A 3-hydroxyacyl-CoA dehydratase (DEH) involved in very long chain fatty acid synthesis is crucial for oocyst mitotic division but not cell size during parasite transmission in *Plasmodium*

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**Running title:** DEH is essential for sporogony

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Abstract
Lipid metabolism is a crucially important area of biochemistry, and cells use fatty acids for membrane biosynthesis, energy storage and the generation of signaling molecules. A 3-hydroxyacyl-CoA dehydratase – DEH – is a key member of this process. Here we further characterized in-depth the location and function of DEH, applying in-silico analysis, live cell imaging, reverse genetics and ultrastructure analysis using the mouse malaria model *Plasmodium berghei*. We show that DEH is evolutionarily conserved across eukaryotic species, with a single DEH in *Plasmodium* spp. and up to three orthologs in the other eukaryotes studied. Using DEH-GFP live-cell imaging, we observed strong GFP fluorescence throughout the life-cycle, with areas of localized expression in the cytoplasm and a circular ring pattern around the nucleus that colocalized with ER markers. Functional analysis showed that while Δ*deh* mutants undergo normal oocyst cell growth during early stages of sporogony, endomitotic cell division and sporozoite development are completely ablated, blocking parasite transmission from mosquito to vertebrate host. Ultrastructure analysis confirmed degeneration of Δ*deh* oocysts, and a complete lack of sporozoite budding. Overall, we show that DEH is evolutionarily conserved, localizes to the ER and plays a crucial role in sporogony, potentially through its involvement in fatty acid production.
Introduction

Malaria remains one of the world’s deadliest infectious diseases. Caused by apicomplexan parasites belonging to genus *Plasmodium*, malaria is responsible for great socio-economic loss to affected countries. According to WHO reports, there were 212 million clinical cases of malaria infection and 429000 deaths in 2015 (Organisation, 2018), and growing resistance against existing drugs has further intensified this problem. Hence, there is a growing need to identify new biological pathways and proteins essential for parasitic growth and development in human hosts, which could act as suitable drug targets. *Plasmodium* parasites have a complex life cycle and require two hosts to complete the life cycle: vertebrates (during asexual stages) and invertebrates (during sexual stages) (Aly *et al.*, 2009). The disease is transmitted to vertebrate hosts by infected female *Anopheles* mosquitoes, which inject sporozoites into the dermis of the vertebrate host during a blood meal. The parasite enters the circulation, and once it invades the liver, and subsequently erythrocytes, undergoes several rounds of atypical closed mitotic cell division through multiple rounds of DNA replication and asynchronous nuclear division (termed schizogony) to produce merozoites that invade erythrocytes. During this period of cyclic asexual proliferation in the blood stream, a subpopulation undergoes gametocytogenesis to develop into male and female gametocytes, which are transmitted to a mosquito during its blood meal. Gamete development, fertilization and zygote formation occur in the mosquito midgut, leading to the differentiation of an infective ookinete, which undergoes meiosis, and penetrates the midgut wall to develop into an oocyst on the basal surface of the midgut, where further rounds of closed mitotic cell division occur. Thousands of sporozoites develop within each oocyst, and then egress into the haemocoel to invade the salivary glands and begin a new life cycle.

