* and *Plasmodium,* but is absent in piroplasms (tick-transmitted apicomlexans, including *Babesia* [35] and *Theileria* [36] and *Cryptosporidum* [37]. A subgroup of Apicomplexa (the Coccidia and *Cryptosporidium*) also harbor a FAS I megasynthase.

The genomes of kinetoplastid parasites encode the enzymes of two pathways to synthesize fatty acids, a FASII pathway and a FAE pathway. Both pathways have been characterized in *T. brucei* using genetic and biochemical approaches. The genomes of other kinetoplastid parasites, namely *T. cruzi* and *Leishmania major*, encode clear homologs of the enzymes of these pathways, suggesting that fatty acid metabolism of these parasites may be similar to that of *T. brucei*. However, their roles have not been studied experimentally. Table 2 provides an overview of the distribution of the three pathways among well-characterized parasite species discussed in this review.

2.1. The FASII pathway in apicomplexans

The FASII pathway is the stereotypical prokaryotic mechanism of fatty acid synthesis. Much of what we know about the pathway comes from studies in *E. coli* [38], however, it is widely distributed among bacteria and typically represents their main and often only source of fatty acids. All enzymes as well as the ACPs are expressed as individual polypeptides. In addition, the FASII pathway is found in some eukaryotes, where it is localized within organelles derived through endosymbiosis involving bacteria. Plants contain this pathway within the chloroplast, which is believed to be derived from a cyanobacterium [39] and represents the sole site of fatty acid *de novo* synthesis in plants. Recent work has also described a FASII pathway in mitochondria of certain eukaryotes, where its role is still unclear but likely includes the synthesis of lipoic acid [40]. In apicomplexan parasites the FASII pathway is found in the apicoplast, a plastid-like organelle that is derived from a red alga through secondary endosymbiosis [41]. Similar to plastids of plants and algae, the apicoplast is not only home to a FASII pathway but also to a non-mevalonate isoprenoid synthesis pathway and a portion of the heme pathway [41, 42]. The enzymes of the FASII pathway have been characterized in several apicomplexan species, with particular attention on *P. falciparum*, from which -ketoacyl-ACP reductase [43], -ketoacyl-ACP synthase [44] and -ketoacyl-ACP dehydratase [45] have been expressed as recombinant enzymes and subjected to kinetic analyses. In addition, there is structural information available for *P. falciparum* 3-oxoacyl-ACP reductase [46] and -hydroxyacyl ACP dehydratase [47].

Since the FASII pathway is not a major source of fatty acids for the mammalian host, it has been suggested that this pathway might represent a parasite-specific drug target against apicomplexan parasites. Consistent with this hypothesis, *Plasmodium* parasites were found

to be sensitive to triclosan inhibition *in vitro* and *in vivo* [48]. Triclosan targets the enoyl-CoA reductase of the bacterial FASII pathway [49] and is a common ingredient of anti-bacterial soaps. Similarly, *Toxoplasma* and *Babesia* were reported to be susceptible to triclosan [50, 51], suggesting that enoyl-CoA reductase may represent a general target to inhibit growth of apicomplexan parasites and prompting further studies to characterize the *Plasmodium* enzyme and to find more potent inhibitors [52–54].

At around the same time, the genomes of several apicomplexans were sequenced, revealing that not all Apicomplexa have a FASII pathway. These include genera that apparently lost the apicoplast, such as *Cryptosporidium*, but also genera that still harbor an apicoplast [35–37]. Importantly, the FASII pathway and the presumptive triclosan target, enoyl-CoA reductase, are absent from *Babesia* and *Theileria* parasites, both of which are susceptible to triclosan at a dose comparable to the malaria parasite [35, 36, 55, 56]. This, together with the observation that some bacteria lacking enoyl-CoA reductase are susceptible to triclosan [57] and the lack of inhibition of enoyl-CoA reductase by triclosan in *T. brucei* [58], raised doubts about the specificity of the compound for the FASII pathway. These concerns were further heightened when new triclosan derivatives were tested. Careful structure-activity relationship studies revealed that the activity of triclosan against enoyl-CoA reductase did not correlate with the activity against parasites [59].

With the pharmacological support for an essential role for FASII weakened, genetic studies were conducted in *Toxoplasma* and *Plasmodium*. A conditional *T. gondii* mutant was constructed in the gene for FASII ACP. When apicoplast ACP expression was blocked in this mutant by exposure to anhydrous tetracycline, parasite growth in culture was significantly reduced, while continued suppression resulted in parasite death [60]. Furthermore, mice challenged with this mutant parasite were cured from a lethal infection by tetracycline treatment. When analyzed biochemically, these mutants showed a pronounced loss of lipoic acid production in the apicoplast. This was consistent with a loss of FASII activity, which is thought to provide octanoic acid-ACP, i.e. the precursor of apicoplast lipoic acid *de novo* synthesis [60–62]. In conclusion, *Toxoplasma* FASII is required for parasite development and pathogenesis.

The importance of FASII was revisited using genetic approaches in *P. falciparum* and two rodent malaria species [59, 63]. The studies demonstrated that the FASII enzymes are not expressed in the blood stages, which are the cause of clinical malaria. Furthermore the genes for several FASII enzymes including the presumptive triclosan target, enoyl-CoA reductase, could be deleted from the genome without impairing parasite growth in the blood stage or their development in the mosquito. Lastly, *P. berghei* enoyl-CoA reductase null mutants were as susceptible to triclosan as wild type parasites and the same was true for parasites expressing enoyl-CoA reductase carrying point mutations that commonly confer robust resistance to triclosan [59]. In conclusion, the anti-parasitic effect of triclosan is not due to inhibition of FASII, and FASII is not a valuable target for anti-malaria therapy to cure blood stage infection. Importantly however, the pathway was found to be essential for the later stage of parasite development in the liver phase of the infection [59, 63]. During liver infection *Plasmodium* generates thousands of merozoites per infected cell. This requires massive organelle and membrane biogenesis [64]. This extraordinary demand for lipid likely cannot be met by import from the host alone and as a consequence parasite lipid synthesis is indispensible. It is also conceivable that the *Plasmodium* FASII pathway may supply the parasite with specialized lipids that cannot be obtained from the liver cell [63].

Taken together, the studies in *Toxoplasma* and *Plasmodium* demonstrate that the importance of the FASII pathway is highly stage- and host cell-dependent. While *Toxoplasma* and the *Plasmodium* liver stage require FASII for survival, other stages and parasite species can

apparently satisfy their needs by salvage of fatty acids from the host. This is consistent with findings in bacteria, where the importance of the FASII pathway is governed by the availability of exogenous fatty acids [65, 66].

While the requirement of the apicoplast FASII pathway is now well understood for life cycle progression of certain apicomplexans, its role in fatty acid metabolism of parasites has remained unclear. Is it a true engine of *de novo* synthesis of fatty acids, as in chloroplasts of plants, or does it have a more specialized and local role [67]? As pointed out above, biochemical analysis of FASII mutants in *T. gondii* suggested a link between FASII and lipoic acid synthesis [60, 61]. Lipoic acid in the apicoplast is required for the activity of the E2 subunit of pyruvate dehydrogenase, which in turn is required to supply the substrate for fatty acid synthesis, acetyl-CoA [68, 69]. In addition, lipoic acid is a cofactor for mitochondrial enzymes and, therefore, may be exported from the apicoplast to mitochondria. However, several studies have shown that mitochondrial lipoylation is independent of *de novo* synthesis of lipoic acid in the apicoplast but instead relies on lipoate salvage from the host [61, 70–72].

The impact of FASII on overall lipid composition and metabolism has been determined in *T. gondii*. Studies on the apicoplast phosphate translocator revealed that FASII is linked to cytoplasmic glycolysis via phosphoenolpyruvate [69, 73]. When parasites are grown in the presence of ^{13}C-labeled glucose, robust incorporation into fatty acids can be detected through gas chromatography/mass spectrometry-based isotopomer analysis. The bulk of parasite myristic and palmitic acid is labeled throughout the carbon chain in a fashion consistent with *de novo* synthesis from glucose. Importantly, this labeling is entirely lost when the FASII pathway is blocked by ablation of ACP [74]. Furthermore, as detailed below, fatty acids produced by FASII are apparently exported from the apicoplast and modified in the endoplasmic reticulum (ER) [74], demonstrating that the apicoplast FASII pathway represents a classical *de novo* synthesis machine that supplies a significant portion of fatty acids in *Toxoplasma*. In addition to the supply of bulk fatty acids, FASII may satisfy additional, and more specialized needs. In *Plasmodium*, the loss of the FASII pathway in liver stage parasites results in a lack of MSP1 (merozoite surface protein 1) [63, 75]. Palmitate and myristate have been shown to be the main lipid components of the MSP1 GPI anchor [76]. In addition, fatty acids are also used to directly modify proteins. In apicomplexans, protein myristoylation and palmitoylation has been shown to be critical for the targeting of a number of membrane-associated proteins [77]. This appears to be particularly important for the inner membrane complex, which not only gives the parasite cell shape and organization, but is also crucial for the gliding machinery the parasite uses to find and invade host cells [78–80].

In summary, the role the FASII pathway in apicomplexan parasites is much more complex and diverse than initially anticipated. Its value as a drug target depends on the parasite species and the life cycle stage under consideration. Parasites harboring this pathway utilize it for *de novo* synthesis of fatty acids as well as to supply the substrate for lipoic acid synthesis (Fig. 2). A recent study has used stable isotope labeling and organelle purification to follow FASII in *P. falciparum* [81]. Consistent with previous genetic studies, the authors do not detect FASII activity in the erythrocyte stage. However, they document robust activity when culturing parasites in minimal lipid media. This adds an additional layer of complexity in that it suggests that the parasites may metabolically adapt to the nutritional and physiological status of the host. This is consistent with the finding that the FASII genes are among those showing transcriptional modulation when comparing different patients with naturally acquired infections [82].

2.2 The FASII pathway in kinetoplastids

The FASII pathway in kinetoplastid parasites appears similar to the FASII pathway from other organisms. An ACP holds the growing acyl chain while the chain is modified by a synthase, dehydratase and two reductases. The major difference between the apicomplexan and kinetoplastid FASII is their organellar localization. While the FASII pathway in apicomplexan parasites localizes to the apicoplast, the FASII pathway in kinetoplastids is found in the mitochondrion [83]. Mitochondrial localization of FASII has been confirmed in *T. brucei* by epitope tagging of the individual TbFASII enzymes. Three of the proteins, ACP, dehydratase and ketoacyl-ACP synthase, have been studied in detail. The presumptive dehydratase component of the TbFASII pathway complements the loss of the corresponding enzyme in yeast, i.e. it restores lipoic acid synthesis and respiration [84]. Genetic analysis in *T. brucei* shows that deletion or RNAi knockdown of ACP or ketoacyl-ACP synthase blocks growth of procyclic and bloodstream forms in culture [83], demonstrating that TbFASII is essential in both life cycle forms. Biochemical analyses using a cell free system and radioactive precursors demonstrate palmitate to be the final product of the TbFASII pathway. While capable of fatty acid production, the pathway shows moderate activity and contributes only about 10% of total parasite fatty acid synthesis activity [83]. This suggests that an alternate mechanism of synthesis may be the major contributor to the overall fatty acid pool in kinetoplastids. A second and maybe more important role for TbFASII appears to be the synthesis of octanoic acid ACP for lipoic acid production. It has been shown that ablation of the FASII pathway in *T. brucei* blocks lipoic acid synthesis [83], indicating that the TbFASII pathway is the main source of octanoic acid for lipoic acid synthesis.

Are there additional roles for FASII-synthesized fatty acids beyond lipoylation? Biochemical analyses indicate reduced phospholipid synthesis in TbFASII mutants [83], and loss of mitochondrial ACP decreases the levels of certain mitochondrial phospholipids. This change in phospholipid composition coincides with changes in morphology and membrane potential of the parasite mitochondrion. Loss of ACP also affects the respiratory complexes and significantly reduces cytochrome-mediated respiration [85]. A more recent report on TbFASII highlights the importance of the pathway for kinetoplastid DNA segregation in *T. brucei* bloodstream forms. This segregation defect is believed to be due to altered phospholipid composition of the mitochondrial membrane, resulting in disturbed assembly and structure of the tripartite assembly complex that is required for kDNA segregation [86].

The importance of the FASII pathway in other kinetoplastids has not been addressed yet. However, it has been demonstrated that expression of *L. major* dehydratase and ketoacyl reductase complements the respiration defect of yeast mutants for the corresponding enzymes (43). In addition, genome mining indicates the presence of additional FASII components in both *L. major* and *T. cruzi* (acyl carrier protein: LmjF.27.0290 and Tc00.1047053511867.140; ketoacyl synthase: LmjF.33.2720 and Tc00.1047053504157.20; enoyl-CoA reductase: LmjF.04.0290 and Tc00.1047053506627.20). All candidate proteins are predicted to localize to the mitochondrion, based on the presence of an N-terminal leader sequence. Together, these data suggest that the FASII pathway is likely conserved among kinetoplastids.

2.3. The FASI pathway in apicomplexans

Several apicomplexan parasites encode a FASI megasynthase. The overall chemistry of FASI is similar to that of FASII. The reaction sequence is initiated by loading an acyl starter moiety to the phosphopantetheinyl arm of the ACP domain, followed by elongation cycles catalyzed by a synthase, a dehydratase and two distinct reductase domains [87, 88]. In contrast to FASII, all activities and domains of FASI are part of a single polypeptide chain. FASI is the fatty acid synthesis mechanism typically found in eukaryotes, including humans

and yeast, however, it has also been described in certain bacteria [89, 90]. Among apicomplexan parasites, candidate genes for the FASI pathway have been reported in *Cryptosporidum* [91], *Eimeria* [92], and *Toxoplasma* [67]. Bioinformatic and chemical analyses of apicomplexan FASI pathways suggest important differences compared to their mammalian counterparts. Structurally, mammalian FASI is a unimodular protein comprised of seven enzymatic domains. These enzymatic domains act sequentially for several cycles of elongation on an acyl moiety attached to ACP. Bioinformatic analysis of *C. parvum* FASI suggests that it is a multimodular protein where each module contains a set of multiple enzymatic domains [91]. The unusual architecture of apicomplexan FASI indicates that the mechanism of chain elongation is quite different from that of mammalian FASI. It is likely that the substrate is undergoing a single round of elongation in each module prior to being transferred to the ACP of the next module. The fate of the elongated product, in both mammals and apicomplexans, depends on the final domain of the enzyme. In mammals, a thioesterase domain releases the elongated product by hydrolytic cleavage [87], whereas in apicomplexan parasites, the thioesterase domain is replaced by a reductase domain, suggesting that the released product may be a fatty alcohol rather than a fatty acid [93].