Lipid metabolism includes essential cellular processes that use fatty acids (FAs) in membrane biosynthesis, energy storage and the generation of signaling molecules. FA elongation and very long chain fatty acid (VLCFA) synthesis occurs in two stages, both consisting of a four-step cyclic process that results in addition of two carbons to the chain with each cycle. In humans, the process involves condensation of acyl-CoA with
malonyl-CoA to produce 3-ketoacyl-CoA (catalyzed by one of seven FA elongases), reduction of 3-ketoacyl-CoA by a 3-ketoacyl-CoA reductase (KAR) to 3-hydroxyacyl-CoA, dehydration of 3-hydroxyacyl-CoA to 2,3-trans-enoyl-CoA (catalysed by one of four 3-hydroxyacyl-CoA dehydratase isoenzymes: HACD1-4), and finally reduction to an acyl-CoA with two additional carbon chain units by 2,3-trans-enoyl-CoA reductase (TER) (Kihara, 2012). HACD1-4 were initially annotated as PTPLA, PTPLB, PTPLAD1 and PTPLAD2, respectively due to their similarities to the yeast Phs1 gene product (Ikeda et al., 2008). However, they were renamed to reflect their function as 3-hydroxyacyl Co-A dehydratases and their relatedness. HACD1 and HACD2 genes restored growth of yeast SAY32 Phs1-defective cells, indicating that they are functional homologues of Phs1, i.e. 3-hydroxyacyl-CoA dehydratases. Further, studies have indicated that HACD1 has an essential role in myoblast proliferation and differentiation (Lin et al., 2012), with HACD1-deficient cell lines displaying S-phase arrest, compromised G2/M transition and retarded cell growth. Studies of the *Arabidopsis thaliana* Phs1 homologue PASTICCINO2 or PAS2 showed the protein has an essential role in VLCFA synthesis (Bach et al., 2008), as well as being essential during cell division, proliferation and differentiation (Bellec et al., 2002). Further, *Arabidopsis* PAS2 complements Phs1 function in a yeast mutant defective for FA elongation (Morineau et al., 2016). PAS2 interacted with FA elongase subunits in the endoplasmic reticulum (ER) and in its absence 3-hydroxyacyl-CoA accumulates, as expected from loss of a dehydratase involved in FA elongation. Similarly, in the yeast *Saccharomyces cerevisiae* VLCFA synthesis is also catalyzed in the ER by a multi-protein elongase complex, following a similar reaction pathway as mitochondrial or cytosolic fatty acid synthesis (Tehlivets et al., 2007).

In Apicomplexans, the process of fatty acid synthesis and assembly into more complex molecules is critical for their growth and development, while also determining their ability to colonize the host and to cause disease. They acquire lipids through *de novo* synthesis and through scavenging from the host (Mazumdar et al., 2007), and simple components like mosquito-derived lipids determine within-host *Plasmodium* virulence by shaping sporogony and metabolic activity, affecting the quantity and quality of
sporozoites, respectively (Costa et al., 2018). Fatty acid synthesis (FAS) occurs in the apicoplast via the type II FAS (FASII) pathway, followed by fatty acid elongation (FAE) on the cytoplasmic face of the ER through the elongase (ELO) pathway (Ramakrishnan et al., 2012, Ramakrishnan et al., 2013). Studies on whether FAS is essential in Plasmodium are contradictory. In Plasmodium yoelii the FASII enzymes are only essential during liver stages (Yu et al., 2008, Vaughan et al., 2009); whereas in Plasmodium falciparum, genetic disruption of the FASII enzymes FabI and FabB/F results in complete abolition of sporogony (van Schaijk et al., 2014). Specifically, day 17 to day 23 after mosquito feeding, FabB/F mutant oocysts appeared to degenerate, and expression of the marker GFP was barely detectable, confirming that VLCFA synthesis is crucial for commencement of sporogony. The enzymatic steps of the ELO process are similar to those in the FASII pathway in the apicoplast (Tarun et al., 2009); however, the growing chain is held by CoA instead of acyl carrier protein (ACP). In Toxoplasma gondii, the activity of the ELO-pathway is considered an alternative route to FASII-independent $^{14}$C-acetate incorporation (Bisanz et al., 2006) and is engaged in conventional elongation rather than de novo synthesis (Mazumdar et al., 2007). Indeed, P. falciparum parasites with no functional FASII pathway can still elongate fatty acids; possibly because of the activity of the ELO pathway (Yu et al., 2008).

In a genome-wide study of Plasmodium berghei (Pb) protein phosphatases, we identified 30 phosphatase genes together with one for a predicted protein tyrosine phosphatase-like protein, PbPTPLA, which was shown to be essential for sporozoite formation and completion of the parasite life cycle, but not fully characterized (Guttery et al., 2014). However, despite the original annotation as an inactive PTP-like protein (Andreeva et al., 2008, Wilkes et al., 2008, Guttery et al., 2014, Pandey et al., 2014), more recent functional studies indicate that it is a key component of the VLCFA elongation cycle – more specifically the ELO pathway as a 3-hydroxyacyl-CoA dehydratase (DEH) (Stanway et al., 2019). Therefore, to investigate further the role of DEH in Plasmodium development, we performed an in-depth genotypic and phenotypic analysis of the protein, using in-silico, genetic manipulation and cell biological techniques. We show evolutionary conservation of DEH in the model organisms
examined here. Furthermore, we show that PbDEH is located at the ER and is essential for cell division and parasite budding within oocyst but not cell growth of oocysts, thereby blocking parasite transmission.