The divergence of the apicomplexan FASI is not limited to its structural organization but may include its substrate specificity. Our current knowledge on the specificity of apicomplexan FASI is limited to data from the *C. parvum* enzyme, CpFASI. Biochemical experiments with recombinant CpFASI indicate that it may act as a fatty acid elongase rather than in *de novo* synthesis. Elongation versus *de novo* activity of FASI is defined by the substrate specificities of the starter and termination domains. The starter domain of CpFASI showed preference for long chain fatty acid substrates, with palmitoyl-CoA showing the highest activity in substrate competition assays [94], whereas the terminator domain showed preference for very long chain fatty acids, i.e. hexacosanoyl-CoA [93]. Expression of enzyme modules in *E. coli* is a remarkable idea to make these giant enzymes tractable, but may have limitations. Heterologous expression and the reliance on CoA-bound model substrates may modulate the specificity of the domains. Also, interaction with other enzymes *in vivo* may not be fully mimicked in an *in vitro* system. Nonetheless, these results strongly support the idea that CpFASI is involved in the production of very long chain fatty alcohols and, thus, is functionally different from mammalian FASI. Instead, CpFASI may resemble bacterial FASI (phylogenetic analyses appear to support a potential direct relationship via horizontal gene transfer). For example, it has been shown that FASI from *Mycobacterium* is capable of generating very long chain fatty acids using palmitate as substrate [95], and pathogenic mycobacteria use their FASI enzyme to elongate fatty acids imported from the host cell [96]. The resulting very long chain fatty acids can then be utilized for mycolic acid biosynthesis. It is likely that *C. parvum* FASI has a similar function, i.e. to elongate palmitate imported from the host cell to generate very long chain fatty acids.

Since the overall architecture of CpFASI is similar to FASI in *Toxoplasma* and *Eimeria*, it is likely that these proteins work in a similar fashion and perform a similar role. It is interesting to note that apicomplexan parasites harboring this pathway also harbor polyketide synthases (PKS) and form oocysts that are shed into the environment. These oocysts are surrounded and protected by an oocyst wall that confers remarkable chemical resistance. Oocysts are impervious to bleach and water chlorination, and the wall has a yet to be characterized lipid component. In contrast, apicomplexan parasites lacking FASI and PKS, like *Plasmodium* and *Babesia* [35, 36, 67], which are transmitted by arthropod vectors, do not form such environmentally stable oocysts. It is tempting to speculate that the products of the FASI enzyme could play an important role in oocyst formation, however, such a link has not been demonstrated experimentally.

PKS, like FASI, are multifunctional polypeptides and the apicomplexan enzymes appear to be type I PKS [97]. While the overall architecture of PKS enzymes resembles that of FASI enzymes, there are important differences. FASI fully reduces each added two-carbon unit. In contrast, PKS megasynthases typically lack certain domains, resulting in chains with multiple keto or hydroxyl groups [98]. These reactive groups can then be precursors for complex secondary products (this can but not always does involve additional enzymes). By varying the domain architecture, a myriad of compounds can be synthesized, including many molecules with importance in medical microbiology, such as toxins [99] and antibiotics [100]. PKS from *C. parvum* is the only characterized apicomplexan PKS so far, but its product is not known [101]. As for FASI, Zhu and colleagues used modular expression to study this enzyme. Interestingly, they found that the loading unit of PKS preferred very long fatty acids as substrates [102]. Dinoflagellates, a phylum of photosynthetic protists most closely related to apicomplexans, are well known to produce a variety of polyketide toxins (the typical cause of shellfish poisoning and red tide massive fish kills). These toxins appear to be the product of PKS-type enzymes and phylogenetic analysis has linked PKS from *Cryptosporidium* and some dinoflagellate PKS [103]. While initial studies in dinoflagellates have focused on toxins, it now appears that there may be additional roles for polyketides and related metabolites in the biology of these organisms [104]. Cysts are not restricted to apicomplexans but also found in dinoflagellates. It is tempting to speculate that FASI and two presumptive PKS megasynthases may collaborate to produce a special lipid of the oocyst wall and that such walls may predate apicomplexan parasitism. A similar collaboration between FASI and PKS has been reported for certain fungal [105] and bacterial [106] species.

2.4. The FAE pathway in apicomplexans

FAE represents the third mechanism of fatty acid synthesis in apicomplexan parasites. Similar to the FASII pathway, the FAE pathway operates using single monofunctional polypeptides to catalyze each elongation cycle. Every elongation cycle starts with a condensation reaction catalyzed by an elongase enzyme, however, in contrast to *de novo* synthesis systems, the FAE begins with a much longer starter (16:0 or longer). The product of this enzyme is modified sequentially by an enoyl reductase, a dehydratase and a ketoacyl reductase to produce a fatty acid that is elongated by a two carbon unit [107]. A major mechanistic difference that distinguishes the FAE pathway from the FASII pathway is the anchor molecule to which the growing acyl chain is attached. Unlike the FASII pathways that use ACPs, the FAE pathway employs CoA as an anchor molecule for fatty acid elongation [107, 108].

The FAE pathway is present in all eukaryotes. In plants [109], animals [110] and yeast [111], the pathway localizes to the ER and is important for proper growth and development [112, 113]. In apicomplexan parasites, elements of the FAE pathway were first characterized in *C. parvum*, which encodes a single fatty acid elongase localized in the parasitophorous vacuole membrane. This implies that the enzyme is secreted from the parasite and could act on substrates derived from the host cell. This may be part of a specialized uptake and modification process. It would be very interesting to localize additional components of the *Cryptosporidium* FAE machinery to further characterize this unusual compartmentalization. Biochemical studies on the recombinant enzyme showed that its preferred substrates are myristoyl-CoA and palmitoyl-CoA. Starting with myristoyl-CoA the enzyme can conduct up to two rounds of elongation generating stearic acid as the final product [114].

In contrast to *C. parvum,* in most organisms the FAE pathway employs multiple elongases with defined substrate and product length specificities that can act successively. While yeast contains three elongase genes [113], mammals contain six [107] and plants as many as twenty-one, highlighting the particular importance very long chain fatty acids, fatty alcohols

and waxes play in the biology of plants [115]. Similar to *S. cerevisiae*, *T. gondii* and *P. falciparum* encode three fatty acid elongases [67]. Metabolic labeling experiments with radioactive myristate and palmitate suggest fatty acid elongation to be active in blood stage malaria parasites [116] and the *T. gondii* fatty acid elongases have been studied in detail [74]. In contrast to *Cryptosporidium,* the *T. gondii* pathway resides in the ER. The specific substrates and products of the *T. gondii* fatty acid elongases, named TgELO-A, TgELO-B and TgELO-C, have been established using a combination of conditional gene ablation and metabolomic analysis [74]. Stable isotope labeling in combination with gas chromatography/ mass spectrometry analysis showed that TgELO-A is required to generate stearate from palmitate. Interestingly, TgELO-B and TgELO-C exhibit elongation activities exclusively towards monounsaturated fatty acids, with TgELO-B required to generate erucic acid (22:1) from oleic acid (18:1) and TgELOC to generate hexacosenoic acid (26:1) from erucic acid [74]. Chain length specificity for products has been previously reported for yeast and plant enzymes. However, how ELOs measure the actual chain lengths of their products was puzzling for some time. A seminal article by Denic and Weismann has elucidated this mechanism [111]. Fatty acid elongases in yeast are membrane proteins of the ER with their active sites exposed on the cytoplasmic surface. A lysine residue on the luminal side of a transmembrane helix is responsible for sensing the methyl end of the fully elongated fatty acid. Using mutational analyses, it was shown that the position of this lysine residue in the sixth transmembrane helix determines the length of the fatty acid released from a fatty acid elongase [111]. A precise residue responsible for the selectivity of *T. gondii* ELOs has not been identified yet.

The products of the FAE pathway are essential in mammals, plants and yeast. Stable isotope labeling shows that a significant proportion of fatty acids in *T. gondii* is the product of the FAE pathway [74]. Thus, it is reasonable to assume that these lipids also play an essential role in *T. gondii*. However, the loss of any individual elongase has no effect on parasite growth. Similarly, in yeast the loss of individual elongase enzymes is tolerated, whereas simultaneous disruption of two fatty acid elongases is lethal [113]. This has been attributed to overlapping specificity of the fatty acid elongases [113]. It is possible that the lack of growth defects in TgELO mutants is equally due to overlap. Our preliminary work showed that disruption of non-redundant enzyme components of the *T. gondii* FAE pathway results in strong inhibition of parasite growth, supporting such a model (S. Ramakrishnan and B. Striepen, unpublished results).

While we now know that apicomplexans use two different systems (FAE and FASI) to synthesize long chain fatty acids, it is not known what the products are used for and why they cannot be taken from the host. In plants [112] and yeast [117], loss of the FAE pathway affects phospholipid synthesis. Long chain fatty acids could be performing a similar role in apicomplexan parasites as well. However, such a link between phospholipid synthesis and fatty acid elongation in apicomplexan parasites has yet to be studied experimentally.

2.5. The FAE pathway in kinetoplastids

The kinetoplastid FAE pathway comprises of multiple elongases, dehydratases and reductases. The best-characterized components of this pathway are the four elongase enzymes of *T. brucei*. The *T. brucei* FAE resides in the membrane of the ER [118] (Fig. 3). A transgenic version of TbElo3 fused to GFP co-localized with blue-white DPX staining, a dye that preferentially accumulates in the ER [118]. While at first glance the enzymes and their localization in *T. brucei* appear to be quite similar to those in other eukaryotes, their functions are remarkably different. Unlike any other FAE pathway studied so far, the TbFAE pathway is capable of synthesizing fatty acids *de novo*. The key to this ability lies in the chain length specificity for the starter. While FAE pathways typically use a medium or long chain fatty acyl-CoA as primer, the TbFAE can use butyryl-CoA to prime the

elongation process. The names of the elongase enzymes in the pathway (ELO1-4) refer to the order in which they act in the pathway. The specific role of each elongase enzyme was determined by analyzing ELO mutants in *T. brucei* bloodstream forms [118]. Fatty acid elongases are generally divided into two categories: elongases that act on saturated and monounsaturated fatty acids and elongases that act on polyunsaturated fatty acids (PUFAs). In the TbFAE pathway, ELO1-3 act on saturated fatty acids, whereas ELO4 is specific for PUFAs. Chain elongation for the TbFAE pathway starts with the action of TbELO1, which uses butyryl-CoA to generate decanoyl-CoA, which is then extended by TbELO2 to myristoyl-CoA. The identification of this synthesis route for myristoyl-CoA solved a long-standing conundrum. The membrane of *T. brucei* bloodstream forms is covered with a dense coat of variant surface glycoproteins, which allows the parasite to evade host immunity through antigenic variation. These proteins are attached to the plasma membrane via a GPI anchor, which represents the first fully characterized GPI structure in eukaryotes [119]. Its composition is unique in that the fatty acyl chains are composed of myristic acid exclusively [120], requiring an elaborate fatty acid remodeling process to ensure this exclusivity [121]. Myristic acid is in low abundance in the serum of the mammalian host and it was puzzling for a long time as to how the parasite satisfies its enormous demand for this fatty acid. Myristoyl-CoA synthesized by the peculiar FAE *de novo* pathway solved this puzzle. Unexpectedly, however, *T. brucei* bloodstream forms lacking the FAE pathway showed no growth defect in culture and were not attenuated *in vivo* in the rat model [118].

Analysis of TbELO3 shows that this enzyme is capable of generating stearoyl-CoA from myristoyl-CoA. Importantly, this enzyme is only expressed in *T. brucei* procyclic forms, which synthesize surface proteins and GPI anchors that differ from bloodstream forms. The major acyl groups on procyclic GPI anchors are palmitate and stearate, and not myristate [122], suggesting that TbELO3 is the main provider of fatty acids for GPI anchors in procyclic trypanosomes. In contrast to the situation in *T. brucei* bloodstream forms, FAE appears to be essential for growth of procyclic parasites in culture. Altogether, these results show FAE in *T. brucei* to be the dominant source of fatty acids–even for shorter species like myristate. Thus, as in Apicomplexa, the importance of a fatty acid synthesis pathway for trypanosome survival depends on the life cycle stage and may be influenced by the availability of fatty acids in the host environment.

Components of the FAE pathway have also been detected in *T. cruzi* and *L. major*. A search of the respective genome sequences suggests the presence of candidate genes for ketoacyl-CoA reductases, dehydratases, and enoyl-CoA reductases in all kinetoplastid parasites. While *T. brucei* harbors four elongase genes, *T. cruzi* seems to contain five and *L. major* as many as fourteen. None of the encoded candidate enzymes has been studied experimentally. However, the elongases detected in *T. cruzi* and *L. major* are believed to be orthologs of TbELO1-3, or TbELO4, based on phylogenetic analysis [123]. The presence of additional elongase enzymes in *L. major* and *T. cruzi* may be related to the fact that the GPI anchors in these parasites have longer fatty acids [124] compared to those in *T. brucei*. In addition, the lipid portion of *Leishmania* lipophosphoglycan is composed of very long (24 and 26 carbon) alkyl chains that are likely derived from very long fatty acids [124].

2.6. Synthesis of unsaturated fatty acids

Unsaturation of fatty acids alters their physical and chemical properties. Incorporation of unsaturated fatty acids into membrane lipids can increase the overall flexibility and fluidity of membranes. The degree to which unsaturated fatty acids are used varies considerably between organisms. Unsaturated fatty acids are synthesized from saturated fatty acids by the action of desaturases [125]. Unsaturated fatty acids are classified into two main categories, monounsaturated fatty acids (MUFAs) and PUFAs. The synthesis of MUFAs occurs by introduction of a single double bond and is universally catalyzed by the enzyme stearoyl-

CoA desaturase (SCD) [126–128]. SCD in the apicomplexan parasite *P. falciparum* was identified only recently [129]. The enzyme seems to be active in blood stage parasites [116] and is required for growth [129]. Data from other systems suggest that expression of SCD can be influenced by certain PUFAs [130]. Whether such an effect on SCD is responsible for PUFA-mediated inhibition of *Plasmodium* growth [131] has yet to be determined. Genome searches in apicomplexan parasites reveal the presence of SCD in *Toxoplasma, Eimeria* and *Neospora*, but not in *Theileria, Babesia* or *Cryptosporidium*. Heavy isotope labeling studies in *T. gondii* indicate that SCD likely acts downstream of FASII and ELO-A and upstream of ELO-B and ELO-C [74]. However, the absence of FASII activity in *Plasmodium* blood stages suggests that precursors for desaturation also may be salvaged from the host.