**Methods**

**Ethics statement**

All animal work was performed following ethical approval and was carried out under United Kingdom Home Office Project Licence 40/3344, in accordance with the UK ‘Animals (Scientific Procedures) Act 1986’ and in compliance with ‘European Directive 86/609/EEC’ for the protection of animals used for experimental purposes. Six- to eight-week old female Tuck-Ordinary (TO) (Harlan) or Swiss Webster (Charles River) outbred mice were used for all experiments.

**Identification of conserved domains and evolutionary lineage**

The deduced amino acid sequence of PBANKA_134650 (PbPTPLA) now classified as DEH in the manuscript, was retrieved from PlasmoDB (release 27) (Aurrecoechea et al., 2009). Conserved domains in PbPTPLA (DEH) were identified using the Conserved Domain Database (CDD) (Marchler-Bauer et al., 2011), the Simple Modular Architecture Research Tool (SMART) (Schultz et al., 1998) and Protein Family Database (PFAM) (Finn et al., 2008). The deduced amino acid sequence and individual conserved domains were used as BLAST (BLASTP) queries to identify orthologues in PlasmoDB and NCBI protein databases. OrthoMCL database (version 5) was used to identify and retrieve *P. berghei* orthologues (Table S1) (Li et al., 2003). Multiple sequence alignment was performed for the retrieved sequences using ClustalW (Larkin et al., 2007). ClustalW alignment parameters included gap opening penalty (GOP) 10 and gap extension penalty (GOE) 0.1 for the pairwise sequence alignment; GOP 10 and GOE 0.2 was used for multiple sequence alignment. A gap separation distance cut-off of 4 and Gonnet protein weight matrix was used for the alignments. Residue-specific penalty and hydrophobic penalties were used, whereas end gap separation and negative matrix were excluded in the ClustalW alignments. The phylogenetic tree was inferred using the neighbor-joining method, computing the evolutionary distances using the Jones-Taylor-
Thornton (JTT) model for amino acid substitution with the Molecular Evolutionary Genetics Analysis software (MEGA 6.0) (Tamura et al., 2013). Gaps and missing data were treated using a partial deletion method with 95% site-coverage cut-off and 1000 bootstrap replicates to generate a phylogenetic tree. iTOL was used to visualize the phylogenetic tree (Letunic et al., 2019). The STRING database was used to identify PTPLA interacting proteins (Franceschini et al.). For STRING search, we used a cut-off of 0.70 for the parameters of neighborhood, gene fusion, co-occurrence, co-expression, experiments, databases and text mining results. Predotar (Small et al., 2004) was used for inferring Pf and PbDEH subcellular localization.

**Generation of transgenic parasites and genotype analysis**
Details of GFP-tagged PTPLA (termed DEH-GFP in this study) and deh (PBANKA_1346500) knockout (KO) parasite lines (Δdeh in this study) are given in (Guttery et al., 2014). For this study, the KO construct was transfected into the GFPCON wild-type line (Janse et al., 2006), with 3 clones produced by serial dilution.

**Parasite development in the mosquito**
*Anopheles stephensi* mosquitoes (3–6 days old) were allowed to feed on anaesthetized mice infected with either wild type or mutant parasites at comparable gametocytemia as assessed by blood smears. Mosquitoes were dissected post-blood meal, on the days indicated. For midgut and salivary gland sporozoites, organs from 10-20 mosquitoes were pooled and homogenized, and released sporozoites were counted using a haemocytometer. For oocyst counts, midguts taken at day 7, 14 and 21 post-infection were harvested, mounted on a slide and oocysts counted using phase or fluorescence microscopy. To quantify sporozoites per oocyst (the ratio of number of sporozoites to number of oocysts), an equal number of mosquitoes from the same cage were used to count the number of oocysts and sporozoites. This number varied among experiments but at least 20 mosquitoes were used for each count. For light microscopy analysis of developing oocysts, at least 20 midguts were dissected from mosquitoes on the indicated days and mounted under Vaseline-rimmed cover slips. ER tracker (ThermoFisher) was used to perform co-localization studies according to manufacturer's
instructions. Images were collected with an AxioCam ICc1 digital camera fitted to a Zeiss AxioImager M2 microscope using a 63x oil immersion objective. Statistical analyses were performed using Graphpad prism software.

**Electron Microscopy**
The guts from mosquitoes harvested at 14 and 21 days post-infection were dissected and fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer and processed for electron microscopy (Guttery et al., 2012).