The action of desaturases is not limited to the formation of MUFAs. Additional desaturases distinct from SCD are responsible for the synthesis of PUFAs. These types of desaturases have not been reported in apicomplexan parasites. The presence of polyunsaturated fatty acids in *T. gondii* [130] and the demonstration of linoleic acid in *C. parvum* [130] and *Eimeria* oocysts [130, 132] suggests that these parasites could have evolved a mechanism to import the required polyunsaturated fatty acids from the host.

Kinetoplastids are able to synthesize PUFAs and they harbor multiple desaturases that can act sequentially. The pathway begins with SCD, which has been characterized experimentally in *T. brucei* and is essential for both bloodstream and procyclic forms [133]. Genome analysis of *Trypanosoma* and *Leishmania* further reveals the presence of delta-12, delta-4 and omega-3 desaturases (the numbers indicate the position of desaturation with respect to the carboxy- (delta) or methyl- (omega) end of the chain respectively). In contrast, delta-6 and delta-5 desaturases are only found in *Leishmania* [134–136], indicating that *Leishmania* but not *Trypanosoma* have a complete pathway to synthesize C22 PUFAs. In trypanosomes, a PUFA-specific elongase (TbELO4) and a delta-4 desaturase may generate C22 PUFAs from host-derived C20 substrates. In addition to high amounts of C22 PUFAs, trypanosomes also contain high amounts of linoleic acid [137, 138]. The omega-3 desaturase present in both *Leishmania* and *Trypanosoma* is responsible for the synthesis of linoleic acid and has received particular attention. The absence of this enzyme in humans makes omega-3 desaturase a potential candidate for parasite-specific intervention [134, 139].

Taken together, these studies show that both apicomplexan and trypanosomatid parasites follow a common paradigm for generating MUFAs using SCD. In both parasite groups this enzyme is important for growth. However, the processes of obtaining PUFAs appear quite different. Apicomplexans parasites rely on fatty acid import whereas trypanosomatids are capable of synthesizing PUFAs.

2.7. Substrates and activation of FA synthesis pathways

The difference in the evolutionary origin and cellular localization of fatty acid synthesis pathways in apicomplexans is also reflected in more peripheral aspects. A first example is the provision of malonyl-CoA, the main substrate for fatty acid synthesis, a second is the activation of ACPs that are critical in both FAS systems. Apicomplexan parasites that harbor both the FASI and FASII mechanism seem to have evolved two independent systems for substrate provision and enzyme activation. The malonyl-CoA substrate is generated by the action of ACC. Two genes encoding ACC have been identified in *T. gondii*. Localization studies suggest that one of these enzymes is recruited to the apicoplast while the other is cytosolic [140, 141]. The apicoplast-localized ACC is probably required to generate malonyl-CoA for the apicoplast-based FASII pathway, whereas cytosolic ACC may supply malonyl-CoA for the FASI pathway. *Plasmodium,* which lacks the FASI pathway, and *Cryptosporidium*, which lacks the FASII pathway, harbor only single genes for ACC [67].

The idea that the apicoplast and the cytoplasm rely on independent pools of malonyl-CoA is also consistent with our recent stable isotope labeling experiments. We found that ^{13}C-acetate is readily incorporated into the products of the cytoplasm- and ER-based FAE pathways (see below). In contract, carbon derived from ^{13}C-glucose but not ^{13}C-acetate is incorporated into fatty acids produced by the FASII pathway [74]. The apicoplast pathway is solely fed through the import of phosphoenolpyruvate [69] from the cytoplasm.

Unlike apicomplexans, kinetoplastids harbor only a single ACC. In *T. brucei*, the enzyme is cytosolic and its main role is to supply the FAE pathway. RNAi experiments demonstrate that TbACC is required in procyclic forms but dispensable in bloodstream forms, thus mirroring the findings for TbELO mutants [142, 143]. It is likely, yet has not been demonstrated experimentally, that malonyl-CoA generated by cytoplasmic TbACC also serves as substrate for the mitochondrial FASII pathway.

Two independent ACP activating enzymes are present in *T. gondii* [67, 144]. In both the FASI and FASII pathway, apo-ACP is activated to the holo-ACP form by addition of a phosphopantetheinyl moiety. This reaction is catalyzed by phosphopantetheinyl transferase [145]. The two phosphopantetheinyl transferases in *T. gondii* have not been characterized in detail yet. However, one of them carries a bipartite leader and is likely localized to the apicoplast and activates the FASII ACP. The second protein is related to the cytoplasmic enzyme previously characterized in *Cryptosporidium* [144] and is probably involved in FASI (and PKS) activation. How *T. brucei* ACP is activated remains unknown, a candidate phosphopantetheinyl transferase gene is not immediately evident in the genome.

3. Phospholipid synthesis

3.1. Phospholipids of Kinetoplastida and Apicomplexa

Growth and proliferation of parasites depends on their ability to recruit the building blocks for membrane assembly. According to the paradigm of parasitism, it has long been thought that Kinetoplastida and Apicomplexa scavenge lipids for membrane formation from their hosts. However, recent genetic, biochemical, and pharmacological data have made it clear that *de novo* lipid synthesis plays a crucial role for parasite viability at different life cycle stages.

Phospholipids are the major lipid components of biological membranes. They comprise of two categories, the glycerophospholipids consisting of hydrophilic head groups linked via phosphate to glycerol-bound fatty acid or fatty alcohol chains and the sphingophospholipids consisting of head groups linked via phosphate to ceramide. The composition of the different phospholipid classes, together with the acyl and alkyl chain composition, determines the biophysical properties of a given membrane. In addition to their roles as membrane constituents, phospholipids are involved in many other biological processes, such as lipid signaling, maintaining and regulating protein structure and function, formation of specialized membrane domains, and attachment of proteins to membranes.

3.1.1. Phospholipids of Kinetoplastida—African trypanosomes have a glycerophospholipid composition similar to that of other eukaryotic cells, with phosphatidylcholine (PC) (45–60% of total lipid phosphorus) and phosphatidylethanolamine (PE) (10–20%) representing the most abundant, and phosphatidylinositol (PI) (6–12%), phosphatidylserine (PS) (<4%), cardiolipin (CL) (2–4%) and phosphatidylglycerol (PG) (<2%) representing minor glycerophospholipid classes [146–150]. Similarly, in *Leishmania*, PC is the most abundant glycerophospholipid class (30–50%), followed by PE (10%) and PI (10%) [151], while PA, CL, and PG, as well as *lyso*-PE and *lyso*-PC, are present in trace amounts only [151–153]. Although the presence of PS in *Leishmania* parasites has been

reported in several studies [151, 154, 155], recent analyses using mass spectrometry and serine labeling failed to detect this lipid in *L. major* promastigotes [156–158] (see also below). Interestingly, the glycerophospholipid classes in *T. brucei* and *Leishmania* consist of high amounts of ether-type molecular species [137, 147, 159]. In particular, PE in *T. brucei* procyclic and bloodstream forms [147, 159] and in *L. major* promastigotes [152] is composed mostly of alkenyl-acyl and alkyl-acyl glycerol, also called plasmenyl or plasmalogen and plasmanyl, respectively, molecular species. The most prominent glycerophospholipids of *Leishmania* promastigotes contain saturated and unsaturated C18 fatty acids [152], and most ether-type phospholipids in *T. brucei* contain C18:0 at the *sn*-1 and mainly unsaturated fatty acids (C18:2, C22:2) at the *sn*-2 position [147]. Phospholipid analysis in *T. cruzi* epimastigotes revealed PC as the most abundant phospholipid class (44%), followed by PE (28%), PI (12%), SM (4%), and small amounts of CL, PA, *lyso*-PC and PS [160].

The sphingophospholipid classes, sphingomyelin (SM), inositol phosphorylceramide (IPC) and ethanolamine phosphorylceramide (EPC), constitute 10–15% of total lipid phosphorus in *T. brucei* [146, 147, 161, 162]. Remarkably, due to stage-specific expression of the enzymes catalyzing the final reactions in SM, IPC and EPC synthesis, the relative amounts of these classes vary considerably between different *T. brucei* life cycle forms, with IPC being primarily present in procyclic, and EPC in bloodstream form parasites [162] (see also below). The presence of SM and IPC has also been demonstrated in *T. cruzi* epimastigotes [160, 163–165]. The ceramide portion of IPC consists primarily of a mixture of d18:0/16:0 dihydroceramide and d18:1/16:0 ceramide [164]. IPC also represents the major sphingophospholipid class in *Leishmania* [166, 167], with d16:1/18:0 IPC representing the major molecular species. The unusual composition of the long chain bases of *Leishmania* IPC suggests that the parasite preferentially uses myristoyl-CoA for ceramide synthesis, instead of palmitoyl-CoA as in *T. brucei* [162], *T. cruzi* [164], mammalian cells and yeast [168, 169]. Unlike trypanosomes, *Leishmania* parasites do not synthesize SM [166, 167].

3.1.2. Phospholipids of Apicomplexa—Membranes of *Plasmodium* are composed of several glycerophospholipid classes, but contain only small amounts of SM and cholesterol [170]. The main glycerophospholipid classes in isolated parasites include PC (40–50% of total lipid phosphorus), PE (35–45%) and PI (4–11%), while PS (<5%), CL (5%) and PA (<0.1%) represent minor classes [170–172]. Interestingly, upon parasite infection, the phospholipid content of erythrocytes was shown to increase 6-fold, indicating the high phospholipid biosynthetic capacity of the parasite [170]. In particular, compared to uninfected erythrocytes, plasma membranes from *P. falciparum*-infected erythrocytes contained more PC (38.7% *versus* 31.7%) and PI (2.1% *versus* 0.8%), but less SM (14.6% *versus* 28.0%) [172]. In addition, membranes of infected erythrocytes showed an altered phospholipid molecular species composition, with a shift in the fatty acyl chain composition towards that of the parasite, with increased amounts of palmitic, oleic and linoleic acid and a decrease in arachidonic acid [172]. Together, these results indicate that *Plasmodium* parasites are capable of modulating the membrane lipid composition of host cells.

Lipid analysis of isolated *T. gondii* tachyzoites revealed a 5-fold higher amount of phospholipid over cholesterol, with the major glycerophospholipid classes being PC (62% of total lipid phosphorus), PE (11%), PS (8%) and PI (6%), and SM representing the major sphingophospholipid class (8% of total lipid phosphorus) [173]. The most abundant fatty acids in tachyzoites are oleic acid > linoleic acid > palmitic acid [173]. In addition, metabolic labeling experiments demonstrated *de novo* synthesis of glycosphingolipids in *T. gondii* tachyzoites [174]. A more recent lipidomic analysis using electrospray ionization mass spectrometry confirmed the presence of hexosylceramide and dihexosylceramide and,

in addition, revealed small amounts of PA and EPC, in addition to the above-mentioned phospholipid classes [175].

3.2. Substrate acquisition for de novo phospholipid biosynthesis

Despite the ability of trypanosomatids to scavenge lipids from their hosts via uptake of fatty acids and *lyso*-glycerophospholipids, or by receptor-mediated endocytosis [138, 176–178], the genomes of *T. brucei*, *T. cruzi* and *Leishmania spp.* contain candidate genes encoding enzymes involved in *de novo* phospholipid biosynthesis [179, 180]. Indeed, work in recent years has demonstrated that many lipid synthesis pathways known from other eukaryotes are also active in trypanosomatids. A prerequisite for *de novo* formation of glycerophospholipids is the availability of substrates for head group attachment, including choline, ethanolamine, *myo*-inositol and serine, which have to be taken up by parasites from their host environments.

Transport of choline has been reported in *L. major* promastigotes [181]. This study showed that choline uptake occurs with a K_m in the low micromolar range, is Na^+-dependent and inhibited by high concentrations of hemicholinium-3, suggesting that *Leishmania* express a transporter related to members of the mammalian CTL transporter family [182]. However, a gene encoding a choline transporter has not been identified yet. In contrast to *Leishmania*, *T. brucei* bloodstream forms were reported to meet their demand for the essential nutrient, choline, by uptake of *lyso*-PC from the medium or host plasma, while direct uptake of choline into parasites was negligible [138, 183]. However, in contrast to a previous report [184], we recently found that [^{3}H]choline can be taken up by *T. brucei* procyclic and bloodstream forms in culture, with K_m values in the low micromolar range, suggesting that *T. brucei* expresses a choline transporter [185].

Uptake of ethanolamine has been reported in *Leishmania* parasites and *T. brucei* bloodstream and procyclic forms, where it is used as precursor for the synthesis of PE [159, 186, 187], GPIs [187–189] and the unique ethanolamine phosphoglycerol modification of eEF1A (eukaryotic elongation factor 1A) [190]. However, an ethanolamine transporter has not been identified.

Although *myo*-inositol can be produced *de novo* from glucose (see below), uptake of *myo*-inositol has been described in *L. donovani* [191], *T. cruzi* [192], and *T. brucei* [193]. Detailed characterization of the transporters, including functional expression in *Xenopus* oocytes, demonstrated that *myo*-inositol uptake in *L. donovani* promastigotes and *T. brucei* procyclic forms is mediated via H^+-coupled electrogenic cotransport [150, 191, 194]. Interestingly, down-regulation of the transporter in *T. brucei* blocked *myo*-inositol uptake and formation of PI and IPC, but had no effect on GPI production [150]. Together with a previous study [195], these results demonstrate that *de novo* synthesized *myo*-inositol and *myo*-inositol taken up from the environment are compartmentalized and used for the formation of different pools of inositol-containing lipids in *T. brucei* (see below). The observation that a hemagglutinin-tagged form of the *T. brucei myo*-inositol transporter localizes to the Golgi in procyclic forms suggests that bulk PI and IPC synthesis may occur in the Golgi [150], whereas PI production for GPI synthesis using endogenously produced *myo*-inositol occurs in the ER [191].