**Subcellular fractionation of parasite lysates and detection of DEH**
Immunoprecipitation and subcellular fractionation of lysates containing GFP tagged DEH was performed as described previously (Guttery et al., 2014). WT-GFP was used as the control protein in all experiments. In summary, cells from mouse blood infected with the DEH-GFP-expressing parasite were pelleted and then lysed in hypotonic buffer (10 mM Tris-HCl pH 8.4, 5 mM EDTA) containing protease inhibitors (Roche), freeze/thawed twice, incubated for 1 hr at 4°C and then centrifuged at 100,000 g for 30 min. The supernatant was collected as the soluble protein fraction (cytosol). The pellet was resuspended and washed in carbonate solution (0.1M Na₂CO₃, pH 11.0) containing protease inhibitors (Roche), and after incubation for 30 min at 4°C the sample was centrifuged again at 100,000 g for 30 min. The supernatant was saved as the peripheral membrane protein fraction (PMF). The residual pellet was solubilized in 4% SDS and 0.5% Triton X-100 in PBS, to form the integral membrane protein fraction (IMF). Samples from these three fractions, containing equal amounts of protein, were then analyzed by western blot using anti-GFP antibody.

**Results**
**Phylogenetic analysis reveals that DEH is highly conserved among eukaryotes**
Genome-wide analysis showed DEH is present in all the eukaryotic organisms studied here, which includes apicomplexans, yeast, fungi, plants, nematodes, insects, birds and mammals. The number of encoded DEH proteins was shown to vary from one to three in the studied organisms, with *Plasmodium* spp coding for a single DEH. Intriguingly,
both Arabidopsis thaliana and Oryza sativa encode three DEHs (PAS2 and 2 HACD isozymes) each, as compared to two (HACD1 and HACD2) in Homo sapiens (plus two sharing relatively weak similarity – HACD3 and HACD4) and two in Mus musculus. Phylogenetic analysis using the neighbor joining method, clustered organisms based on their evolutionary relatedness (Figure S1, Table S1). In addition, the phylogenetic analysis suggests that gene duplication in non-chordata, chordata and plants where there are multiple DEH copies, may have happened from a single DEH gene independently to perform specific functions after divergence during evolution, based on the clustering of all DEH isoforms in the same cluster.

Plasmodium DEH does not contain the canonical PTPLA CXXGXXP motif and is predicted in silico to interact with factors associated with FAE

P. berghei (PBANKA_1346500) and P. falciparum (Pf; PF3D7_1331600) DEH genes are annotated as PTPLA (pfam04387) (Andreeva et al., 2008, Wilkes et al., 2008, Guttery et al., 2014, Pandey et al., 2014), the criterion for PTPLA being the presence of a PTP active site motif (CXXGXXR) but with arginine replaced by proline (CXXGXXP). However, CLUSTALW alignment of Pb and Pf protein sequences with the human and mouse HACD1 and HACD2 shows this motif is absent (Figure S2), indicating that Plasmodium DEHs cannot be classified as PTP-like proteins. Furthermore, STRING database analysis predicts that PfDEH interacts with FAE and FAS proteins, and many other proteins with an ER location (Figure S3). These proteins include 3-oxo-5-α-steroid 4-dehydrogenase (PBANKA_09127; PF3D7_1135900), Stearoyl-CoA δ-9 desaturase (PBANKA_1110700; PF3D7_0511200), putative long chain polyunsaturated fatty acid elongation enzyme (ELO-B, PBANKA_0104700; PF3D7_0605900 - involved in the FAE pathway) as well as β-hydroxyacyl-(Acyl-carrier-protein) dehydratase (FabZ), involved in stage 3 of fatty acid synthesis in the FASII pathway (Stanway et al., 2019). In addition, interactome analysis revealed DEH interaction with a putative ER membrane protein, Acetyl-CoA transporter protein (PF10_0360).

DEH is expressed throughout the Plasmodium life-cycle stages and localized to the ER
To determine the expression profile and location of PbDEH, we used a single homologous recombination strategy to tag the 3’ end of the endogenous deh locus with sequence coding for GFP (Guttery et al., 2014), and then analyzed blood and mosquito stages of the life-cycle for GFP. Strong GFP fluorescence was observed throughout the life-cycle, with areas of localized expression in the cytoplasm and a circular ring formation around the nucleus (Figure 1). Predotar analysis (Small et al., 2004) predicted an ER localization for both PbDEH and PfDEH. Colocalization with ER tracker confirmed the DEH-GFP location at the ER, in all parasite stages analyzed (Figure 2A), with subcellular fractionation of blood stage parasites confirming its integral membrane location (Figure 2B).

**PbDEH is essential for mitotic cell division but not cell size of *Plasmodium* during oocyst development**

Previous comparison of Δdeh and WT parasite lines highlighted the non-essential role of this gene for blood stage development (Guttery et al., 2014). In this study we confirmed it is not essential during asexual blood stages, or for zygote development (Figure 3A). However, while the overall number of oocysts observed in Δdeh and WT lines was not significantly different (Guttery et al., 2014), there was a significant reduction in Δdeh GFP-expressing oocysts beginning at day 7 and continuing through to day 21 post-infection (Figure 3B, C), with many appearing to be degenerating. Analysis of oocyst size revealed a small decrease from day 10 onwards in Δdeh lines compared to WT (Figure 3D), and by day 21 the vast majority of Δdeh oocysts expressed GFP no longer, and in the few that did GFP was present at very low levels or in fragmented patterns. However, it is important to note that Δdeh oocysts that continued to express GFP and showed faint DAPI staining of DNA, were similar in size to WT oocysts; whereas the vast majority of oocysts that reduced in size did not express GFP or stain with DAPI, suggesting they were dead. Analysis of salivary glands from mosquitoes infected with Δdeh parasites revealed no sporozoites, in contrast with the normal numbers of sporozoites in glands from WT-parasite infected mosquitoes (Figure 3E). Representative examples of Δdeh oocyst morphology and lack of sporozoite
development at all stages are shown in Figures 3F and G, highlighting oocyst degeneration, fragmented GFP expression and failure to form sporozoites.

**Ultrastructure analysis confirmed oocyst degeneration and apoptotic nuclear chromatin condensation in Δdeh lines**

To investigate further the marked differences in oocyst morphology and complete lack of sporozoite formation, we used electron microscopy to compare Δdeh and WT lines at 10-, 14- and 21-days post-infection. At 10 days, the oocysts of Δdeh and WT parasites were of a similar size. However, Δdeh oocysts showed numerous cytoplasmic vacuoles (Figure 4a) with evidence of dilatation of the nuclear membranes (Fig 4b), in contrast to WT oocysts which were completely filled with cytoplasm with numerous nuclear and mitochondrial profiles (Figure 4g, h). At fourteen days post-infection the WT parasite showed a mixture of early, mid and late stage oocysts with sporozoites at various stages of development (Figure 4i, j). In contrast, the majority of Δdeh oocysts exhibited advanced stages of degeneration with increased cytoplasmic vacuolation, dilated nuclear membranes, and evidence of mitochondrial abnormalities (Fig 4c, d). There was little evidence of retraction of the plasmalemma from the oocyst wall and no evidence that sporozoite inner membrane complex (IMC) formation had been initiated in any of the oocysts. At day 21 post-infection, all Δdeh oocysts were in an advanced stage of degeneration - almost completely vacuolated with a few nuclei appearing to have undergone apoptotic-like nuclear chromatin condensation (Fig 4e, f), in contrast to WT oocysts, which were mostly mature with numerous fully formed and free sporozoites (Fig 4k, l) although a few degenerate oocysts were observed.

**Discussion**

Lipid metabolism is essential for cellular function, and includes critical pathways for FA synthesis and elongation. DEH is a 3-hydroxyacyl-CoA dehydratase involved in VLCFA synthesis, which interacts with several elongase units, is located at the ER (Beaudoin *et al.*, 2009, Morineau *et al.*, 2016) and has an essential role during development, differentiation, and maintenance of a number of tissue types (Li *et al.*, 2000, Bellec *et
In this study, we examined the location and function of *Plasmodium* DEH using *in silico*, genetic manipulation and cell biological techniques.

In our previous phosphatome study, a putative, catalytically inactive, PTP-like protein with an essential role during sporogony was identified (Guttery *et al.*, 2014), which had been classified as a putative PTPLA by others (Andreeva *et al.*, 2008, Wilkes *et al.*, 2008, Pandey *et al.*, 2014) based on high sequence similarity and e-score values. However, a recent genome-wide functional screen in *P. berghei* showed that PbPTPLA has an essential role in lipid metabolism, specifically during the ELO pathway as a 3-hydroxyacyl dehydratase (DEH) (Stanway *et al.*, 2019). The specific criterion for a PTP-like protein is the presence of a CXXGXXP motif (i.e. the CXXGXXR motif of PTPs, but with the arginine replaced by proline). However, we show here that this motif is not present in either *P. falciparum* or *P. berghei* protein and this, along with its proven function in lipid metabolism (Stanway *et al.*, 2019), suggests that the classification as a phosphatase-like protein is erroneous. Our *in silico* interactome analysis suggests that PfDEH interacts with a number of proteins involved in lipid metabolism, confirming previous functional findings (Stanway *et al.*, 2019) and adding further weight to its annotation as a 3-hydroxyacyl-CoA dehydratase.