Uptake of exogenous lipids and lipid precursors has also been reported in apicomplexan parasites. In *Plasmodium*, incorporation of exogenous fatty acids [196–198] and *lyso*-PC [199] is necessary for parasite growth and to sustain phospholipid synthesis during the intraerythrocytic stage. In addition, PC is readily transferred from the erythrocyte membrane to intraerythrocytic parasites, indicating the presence of a selective PC transport mechanism providing PC from the host cell to the parasite [200]. Furthermore, it has been shown that *P.*

falciparum efficiently take up ethanolamine and, to a lesser extent choline and *myo*-inositol, from the host erythrocyte [201, 202]. Choline transport into *P. falciparum* has been shown to be Na^+-independent and mediated by an H^+-motive force [202]. However, a choline transporter has not yet been identified. Interestingly, uptake of choline can be inhibited by a set of choline analogs, which are toxic for malaria parasites [202, 203]. At present, it is unclear if the anti-malarial activity of the compounds is related to their potency to block choline uptake or inhibit PC synthesis [204–206].

Similarly, intracellular *T. gondii* parasites are able to take up glycerophospholipid precursors, in particular choline for PC synthesis, but they also divert host cell PC for membrane formation [207]. In addition, uptake of choline, ethanolamine and serine with subsequent incorporation into phospholipid classes has also been reported in extracellular *T. gondii* parasites [208].

Finally, serine, which is used as head group for PS formation and as precursor for sphingolipid synthesis, is taken up by protozoan parasites via amino acid transporters. Although no serine-specific transporter has been identified, serine uptake from the environment and metabolism into membrane lipids has been demonstrated in *Leishmania* and *T. brucei* parasites [156, 159, 161, 186]. In addition, intraerythrocytic *Plasmodium* parasites use serine from the blood or from hemoglobin catabolism after import into the acidic food vacuole [209] for phospholipid formation.

3.3. Phospholipid synthesis pathways

The major routes for phospholipid synthesis in eukaryotes are summarized in Fig. 4; only those pathways that are active in Apicomplexa and Kinetoplastida are shown (for explanations see below).

3.3.1. Synthesis of PC, PE and PS—Eukaryotes possess several pathways for the synthesis of the two most abundant phospholipid classes, PC and PE. A major route involves formation of the CDP-activated precursors, CDP-choline and CDP-ethanolamine, and is known as the Kennedy pathway after its discovery more than 55 years ago by Kennedy and Weiss [210]. In the CDP-choline branch, choline is first phosphorylated by choline kinase (see Table 3 for names of enzymes of phospholipid synthesis and their corresponding genes in selected parasites) and then activated to CDP-choline by choline-phosphate cytidylyltransferase. The latter reaction often represents the rate-limiting step in PC synthesis by the Kennedy pathway [211]. The third step is mediated by choline phosphotransferase, transferring CDP-choline to diacyl glycerol or the corresponding ether-type glycerol species, to generate diacyl or alk(en)yl-acyl PC, respectively [211]. Since in most cells this enzyme shows dual specificity for CDP-choline and CDP-ethanolamine, i.e. the corresponding metabolite of the PE branch of the Kennedy pathway, it will be named choline/ethanolamine phosphotransferase (CEPT) throughout this review. In the CDP-ethanolamine branch of the pathway, ethanolamine is phosphorylated by ethanolamine kinase and activated by ethanolamine-phosphate cytidylyltransferase to CDP-ethanolamine [212, 213]. An alternative route for ethanolamine-phosphate formation involves degradation of sphingoid bases by sphingosine-1-phosphate lyase [214]. The last step of the CDP-ethanolamine pathway is catalyzed by ethanolamine phosphotransferase (EPT), leading to the formation of diacyl- and ether-type PE molecular species, using diacyl or alk(en)yl-acyl glycerol, respectively, as substrates [212, 213] (Fig. 4).

Alternatively, PC can be generated by methylation of ethanolamine-phosphate by phosphoethanolamine N-methyltransferase and subsequent PC formation via the Kennedy pathway, or by methylation of PE involving PE N-methyltransferase [211]. In addition, PC can be formed by head group exchange with PS [211]. Furthermore, in most eukaryotes PE

can be synthesized by irreversible decarboxylation of PS, or reversible head group exchange with PS [213, 215, 216] (Fig. 4).

Finally, PS in eukaryotes can be synthesized in single step reactions via head group exchange with PC and PE, catalyzed by PS synthase 1 and 2, respectively, or from serine and CDP-diacyl glycerol via prokaryotic-type PS synthase [213] (Fig. 4). The contributions of the different routes to PC, PE and PS formation greatly vary between eukaryotes, with certain routes being absent in some organisms.

PC synthesis in Kinetoplastida: The most abundant phospholipid class in *T. brucei* bloodstream and procyclic forms, PC, is synthesized via the CDP-choline branch of the Kennedy pathway (Figs 4, 5). The *T. brucei* enzyme catalyzing the first step in this pathway, choline/ethanolamine kinase, has been expressed as a recombinant protein in *E. coli* and characterized biochemically [217]. The purified enzyme shows dual specificity for choline and ethanolamine, with a 80-fold lower K_m value for choline [217]. Expression of choline/ ethanolamine kinase in *T. brucei* bloodstream forms has been reported to be essential (cited as unpublished data in [179]). Homologs of the *T. brucei* enzyme have been identified and annotated in the *T. cruzi* and *Leishmania spp.* genome databases. The second enzyme of the Kennedy pathway, choline-phosphate cytidylyltransferase, catalyzes the activation of choline-phosphate to CDP-choline. Candidate enzymes have been identified in all trypanosomatids, however, none of them has been characterized experimentally so far. The final reaction in the CDP-choline branch of the Kennedy pathway is mediated by CEPT and has been characterized in *T. brucei* procyclic forms [159]. The enzyme is essential for parasite growth in culture and shows dual specificity for CDP-choline and CDP-ethanolamine [159]. Homologs of CEPT have been identified and annotated in the *T. cruzi* and *Leishmania spp.* genome databases.

In *T. brucei* and *T. cruzi*, the Kennedy pathway represents the only pathway for PC synthesis (Fig. 5). The respective genomes lack candidate genes encoding PE N-methyltransferases and (plant-like) phosphoethanolamine N-methyltransferases, catalyzing the methylation of PE to PC and ethanolamine-phosphate to choline-phosphate, respectively. The lack of PC formation from PE has been confirmed experimentally in *T. brucei* bloodstream and procyclic forms by labeling experiments using ethanolamine [186, 189] or serine [159]. These findings demonstrate that trypanosomes are auxotrophic for choline, i.e. they have to take up choline, or choline-containing metabolites, from the environment. In contrast, the *Leishmania spp.* genomes contain genes encoding candidate PE N-methyltransferases [180], suggesting that *Leishmania* parasites possess another route for PC synthesis. However, the corresponding enzymes or pathways have not been characterized experimentally.

PC synthesis in Apicomplexa: In apicomplexan parasites, synthesis of PC occurs via multiple routes (Figs 4, 5). The first enzyme of the CDP-choline branch of the Kennedy pathway, choline kinase, has been identified and characterized more than 25 years ago in *P. falciparum* and *P. knowlesi* [218–220] and, more recently, in the rodent malaria parasites, *P. berghei* and *P. vinckei* [221]. The enzymes catalyzing the two subsequent reactions in the Kennedy pathway, choline-phosphate cytidylyltransferase and CEPT, have been demonstrated in several *Plasmodium* species [221–223]. CEPT, which shows dual specificity towards CDP-choline and CDP-ethanolamine as substrates, has been studied in *P. knowlesi*-infected erythrocytes [218, 221], and corresponding genes are found in all *Plasmodium* genomes. In *P. berghei*, CEPT localizes to the ER [221]. Unsuccessful attempts to disrupt choline kinase, choline-phosphate cytidylyltransferase or CEPT suggest that PC synthesis by the CDP-choline pathway is essential in *P. berghei* [221].

P. falciparum is also able to use serine as substrate for PC formation, via decarboxylation to ethanolamine and phosphorylation to ethanolamine-phosphate, followed by three methylation reactions to form choline-phosphate [221] (Figs 4, 5). Subsequently, choline-phosphate is used to generate PC via the CDP-choline pathway [221, 224]. The formation of choline-phosphate is mediated by *P. falciparum* phosphoethanolamine N-methyltransferase, an enzyme that selectively methylates ethanolamine-phosphate, but not ethanolamine or PE [224, 225]. Interestingly, phosphoethanolamine N-methyltransferase activity is inhibited by the reaction product, phosphocholine, and the choline-phosphate analog, miltefosine [225], an anti-malarial that has been used to treat visceral and cutaneous Leishmaniasis [226–228]. Inhibition of PC formation by miltefosine has also been reported in *L. donovani* [229]. Disruption of the phosphoethanolamine *N*-methyltransferase gene in *P. falciparum* results in a complete loss of PC synthesis via serine decarboxylation and ethanolamine-phosphate methylation [230]. The *P. falciparum* enzyme has been localized to the Golgi [231]. Interestingly, the gene encoding phosphoethanolamine *N*-methyltransferase is absent from the genomes of the rodent malaria, *P. berghei*, *P. yoelli* and *P. chabaudi* ([171]; reviewed by [232]).

In contrast, *Toxoplasma* parasites lack candidate genes, and enzymatic activities, for phosphoethanolamine *N*-methyltransferase and PE *N*-methyltransferase [180, 208, 233] and, thus, depend on the CDP-choline pathway for PC synthesis (Fig. 5). In a recent report, *T. gondii* was shown to express a novel choline kinase harboring a unique hydrophobic N-terminus that is dispensable for enzyme activity but required for protein oligomerization [233]. Interestingly, expression of two additional choline kinase isoforms through a cryptic promoter compensated for the loss of choline kinase activity after conditional mutagenesis of the unusual choline kinase gene [233].

PE synthesis in Kinetoplastida: PE synthesis has been extensively studied in *T. brucei* [234] (Figs 4, 5). All three enzymes of the CDP-ethanolamine pathway have been identified and experimentally characterized. Down-regulation of ethanolamine kinase, ethanolamine-phosphate cytidylyltransferase and EPT using RNAi in *T. brucei* procyclic forms resulted in changes in mitochondrial morphology, formation of multinucleate cells [235], and growth arrest [159, 190], demonstrating that the CDP-ethanolamine branch of the Kennedy pathway is essential for parasite survival. In addition, depletion of ethanolamine-phosphate cytidylyltransferase was shown to cause a distinct defect in cytokinesis, reflected by parasites being unable to separate from each other during the final step of cytokinesis, suggesting that a lower PE content may inhibit membrane fusion and fission events [234, 235]. Ethanolamine-phosphate cytidylyltransferase is also essential for survival of *T. brucei* bloodstream forms [186]. Interestingly, EPT was shown to mediate the formation of alkenyl-acyl PE molecular species, while diacyl-type PE species are synthesized primarily by the dual-specificity enzyme, CEPT [159]. To date it is unclear if the role of EPT and CEPT in the synthesis of different PE sub-classes is the result of their substrate specificity for alkenyl-acyl glycerol and diacyl glycerol species, respectively, or of distinct sub-cellular localization. At present, cytosolic ethanolamine-phosphate cytidylyltransferase represents the only enzyme in PE synthesis that has been localized [186].

The *T. cruzi* and *Leishmania* genomes contain candidate genes for all three enzymes of the CDP-ethanolamine pathway [180, 236], indicating that this route for PE synthesis is present in other Kinetoplastida as well (Fig. 5). Depletion of EPT in *L. major* inhibits the synthesis of alkenyl-acyl PE molecular species (M. Pawlowic and K. Zhang, personal communication), indicating that EPT shows a similar specificity for ether-type PE formation in both *T. brucei* and *Leishmania spp.* Other enzymes of the CDP-ethanolamine pathway in *Leishmania* have not been characterized experimentally.

Alternatively, PE in kinetoplastids can be produced by decarboxylation of PS (Figs 4, 5). Metabolic labeling experiments have shown that PS decarboxylation activity is present in *T. brucei* procyclic [159] and bloodstream forms [186]. However, this reaction is unable to rescue a block in PE synthesis by the CDP-ethanolamine pathway [159, 186] and, thus, it is unclear at present if PS decarboxylation contributes to *de novo* PE formation (see below). The subcellular localization of PS decarboxylase in *T. brucei* has not been determined. Genes encoding predicted PS decarboxylases are also present in the genomes of *T. cruzi* and *Leishmania spp.* (Fig. 5), however, the roles of the enzymes have not been investigated.

A third pathway for PE synthesis in kinetoplastids involves ethanolamine-phosphate formation from sphingolipid degradation, with subsequent use in the CDP-ethanolamine pathway (Figs 4, 5). Sphingosine-1-phosphate lyase catalyzes the irreversible catabolism of phosphorylated sphingoid bases to produce ethanolamine-phosphate and a long chain fatty aldehyde [237]. *L. major* promastigotes, in which the gene encoding sphingosine-1-phosphate lyase was disrupted, were viable but defective in stationary phase differentiation and showed attenuated virulence [156]. Interestingly, parasite growth was restored by supplementing ethanolamine in the culture medium, indicating that a primary role of sphingolipid turnover in *L. major* promastigotes is to provide ethanolamine-phosphate for PE synthesis [156]. Gene orthologs of sphingosine-1-phosphate lyase are also present in the genomes of *T. brucei* and *T. cruzi* (Fig. 5), however, at present it is not known if ethanolamine-phosphate production via sphingosine-1-phosphate breakdown plays a similar role in trypanosomes. Our preliminary results using RNAi to down-regulate sphingosine-1-phosphate lyase expression in *T. brucei* indicate that the enzyme is not essential for parasite growth in culture (L. Farine and P. Bütikofer, unpublished results).

PE synthesis in Apicomplexa: The CDP-ethanolamine branch of the Kennedy pathway is also active in several *Plasmodium* species (Figs 1, 2). Ethanolamine kinase activity has been demonstrated in *P. falciparum* and *P. knowlesi* [220, 238], as well as rodent *P. berghei* and *P. vinckei* [221]. The second enzyme of the pathway, ethanolamine-phosphate cytidylyltransferase, represents the rate-limiting step in PE synthesis by the Kennedy pathway in *P. falciparum* and *P. knowlesi* [223]. In contrast to *Trypanosoma* and *Toxoplasma*, which contain separate genes encoding EPT and CEPT to catalyze the last steps in the two branches of the Kennedy pathway, the genome of *Plasmodium* contains a single gene encoding the dual-specificity enzyme, CEPT [180, 218, 221, 223, 239]. Attempts to disrupt the genes involved in PE synthesis by the Kennedy pathway in *P. berghei* failed, indicating that the pathway is likely essential for parasite survival in the blood stages [221].

PE synthesis by the Kennedy pathway has also been demonstrated in *Toxoplasma* (Fig. 5). A cytosolically located ethanolamine kinase in *T. gondii* phosphorylates ethanolamine only, whereas a choline kinase showing punctate intracellular distribution mediates phosphorylation of both choline and ethanolamine [233]. Similar to the situation in *Plasmodium*, the *T. gondii* choline kinase gene cannot be disrupted, unless using a conditional knock-out approach, indicating that the enzyme is essential for parasite viability [233]. Alternatively, PE in *Plasmodium*-infected erythrocytes can also be generated by PS decarboxylation [198], or from serine via decarboxylation to ethanolamine followed by synthesis by the CDP-ethanolamine pathway [223] (Figs 4, 5).

PS decarboxylases have been identified in *P. falciparum* [240] and *P. knowlesi* [241]. In *P. falciparum*, the enzyme was found to localize to the ER [240]. A gene encoding serine decarboxylase, a soluble and pyridoxylphosphate-dependent enzyme widely expressed in plants and algae [208, 233], has not been identified in the *Plasmodium* genome [180]. In *T. gondii*, PS decarboxylation represents a major contributor to PE formation [208, 242],

establishing PS as an important precursor for PE in *Toxoplasma* (Fig. 5). Remarkably, *T. gondii* PS decarboxylase is a soluble enzyme localizing to dense granules or the parasitophorous vacuole of replicating parasites [242]. This is the first report of a soluble form of PS decarboxylase in any organism. Similar to *Plasmodium*, no serine decarboxylase gene has been detected in *Toxoplasma*. In contrast to Kinetoplastida, PE synthesis via ethanolamine-phosphate formation from sphingolipid degradation has not been reported in Apicomplexa (Fig. 5). A candidate gene encoding sphingosine-1-phosphate lyase has not been identified in the genomes of *P. falciparum*, *T. annulata* and *T. gondii*.

PS synthesis in Kinetoplastida: Synthesis of PS has been demonstrated in *T. brucei* procyclic [159] and bloodstream [186] forms using labeled serine as precursor. Bioinformatic analyses suggest the presence of a gene encoding PS synthase 2 in *T. brucei* (Figs 4, 5), however, the enzymatic reaction has not been characterized. A PS synthase 2 homolog is also found in the genomes of *T. cruzi* and *Leishmania spp* (Fig. 5). Interestingly, however, although the presence of PS in *Leishmania* has been suggested, in part based on indirect assays [155, 243, 244], other reports failed to detect PS in *Leishmania* promastigotes using several analytical methods, including mass spectrometry [153, 156, 158]. The findings that annexin V and anti-PS antibody binding is also observed in parasites lacking PS questions the specificity of these assays, which have been widely used to demonstrate PS exposure in the outer leaflet of the plasma membrane in eukaryotic cells, including *T. brucei* [245]. An overview on the methods and their limitations to detect PS exposure on the surface of eukaryotic cells has recently been published [246].

PS synthesis in Apicomplexa: In *Plasmodium*-infected erythrocytes, serine is used as substrate for PS synthesis via bacterial-type PS synthase [223, 247], while PS formation by head group exchange with PC or PE is absent (Figs 4, 5). In addition, in *P. falciparum* and *P. knowlesi*, serine can be decarboxylated to ethanolamine [223], making serine an important precursor for PE and PC formation [247]. The genes encoding PS synthase and serine decarboxylase have not been identified. In *T. gondii*, [^{3}H]serine is rapidly incorporated into PS, which is then decarboxylated to PE [208]. In addition, small amounts of radioactivity can be detected in base-resistant minor lipid classes, tentatively identified as sphingolipids [248]. In contrast to *Plasmodium*, PS synthesis in *T. gondii* most likely occurs via head group exchange with PE [208]. In addition, direct decarboxylation of serine has not been reported in *Toxoplasma* (Fig. 5).

3.3.2. Synthesis of PI—PI contains a head group consisting of the cyclic polyalcohol, *myo*-inositol, which can be taken up from the environment via *myo*-inositol transporters (see above), or synthesized *de novo* from glucose. Biosynthesis of *myo*-inositol involves generation of *myo*-inositol–3-phosphate from glucose-6-phosphate by inositol–3-phosphate synthase, followed by dephosphorylation to *myo*-inositol by inositol–3-phosphate monophosphatase [249] (Fig. 4). The final step in PI synthesis, converting CDP-diacyl glycerol and *myo*-inositol to PI, is catalysed by PI synthase and is conserved in all eukaryotes [250] (Fig. 4). PI can then be used to generate the different phosphorylated forms of this phospholipid, many of which represent important signalling molecules in eukaryotic cells [249]. One particular form of phosphoinositide, phosphatidylinositol 3-phosphate, has recently been implicated in apicoplast biogenesis in *Toxoplasma* [251] and host targeting of *P. falciparum* proteins to the erythrocyte [252]. Phylogenetic analyses of microbial phosphoinositide kinases and phosphatases have revealed distinct amino acid sequence and catalytic characteristics from human homologs, suggesting they could be targeted for drug development against infectious diseases [253, 254]. In addition, PI represents the precursor for GPIs, prominent cell surface glycophospholipids and anchors for plasma membrane proteins, in particular in protozoan parasites [255–257].

PI synthesis in Kinetoplastida: The discovery that inositol–3-phosphate synthase is essential for viability of *T. brucei* bloodstream forms indicated that *de novo myo*-inositol synthesis is required for parasite survival and that *myo*-inositol taken up from the environment is unable to substitute for endogenous *myo*-inositol production [258] (Figs 4, 5). The observations that i) parasite GPI-anchored proteins are poorly labeled *in vivo* using exogenous [³H]inositol [150, 179], ii) *de novo myo*-inositol synthesis is required for GPI synthesis in the ER [195, 258], and iii) PI synthase in *T. brucei* bloodstream forms shows a dual localization in the ER and Golgi [193], lead to the hypothesis that two distinct cellular pools of *myo*-inositol exist in *T. brucei* [193]. This hypothesis was experimentally supported by recent observations in *T. brucei* procyclic forms showing that exogenous *myo*-inositol is essential for bulk PI and IPC production, but not GPI formation, and by the identification of a *myo*-inositol transporter located in the plasma membrane and Golgi [150]. Together, these finding suggest that *de novo* synthesis of *myo*-inositol for PI and GPI production is catalyzed by PI synthase in the ER, whereas uptake of exogenous *myo*-inositol into the Golgi is required for bulk PI formation by Golgi PI synthase.

Gene orthologs for *myo*-inositol–3-phosphate synthase and PI synthase have been identified in *T. congolense*, *T. vivax*, *T. cruzi* and *Leishmania spp.*, suggesting that *de novo* synthesis of *myo*-inositol occurs in all kinetoplastids (Fig. 5). Similarly, *myo*-inositol uptake has been reported in several *Leishmania* species [191, 259], and orthologs of the *T. brucei myo*-inositol transporter have been found in most kinetoplastids [150].

PI synthesis in Apicomplexa: PI synthase activity has been detected in *Plasmodium*-infected erythrocytes [260], and the enzyme was characterized in *P. falciparum* and *P. knowlesi* [261]. Amino acid sequence comparison with predicted proteins suggests that PI synthase is also present in *P. berghei*, *P. chabaudi* and *P. vivax* [261] (Fig. 5). In *T. gondii*, two cDNA clones encoding functional PI synthases have been identified [262]. The two genes are expressed stage-specifically in tachyzoites and bradyzoites, with the tachyzoite-specific PI synthase sharing a high degree of identity to human, plant and yeast PI synthases [262]. PI synthases from both *T. gondii* and *P. falciparum* were able to complement PI synthase-deficient yeast mutants, demonstrating functional equivalence between PI synthases from different eukaryotes [261, 262] (Fig. 5).

Genes encoding putative *myo*-inositol–3-phosphate synthases have been annotated in the genomes of *Plasmodium*, but not *T. gondii* and *T. annulata*, suggesting that *de novo* synthesis of *myo*-inositol may be absent in the latter organisms (Fig. 5).

3.3.3. Synthesis of mitochondria-specific lipids—PG and CL are abundant glycerophospholipid classes in bacteria [263]. In eukaryotes, they are confined to the inner mitochondrial membrane, where they play important roles in maintaining protein structure and function [264]. The synthesis of PG and CL starts with PA, which is activated by CTP to CDP-diacyl glycerol [265]. After phosphatidyl group transfer to glycerol–3-phosphate, mediated by phosphatidylglycerophosphate (PGP) synthase, PGP is dephosphorylated to PG by PGP phosphatase (Figs 4, 5). In most prokaryotes, PG receives an additional phosphatidyl group from another PG to form CL, whereas in most eukaryotes, a phosphatidyl group from CDP-diacyl glycerol is transferred onto PG. The reactions are catalyzed by prokaryotic- and eukaryotic-type CL synthases, respectively, which can be distinguished based on their catalytic site motifs [265].

PG and CL synthesis in Kinetoplastida: Low levels of PG and CL have been demonstrated in *T. brucei* procyclic and bloodstream forms [137, 149] and *Leishmania* [151, 158, 229]. Very recently, a protein homologous to PGP synthase from mammalian cells and yeast has been identified and experimentally characterized in *T. brucei* [266]. RNAi-

mediated down-regulation of PGP synthase activity resulted in decreased PG and CL levels, alterations in mitochondrial morphology, and growth arrest of procyclic and bloodstream forms in culture, indicating that PG and CL are indispensable for parasite viability in both life cycle forms [266] (Fig. 5).

Surprisingly, CL synthase in *T. brucei* was found to contain the characteristic prokaryotic-type, rather than eukaryotic-type, CL synthase motif consisting of two phospholipase D domains [149, 180]. Conditional gene knock-out of *T. brucei* CL synthase in procyclic forms demonstrated that it is essential for parasite survival in culture [149]. Ablation of CL synthase activity inhibited CL formation and resulted in impaired mitochondrial function [149]. Both PGP synthase and CL synthase localize to the mitochondrion, where they associate with high molecular mass protein complexes [149, 266]. It is possible that both enzymes are present in the same complex, forming a mitochondrial lipid synthesis complex.

Genes encoding homologous proteins to *T. brucei* PGP synthase and CL synthase are present in all kinetoplastids (Fig. 5). In contrast, a candidate gene encoding PGP phosphatase has not been identified in any protozoa (Fig. 5).

PG and CL synthesis in Apicomplexa: PG and CL synthesis has not been studied experimentally in apicomplexans. Predicted proteins sharing sequence similarities with mammalian and yeast PGP synthases and prokaryotic-type CL synthases are annotated in the genomes of *Plasmodium*, *Theileria* and *Toxoplasma* (Fig. 5). The putative enzymes show high homologies to the experimentally characterized PGP synthase and CL synthase of *T. brucei* [149].

The presence of prokaryotic-type CL synthases in Apicomplexa and Kinetoplastida indicates evolutionary conservation of the reaction mechanism into the eukaryotic kingdom [180]. The presence of CL synthases of bacterial origin in protozoan parasites validates this enzyme as potential drug target, however at present, functional studies addressing the essentiality of CL synthase in Apicomplexa are lacking.

3.3.4. Sphingophospholipids—The major higher-order sphingophospholipids include SM, IPC and EPC. In mammalian cells, SM is by far the most abundant sphingophospholipid class, whereas IPC is abundant in fungi and plants and EPC in *Drosophila melanogaster* [267].

Sphingophospholipid biosynthesis is initiated by condensation of L-serine and palmitoyl-CoA to 3-ketosphinganine by serine palmitoyltransferase, followed by reduction to dihydrosphingosine. Subsequently, ceramide synthase acylates dihydrosphingosine to dihydroceramide, which is then desaturated to ceramide, i.e. the common metabolite for the synthesis of sphingophospholipids (Fig. 4). The initial reactions in sphingolipid synthesis leading to ceramide formation occur in the ER [268]. After transport of ceramide to the Golgi, a set of sphingolipid synthases mediates transfer of polar head groups from glycerophospholipid donors to ceramide to produce sphingophospholipids. SM synthases catalyze transfer of phosphocholine using PC as substrate, whereas IPC and EPC synthases transfer phosphoinositol from PI and phosphoethanolamine from PE, respectively [267] (Fig. 4). Alternatively, ceramide can be modified by sugar residues to generate several glycosphingolipid classes.

Degradation of sphingolipids can occur via sphingosine-1-phosphate lyase, which breaks down phosphorylated sphingoid bases into ethanolamine phosphate and fatty aldehydes [214] (Fig. 4) (see also above).

Sphingolipid metabolism in Kinetoplastida: In contrast to mammals and plants, *Leishmania* parasites do not synthesize SM. Instead, the most abundant sphingophospholipid class in *Leishmania* is IPC, accounting for up to 10% of total membrane lipids [269]. Several enzymes involved in sphingophospholipid synthesis in *Leishmania* have been characterized experimentally (Fig. 5). The role of heterodimeric serine palmitoyltransferase, consisting of subunits Spt1 and Spt2, with Spt2 being the catalytic subunit, has been studied in *L. major*. Spt2 deletion mutants completely lacking ceramide and IPC were found to be viable, yet unable to differentiate into metacyclic forms [166]. In addition, a similar defect in differentiation into metacyclic forms has been observed in sphingosine-1-phosphate lyase knock-out strains [156]. These parasites are able to incorporate [^{3}H]serine into ceramide and IPC, but show greatly reduced labeling of PE and PC. Surprisingly, supplementing the culture media with ethanolamine fully restored the defects, indicating that *Leishmania* use sphingolipid metabolism for bulk ethanolamine phosphate production for glycerophospholipid synthesis [156]. Furthermore, candidate genes encoding IPC synthases have been identified in all kinetoplastids [270] (Fig. 5). The *L. major* enzyme, which shows little sequence homology to fungal IPC synthases, was able to complement a yeast strain deficient in IPC synthase and showed activity in human cells, where it localizes to the Golgi [270]. Finally, *Leishmania* parasites were found to remodel sphingophospholipids acquired from the host to retain normal IPC levels [271].

In *T. brucei* procyclic forms, down-regulation of Spt2 expression by RNAi, or inhibition of Spt2 activity by myriocin, showed that sphingolipid metabolism is essential for parasite growth in culture [272] (Fig. 5). Spt2-depleted cells showed reduced IPC levels, dysfunctional cytokinesis and a defect in kinetoplast segregation, resulting in parasites with aberrant DNA content [272]. Addition of the downstream metabolite, 3-ketosphinganine [272], but not ethanolamine [161], was able to rescue the growth defect of Spt2 knock-down cells, indicating that unlike *Leishmania*, *T. brucei* uses sphingoid base synthesis not primarily for ethanolamine-phosphate production.

Remarkably, a sphingolipid synthase gene family consisting of four tandemly linked genes, TbSLS1–4, which are orthologs of *L. major* IPC synthase, is present in *T. brucei* [162]. Using RNAi against a sequence common to all four sphingolipid synthase genes demonstrated that sphingolipid synthesis is essential for growth of *T. brucei* bloodstream forms in culture [162]. Expression of the enzymes in a cell-free system revealed that TbSLS1 and TbSLS2 produce IPC and EPC, respectively, whereas TbSLS3 and TbSLS4 are bi-functional and generate SM and EPC [273]. In addition, expression of TbSLS4 in *L. major* leads to the production of SM and EPC, confirming its bi-functional activity [162]. Interestingly, sphingophospholipid synthesis in *T. brucei* is developmentally regulated, with IPC and SM being produced in procyclic forms, while bloodstream forms lack IPC but instead contain small amounts of EPC [162]. TbSLS4 was found to co-localize with galactosyltransferase, suggesting Golgi localization [162]. In addition, cell-free expression of the single *T. cruzi* sphingolipid synthase ortholog, TcSLS1, showed that it is a dedicated IPC synthase [273]. It is worth mentioning that, in contrast to *T. brucei* and *Leishmania*, IPC in *T. cruzi* is of special importance as it serves as membrane anchor for most GPI proteins and GPI lipids [274].

Sphingolipid metabolism in Apicomplexa: Labeling experiments using [^{3}H]serine demonstrated *de novo* synthesis of SM and glycosphingolipids, but not IPC, in *P. falciparum* [248] (Fig. 5). More recently, using a combination of isotope labeling and mass spectrometry, several sulfoglycosphingolipid species were detected in intraerythrocytic stages of *P. falciparum* [275]. In *T. gondii*, *de novo* synthesis of ceramide, SM and complex glycosphingolipids has been demonstrated using [^{3}H]serine and [^{3}H]galactose labeling [174] (Fig. 5). The identification of an inositol-containing lipid resisting alkaline treatment

suggested the presence of IPC in *T. gondii* [276], however, a recent lipidomic analysis using mass spectrometry found no evidence of IPC [175]. Instead, the analysis revealed the presence of EPC, which was estimated to account for approximately 2% of total polar lipid content [175]. The genomes of *P. falciparum* and *T. gondii* contain two and one, respectively, putative SM synthase genes. However, it is not known if the encoded synthases are capable of synthesizing sphingolipids other than SM.

3.4. Lipid composition of parasite organelles

—It is well known that the lipid composition of the plasma membrane is different from that of intracellular organelles and, in addition, that each organelle has its own characteristic lipid profile [277]. This is related in part to the localization of the enzymes involved in lipid biosynthesis in different organelles and to the existence, or the absence, of lipid trafficking pathways between organelles [278, 279]. Reliable and detailed data on the lipid composition of eukaryotic organelles is scarce, mostly because it has been difficult to isolate organelles in sufficient amounts and with high enough purity, i.e. without contamination by other membranes, for lipid analysis. The situation in parasitic protozoa is no different. However, recent studies have reported the isolation of highly purified preparations of organelles from kinetoplastids and apicomplexans for proteomic studies [280–282]. This together with the latest improvements in lipid analysis, in particular in mass spectrometry, is expected to result in valuable new information on the lipid composition of parasite organelle membranes in the near future.

Lipids of subcellular compartments of Kinetoplastida: Analysis of the phospholipid composition of purified glycosomes from *T. brucei* bloodstream forms revealed the presence of only two phospholipids, PC and PE, with PC being twice as abundant as PE [283]. In contrast, purified glycosomes from *T. cruzi* epimastigotes showed the presence of all major phospholipid classes, PC, PE, PI, SM and PS [163]. Relative to total cell lipid, *T. cruzi* glycosomes contained increased levels of PC, PI and SM, as well as endogenously synthesized sterols [163]. In addition, mass spectrometric analysis of acidocalcisomes, acidic calcium-storage organelles found in many protozoan parasites [284], from *T. cruzi* epimastigotes showed the exclusive presence of PC, PE and PI molecular species [285]. Furthermore, labeling experiments using [³H]mannose revealed the presence of GPI lipids in isolated acidocalcisomes [285].

Although no direct lipid analyses have been performed, experiments measuring association of lipid-modified proteins with the flagellar membrane in *T. cruzi* [286], *T. brucei* [287] and *L. major* [288], together with laurdan microscopy to measure changes in liquid order of membrane domains in *T. brucei* [287], suggest that the lipid composition of the flagellum may be different, i.e. more raft-like, compared to the plasma membrane of the main cell body.

Lipids of subcellular compartments of Apicomplexa: Analysis of *T. gondii* tachyzoites revealed increased levels of PC (75% *versus* 62%) and PS (12% *versus* 8%) in an enriched pellicle fraction compared to total cells, whereas PE (7% *versus* 11%) and SM (1% *versus* 8%) levels were decreased [173]. The study also showed a decreased ratio of saturated to unsaturated fatty acids in phospholipids of enriched pellicle fractions compared to whole cells [173]. In addition, a study to determine the lipid composition of isolated rhoptries from *T. gondii* tachyzoites revealed PC as major phospholipid class (75% of total lipid), with small amounts of PE (9%), PA (6.5%) and lysophospholipids (1.8%), while PI, PS and SM were absent [289]. Remarkably, the cholesterol to phospholipid ratio in purified rhoptries was significantly increased compared to whole cells (1.48 *versus* 0.2), suggesting that the altered lipid composition may be important for host cell invasion [289].

4. Fatty acid and phospholipid synthesis pathways as drug targets

Because lipid biosynthetic pathways have long been considered of little importance for parasite survival, their potential as drug targets has not been systematically addressed. After a brief discussion of selected compounds known to affect lipid metabolism in Kinetoplastida and Apicomplexa, we highlight potentially interesting lipid pathways unique to certain members of these groups of parasites, or alternatively, are common to several parasite species but differ from the corresponding pathways in mammals.

4.1. Drugs targeting fatty acid synthesis

Drugs targeting FASII: Triclosan [49] and thiolactomycin [290, 291] are well studied inhibitors of type II FAS. Soon after the discovery of a FASII pathway in apicomplexan parasites, these drugs were tested for their efficacy against *Plasmodium*. Both triclosan [48, 50] and thiolactomycin [292] inhibit the growth of blood stage *Plasmodium* parasites. Additional drugs known to target bacterial fatty acid synthesis include epigallocatechin gallate [293], NAS-91 [294], and flavonoids [295], all of which were evaluated as anti-malarials. Green tea extracts containing epigallocatechin gallate, NAS-91 [45] or flavonoids [296] inhibit the growth of *P. falciparum* parasites *in vitro*. However, as detailed above, molecular analysis has shown the FASII pathway to be dispensable for growth of blood stage *Plasmodium* parasites. Therefore the anti-malarial activities of these drugs are likely due to mechanisms independent of FASII. This conclusion is further supported by the activity of epigallocatechin gallate against *Babesia,* an apicomplexan parasite lacking FASII [51, 297]. Similarly, NAS-91, which inhibits the dehydratase of mycobacterial FASII, was initially thought to kill *Plasmodium* by the same mechanism. However, a more recent study demonstrates that it may also inhibit mycobacterial SCD [298]. In contrast to FAS II, *Plasmodium* SCD is required for intraerythrocytic development of the parasite [129]. Thus, SCD rather than FASII dehydratase appears to be the likely target of NAS-91.

Thiolactomycin, which is thought to be a more specific FAS II inhibitor, as well as several compounds derived from thiolactomycin were found to inhibit growth of *T. gondii* [299], *T. cruzi, T. brucei* [300, 301], and *L. donovani* [301]. In *T. gondii* and *T. brucei*, where this has been studied in more detail, the activity of thiolactomycin is consistent with molecular data indicating that the FASII pathway is essential for growth [74, 83]. Similar to their activity in *Plasmodium*, green tea extracts containing catechins also inhibit *T. cruzi* [302]. Whether this effect was due to the activity of catechins against *T. cruzi* FASII pathway is not known.

Drugs targeting FASI and FAE: Cerulenin is a more general fatty acid synthesis inhibitor, whose activity is not limited to a particular type of FAS pathway. It is effective against human cancer cells by inhibiting mammalian FASI [303, 304]. Cerulenin has also been tested and proven effective in inhibiting *C. parvum* FASI [94]. In addition, in kinetoplastids lacking FASI but containing the FAE pathway, cerulenin has been suggested to be effective by inhibiting ELO-2, but not ELO-1 [118, 305]. However, cerulenin shows little activity against *C. parvum* fatty acid elongase [114]. A small number of specific inhibitors of fatty acid elongation has been identified. However, the activity of these inhibitors appears limited to a specific subtype of plant fatty acid elongases [115].

Drugs targeting FA desaturation: The identification of an active metabolism for unsaturated fatty acids in protozoan parasites has stimulated interest in desaturase inhibitors as anti-parasitic compounds. Isoxyl, which has been used for a long time in the treatment of tuberculosis, is known to inhibit bacterial SCD, and also inhibits growth of *T. cruzi* epimastigotes with an EC_{50} of 2 μM [306]. However, this drug typically has to be activated by a bacterial monooxygenase to be effective [307]. A homolog of this enzyme is not

evident when searching the genomes of apicomplexans and kinetoplastids. Further studies are needed to establish the target and mode of activation of isoxyl in parasites. Methylsterculate, a methylester of sterculic acid, inhibits the intraerythrocytic growth of *P. falciparum*. The anti-malarial activity of methylsterculate can be reversed by oleic acid supplementation, strongly implicating *P. falciparum* SCD as the likely target of this drug [129].

Thiastearates (TS) are another class of compounds that target the synthesis of unsaturated fatty acids. 10-TS has been shown to inhibit *T. cruzi* SCD [306], whereas 12-TS and 13-TS prevent the conversion of oleate to linoleate and inhibit the growth of *T. cruzi* [306] and *T. brucei* [308]. The enzyme responsible for conversion of oleate to linoleate is delta-12 oleate desaturase. This enzyme is present in kinetoplastid parasites but absent in the human host, making it a strong candidate for a parasite-specific target. Note that based on bioinformatic analyses, this enzyme is absent in apicomplexan parasites, suggesting that 12-TS and 13-TS are likely not effective against Apicomplexa.

4.2. Drugs targeting phospholipid synthesis

Miltefosine (hexadecyl phosphocholine) represents the first orally active anti-leishmanial and one of only two compounds that have been developed and approved to treat visceral leishmaniasis in the last 25 years [309]. It is a member of a class of synthetic alkylphospholipids showing anti-proliferative effects on parasites and tumor cells, by interfering with lipid metabolism [310, 311]. Possible modes of action of miltefosine in *Leishmania* may involve inhibition of PE *N*-methyltransferase and CDP-phosphocholine cytidylyltransferase activity [229], or by blocking choline uptake from the environment [181], resulting in changes in parasite PC and PE contents. Similar effects have also been reported after miltefosine treatment of *T. cruzi* epimastigotes [312]. In addition, miltefosine also inhibits recombinant *P. falciparum* PE *N*-methyltransferase and shows anti-malarial activity on intraerythrocytic parasites with an IC_{50} of approximately 80 μM [225].

The observation that *Leishmania* and *Trypanosoma* synthesize IPC, a sphingophospholipid class typical for fungi, prompted a series of studies to investigate the effects of the anti-fungal drug, aureobasidin A, an inhibitor of fungal IPC synthase at sub-nanomolar concentrations [313], on Kinetoplastida. The results showed that although the drug at high concentrations showed moderate effects on proliferation of *L. major* promastigotes and on differentiation of *T. cruzi* amastigotes, it did not act on IPC formation *in vivo* or *in vitro* [270, 271, 314]. In addition, although it has been reported that aureobasidin A blocks the activity of TbSLS4 expressed in yeast and inhibits growth of *T. brucei* bloodstream forms in culture [315], the drug showed no effect on TbSLS4 expressed in a cell-free system [273]. Conversely, aureobasidin A treatment inhibited general sphingolipid synthesis and replication in *T. gondii* parasites lacking IPC [276], suggesting unspecific, i.e. off-target effects of the drug.

Sphingophospholipid synthesis has also been proposed as drug target against malaria parasites. Earlier work reported that 1-phenyl-2-palmitoylamino-3-N-morpholine-1-propanol blocks SM synthase activity and inhibits intraerythrocytic maturation of *P. falciparum* [316]. However, more recent work demonstrated that other well-characterized inhibitors of ceramide synthesis, such as fumonisin B1 and myriocin, showed no effect on intraerythrocytic parasite development, and only partly affected *Plasmodium* sphingolipid synthesis [248], suggesting that pathways other than *de novo* synthesis may provide *Plasmodium* parasites with sphingolipids. One such alternative route may involve hydrolysis of host sphingophospholipids and uptake of sphingolipid intermediates [316, 317].

Recently, a phospholipid analog affecting *P. falciparum* development and intraerythrocytic maturation through inhibition of PC synthesis via blocking CTP-phosphocholine cytidylyltransferase has been described [318]. Treatment of parasitized erythrocytes with the novel compound, PG12, lead to strongly reduced PC synthesis using choline or ethanolamine as substrates, indicating that both the CDP-choline and the serine-decarboxylase phosphoethanolamine N-methyltransferase pathways are affected by PG12 [318]. In a different study aimed at identifying inhibitors of *P. falciparum* phosphoethanolamine N-methyltransferase, two compounds were found to inhibit the enzyme both *in vitro* and *in vivo*, using a choline auxotroph yeast strain complemented with the plasmodial enzyme [319]. Finally, PC synthesis has been evaluated as drug target in *Plasmodium* and *Trypanosoma* by generating a library of compounds mimicking the choline structure. Some of these drugs showed strong anti-malarial activity, by interfering with the synthesis of PC via the Kennedy pathway [205, 206, 320, 321], and were able to cure malaria-infected monkeys [322]. More recently, a selection of these first-, second- and third-generation choline analogs were found to also have anti-leishmanial and anti-trypanosomal activity [184]. However, in contrast to their mode of action in *Plasmodium*, they did not affect lipid metabolism in *L. major* and *T. brucei* bloodstream forms but disrupted mitochondrial function.

Acknowledgments

Abbreviations

ACC	acetyl-CoA carboxylase
ACP	acyl carrier protein
CEPT	CDP-choline/ethanolamine:diacylglycerol phosphotransferase
CL	cardiolipin
EPC	ethanolamine phosphorylceramide
EPT	CDP-ethanolamine:diacylglycerol phosphotransferase
ER	endoplasmic reticulum
FAE	fatty acid elongation
FASI	fatty acid synthase type I
FASII	fatty acid synthase type II
GPI	glycosylphosphatidylinositol
IPC	inositol phosphorylceramide
MSP1	merozoite surface protein-1
MUFAs	monounsaturated fatty acids
PC	phosphatidylcholine
PE	phosphatidylethanolamine
PG	phosphatidylglycerol

PGP	phosphatidylglycerophosphate
PI	phosphatidylinositol
PKS	polyketide synthase
PS	phosphatidylserine
PUFAs	polyunsaturated fatty acids
RNAi	RNA interference
SM	sphingomyelin
SCD	stearoyl-CoA desaturase
TS	thiastearates

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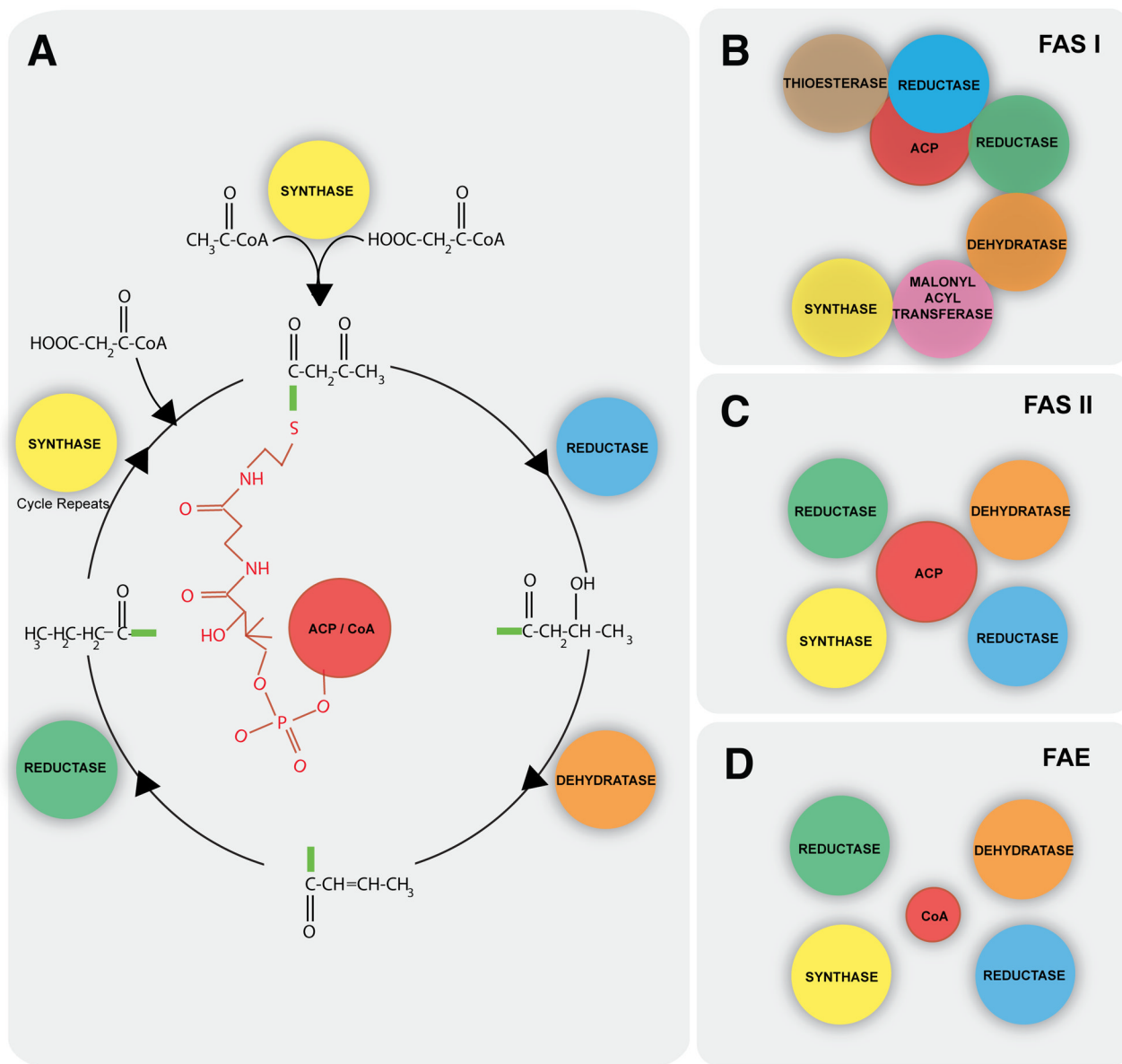
Figure 1. Three mechanisms of fatty acid synthesis
A, All fatty acid synthesis mechanisms follow a similar sequence of enzymatic reactions. A starter molecule is transferred to the phosphopantetheinyl group on ACP or CoA. The starter is then elongated by two carbon atoms and involving a four-reaction mechanism: decarboxylative condensation with a malonyl group by a synthase, reduction by a ketoreductase, dehydration by a dehydratase and reduction by an enoyl reductase. The product is released, or condensation with another malonyl group initiates the next round of elongation. **B**, Fatty acid synthase type I. All enzymes are domains of a single polypeptide (note that the apicomplexan FASI has a more complex multimodular architecture). **C**, Fatty acid synthase type II. All enzymes are encoded as individual proteins. **D**, Fatty acid elongation pathway. The system consists of enzymes encoded as individual proteins, acting on a CoA-bound starter molecule–typically a longer fatty acid (16 carbon or longer). ACP, acyl carrier protein; CoA, Coenzyme-A; FASI, fatty acid synthase type I pathway; FASII, fatty acid synthase type II pathway; FAE, fatty acid elongation pathway.
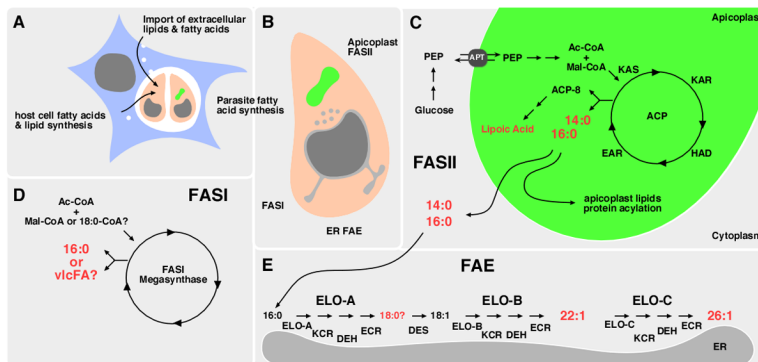
Figure 2. Apicomplexans can acquire fatty acids through a complex network of synthesis and uptake

A, *T. gondii* is shown as a representative apicomplexan parasite (pink) and intracellular pathogen capable of fatty acid and lipid salvage from the host cell (blue). This process can intersect host cell import as well as synthesis routes. **B**, In addition, the parasite harbors three fatty acid synthesis pathways that are localized to different cellular compartments. **C**, Apicoplast (green)-localized FASII pathway produces significant amounts of myristic and palmitic acid, in addition to lipoic acid relying on cytoplasmic glycolysis for precursors. **E**, Apicomplexan parasites also maintain an ER-associated elongase system that synthesizes very long chain monounsaturated fatty acids, subsequently using the activity of ELO-B and ELO-C. **D**, Apicomplexan FASI remains largely uncharacterized. Its stage-specific expression pattern and localization are not established. It is also unclear whether this megasynthase synthesizes fatty acids *de novo*, like the FASI of humans, or acts as an elongase for saturated fatty acids, as demonstrated for FASI of *C. parvum*.

Major products are highlighted in red. Des, desaturase; PEP, phosphoenolpyruvate; Mal, malonate; Ac, acetate; vlcFA, very long chain fatty acids; Ac-CoA, acetyl-CoA; Mal-CoA, malonyl-CoA; ER, endoplasmic reticulum; KAS, ketoacyl-ACP synthase; KAR, ketoacyl-ACP reductase; HAD, hydroxyacyl-ACP dehydratase; EAR, enoyl-ACP reductase; ELO, elongase; KCR, ketoacyl-CoA reductase; DEH, acyl-CoA dehydratase; ECR, enoyl-CoA reductase. Other abbreviations are as in the legend of Fig. 1. Reproduced with minor modifications from [74].
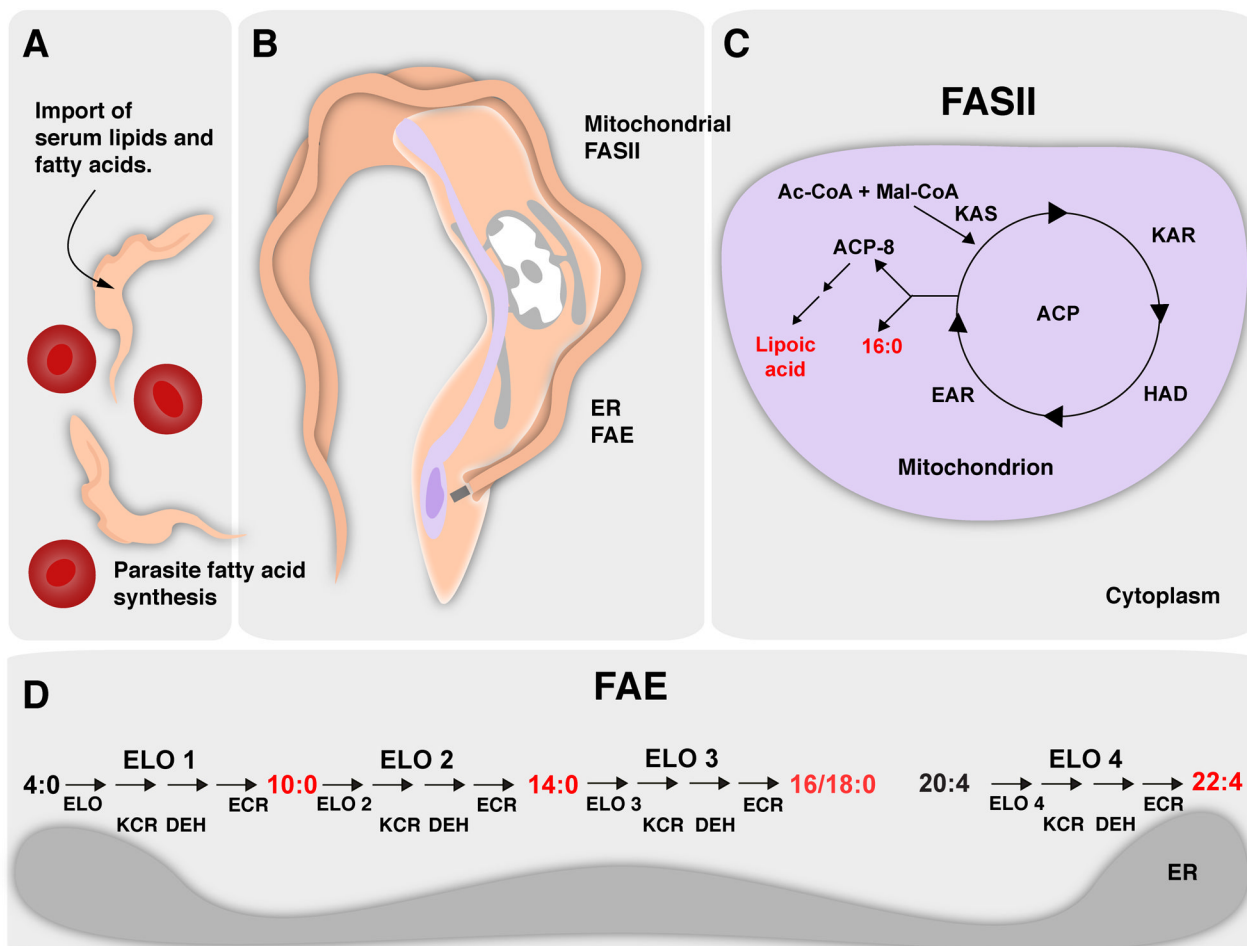
Figure 3. Kinetoplastid fatty acid synthesis occurs in the mitochondrion and the ER

Kinetoplastids obtain fatty acids using synthesis and import mechanisms. **A**, Kinetoplastid parasites replicate extracellularly in the bloodstream of the mammalian host, red blood cells are also shown (red). **B**, Additionally, the parasite harbors two mechanisms of fatty acid synthesis that are localized in two different organelles (redrawn in part after [323]). **C**, A FASII pathway localizes to the mitochondrion (light violet), where it is required for the synthesis of lipoic acid and palmitic acid. **D**, Kinetoplastid parasites also harbor an ER-localized fatty acid elongase system. Unlike all other organisms, kinetoplastid FAE is used for *de novo* synthesis of fatty acids. The kinetoplastid FAE uses butyrate and malonate as substrates to generate myristate/stearate and adrenate as products. Major products are highlighted in red. Mal, malonate; Ac, acetate. Other abbreviations are as in the legend of Fig. 2.
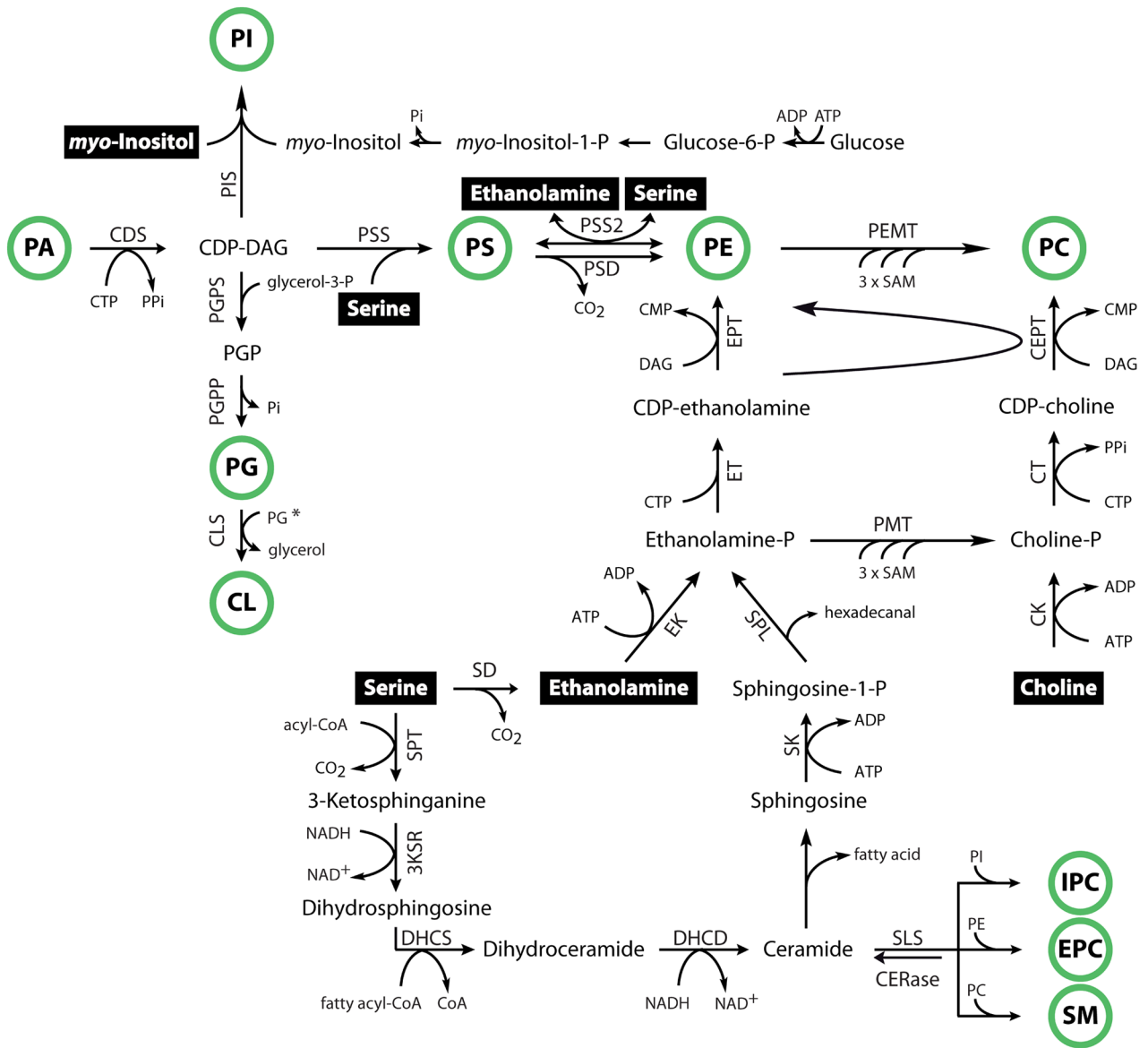
Figure 4. Phospholipid synthesis pathways in kinetoplastids and apicomplexans
Schematic overview on the major pathways for phospholipid synthesis in kinetoplastid and apicomplexan parasites. Metabolites taken up from the environment appear in a black box, major phospholipid classes are indicated by a green circle. 3KSR, 3-ketosphinganine reductase; CDS, cytidine diphosphate diacylglycerol synthase; CEPT, choline/ethanolamine phosphotransferase; CK, choline kinase; CLS, cardiolipin synthase; CT, choline-phosphate cytidylyltransferase; DAG, diacylglycerol; DHCD, dihydroceramide desaturase; DHCS, dihydroceramide synthase; EK, ethanolamine kinase; PMT, phosphoethanolamine N-methyltransferase; EPT, ethanolamine phosphotransferase; ET, ethanolamine-phosphate cytidylyltransferase; PEMT, PE N-methyltransferase; PGPP, PGP phosphatase; PGPS, PGP synthase; PIS, PI synthase; PSD, PS decarboxylase; PSS/PSS2, PS synthase/PS synthase-2; SAM, S-adenosyl methionine; SD, serine decarboxylase; SLS, sphingolipid synthase; SPL, sphingosine-1-phosphate lyase; SPT, serine palmitoyltransferase. * the substrate for prokaryotic-type CLS in protozoan parasites has not been confirmed experimentally.
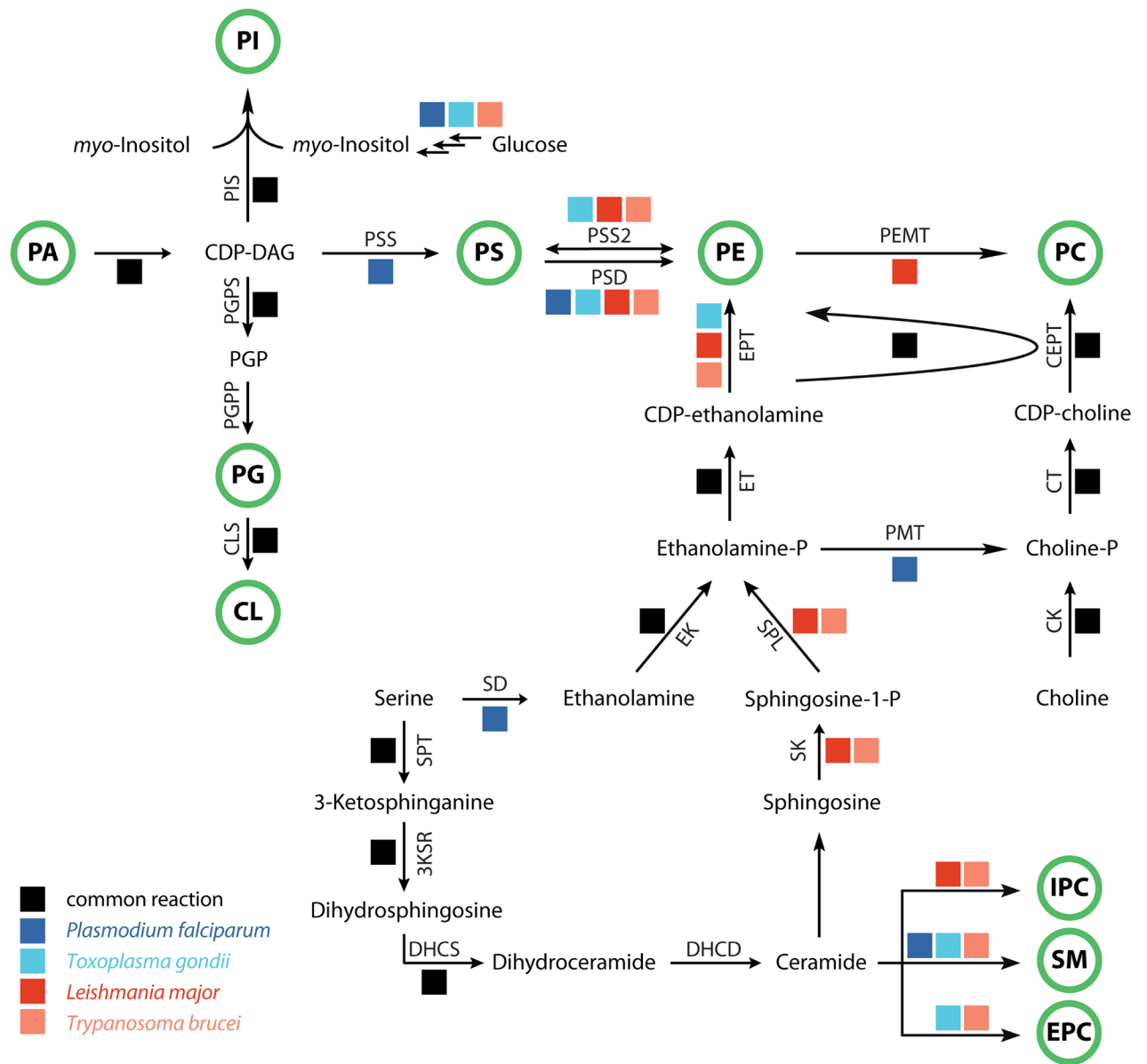
Figure 5. Common and unique phospholipid synthesis pathways in kinetoplastids and apicomplexans

Schematic representation of lipid biosynthetic pathways that are common (black squares) or unique (colored squares) to selected apicomplexan and kinetoplastid parasites to indicate their potential as drug targets. The colored squares indicate experimental evidence of enzyme activity or the presence of a predicted gene in the genome of a given parasite species. The absence of a square indicates that no enzyme activity or candidate gene has been reported. Abbreviations are as in the legend of Fig. 4.

Table 1

Names and EuPathDB gene identification numbers for enzymes of fatty acid synthesis and modification pathways in representative kinetoplastids and apicomplexan parasites.

		T. brucei	*L. major*	*P. falciparum*	*T. gondii*
ELO pathway					
ELO1	Fatty acid elongase A	Tb927.7.4180	Lmjf.14.0640 Lmjf.14.0650 Lmjf.14.0660	PF3D7_0920000	TGME49_042380
ELO2	Fatty acid elongase B	Tb927.7.4170	Lmjf.14.0670	PF3D7_0605900	TGME49_005350
ELO3	Fatty acid elongase C	Tb927.7.4160	Lmjf.14.0680 Lmjf.14.0690	PF3D7_0109300	TGME49_053880
ELO4	Fatty acid elongase D	Tb927.5.4530	Lmjf.05.1170	-	-
KCR	Ketoacyl CoA reductase	Tb927.5.1210	LmjF.34.0010	PF3D7_0422000	TGME49_071890
HCD	Hydroxyacyl CoA reductase	Tb927.10.10610	LmjF.05.0280	PF3D7_1331600	TGME49_111290
ECR	Enoyl CoA reductase	Tb927.3.1840	LmjF.25.1770	PF3D7_1135900	TGME49_085240
FASII pathway					
ACP	Acyl carrier protein	Tb927.1.2100	LmjF.27.0290	PF3D7_0208500	TGME49_064080
KAR	Ketoacyl ACP reductase	Tb927.2.5210	LmjF27.2440	PF3D7_0922900	TGME49_017740
HAD	Hydroxyacyl ACP dehydratase	Tb927.8.1440	LmjF07.0430 LmjF07.0440	PF3D7_1323000	TGME49_121570
EAR	Enoyl ACP reductase	Tb927.10.11930	LmjF.04.0290	PF3D7_0420200	TGME49_051930
PPTase	Phosphopantetheinyl transferase	Tb927.3.1840	LmjF.25.1770	PF3D7_0420200	TGME49_003420
FASI pathway					
FASI	Fatty acid synthase type I	-	-	-	TGME49_094820
PPTase	Phosphopantetheinyl transferase	-	-	-	TGME49_014440

Table 2

Comparison of fatty acid synthesis and modification pathways in apicomplexan and kinetoplastid parasites. The presence (+) or absence (−) of specific pathways is shown for well-studied model species.

Species	FASII		FASI	PKS	FAE	
	Apicoplast	Mitochondrion			De novo	Elongation
Trypanosoma brucei	−	+	−	−	+	−
Trypanosoma cruzi	−	+	−	−	+	−
Leishmania major	−	+	−	−	+	−
Theileria annulata	−	−	−	−	−	−
Babesia bovis	−	−	−	−	−	−
Plasmodium falciparum	+	−	−	−	−	+
Toxoplasma gondii	+	−	+	+	−	+
Eimeria tenella	+	−	+	+	−	+
Cryptosporidium parvum	−	−	+	+	−	+

Table 3

Names and EuPathDB gene identification numbers for enzymes of phospholipid synthesis pathways in representative kinetoplastids and apicomplexan parasites.

Phospholipid pathways		*T. brucei*	*L. major*	*P. falciparum*	*T. gondii*
3KSR	3-Ketosphinganine reductase	Tb927.10.4040	LmjF.35.0030	?	?
CEPT	Choline/ethanolamine phosphotransferase	Tb927.10.8900	LmjF.36.5900	PF3D7_0628300	TGME49_261760
CK	Choline kinase	Tb11.18.0017	LmjF.27.1420	PF3D7_1401800	TGME49_320630
CLS	Cardiolipin synthase	Tb927.4.2560	LmjF.34.2110	PF3D7_0609400	TGME49_309940
CT	Choline-phosphate cytidylyltransferase	Tb927.10.12810	LmjF.18.1330	PF3D7_1316600	TGME49_216930
DHCS	Dihydroceramide synthase	Tb927.8.7730 Tb927.4.4740	LmjF.31.1780	?	?
EK	Ethanolamine kinase	Tb927.5.1140	LmjF.35.1470	PF3D7_1124600	TGVEG_081320
EPT	Ethanolamine phosphotransferase	Tb927.10.13290	LmjF.18.0810	-	?
ET	Ethanolamine-phosphate cytidylyltransferase	Tb11.01.5730	LmjF.32.0890	PF3D7_1347700	TGME49_310280
INO	*myo*-Inositol-3-phosphate synthase	Tb927.10.7110	LmjF.14.1360	PF3D7_0511800	-
PEMT	Phosphatidylethanolamine N- methyltransferase	-	LmjF.31.2290 LmjF.31.3120	-	-
PGPP	Phosphatidylglycerophosphate phosphatase	?	?	?	?
PGPS	Phosphatidylglycerophosphate synthase	Tb927.8.1720	LmjF.07.0200	PF3D7_0820200	TGVEG_005460
PIS	Phosphatidylinositol synthase	Tb09.160.0530	LmjF.26.2480	PF3D7_1315600	TGME49_207710
PMT	Phosphoethanolamine N- methyltransferase	-	-	PF3D7_1343000	-
PSD	Phosphatidylserine decarboxylase	Tb09.211.1610	LmjF.35.4590	PF3D7_0927900	TGME49_269920 TGME49_225550
PSS	Phosphatidylserine synthase	-	-	PF3D7_1366800	-
PSS2	Phosphatidylserine synthase 2	Tb927.7.3760	LmjF.14.1200	-	TGME49_261480
SD	Serine decarboxylase	-	-	?	-
SK	Sphingosine kinase	Tb927.7.1240	LmjF.26.0710	-	-
SLS	Sphingolipid synthase	Tb09.211.1000 Tb09.211.1010 Tb09.211.1020 Tb09.211.1030	LmjF.35.4990	PF3D7_0625000 PF3D7_0625100	?
SPL	Sphingosine-1-phosphate lyase	Tb927.6.3630	LmjF.30.2350	-	-
SPT	Serine palmitoyltransferase	Tb927.4.1020	LmjF.34.3740	PF3D7_1415700	TGVEG_084380