Studies in mammalian systems have suggested that the ER-bound DEH catalyzes the third of four reactions in the long-chain FA elongation cycle (Ikeda *et al.*, 2008). Our detailed GFP-based localization analyses showed that the protein is expressed strongly throughout the life-cycle, with protein expression at localized areas in the cytoplasm and as a circular ring-like structure around the nucleus. *In-silico* analysis using Predotar and microscopy-based co-localization using ER tracker confirmed the ER location, consistent with previous studies suggesting a role in FAS in *Plasmodium* (Stanway *et al.*, 2019). Phenotypic analysis of DEH function throughout the life cycle confirmed the results of our previous study (Guttery *et al.*, 2014), highlighting that it is essential for oocyst maturation and sporozoite development, but dispensable for asexual blood stage development (Bushell *et al.*, 2017). Time-course analysis at days 7, 14 and 21 after mosquito infection showed that while early-stage Δ*deh* oocysts were comparable in size
to WT oocysts, they begin to degenerate at an early stage of development, with a significant decrease in GFP-expressing oocysts even at day 7 post-infection, and as seen previously in other FAE-critical mutant parasites (Stanway et al., 2019). Ultrastructure analysis confirmed that at 14 days post-infection, Δdeh oocysts were at an advanced state of degeneration, with no evidence of sporozoite development. Retraction of the oocyst plasmalemma (the parasite plasma membrane) from the oocyst capsule is a crucial first stage in sporozoite development, where sporoblast formation is followed by thousands of sporozoites budding off into the space delineated by the capsule (Aly et al., 2009). A model of this process is detailed in (Burda et al., 2017). Our study suggests that initiation of mitosis, which results in sporozoite development, does not even commence in Δdeh oocysts, since retraction of the plasmalemma and initiation of daughter IMC formation is ablated. The phenotype is similar to that of a cyclin-3 mutant (Roques et al., 2015), with defects leading to abnormalities in membrane formation, vacuolation and subsequent cell death during the later stages of sporogony. However, in contrast to the cyclin-3 mutant, oocyst growth was not affected but sporogony was completely ablated in Δdeh parasites, and no transmission was observed in bite-back experiments. This suggests that the parasite cannot scavenge VLCFA from its mosquito host environment, and that DEH (and therefore the ELO pathway) is critical for oocyst mitotic maturation and differentiation. The cells were unable to progress further to form additional lobes and start sporozoite budding in its absence, although the oocyst size was not grossly affected, suggesting that two independent processes drive oocyst formation and sporogony, respectively.

While FASII activity is exclusively in the apicoplast (Shears et al., 2015), our study showed that DEH-GFP is located at the ER, suggesting that it is an active component of the ELO pathway. The genes involved in the ELO pathway include members of the ELO family (1, 2 and 3), of which ELO2 and ELO3 are involved in keto- and enoyl-reduction (Kohlwein et al., 2001). In yeast, the dehydratase step is carried out by the DEH-like homologue, Phs1, which has also been characterized as a cell cycle protein with mutants defective in the G2/M phase (Yu et al., 2006). Gene knockout studies for any ELO proteins are few, with a single genome-wide functional analysis showing that the P.
bergheri homologue of PF3D7_0605900 (a putative long chain polyunsaturated fatty acid elongation enzyme) is dispensable during the asexual blood stages (Bushell et al., 2017). In addition, the comprehensive analysis of FAE in Plasmodium by Stanway et al. (Stanway et al., 2019) showed that mutants of a ketoacyl-CoA reductase (KCR) have an identical phenotype to our DEH gene knockout lines, with normal development of ookinetes and oocysts gradually disappearing over the course of development, resulting in the complete ablation of sporogony. In contrast, ELO-A (stage 1 of VLCFA synthesis) mutants were critical for liver stage development. This suggests that reduction of ketoacyl-CoA to hydroxyacyl-CoA and subsequent dehydration of hydroxyacyl-CoA to enoyl-CoA (i.e. stages 2 and 3 of VLCFA synthesis) are the most crucial stages for oocyst maturation and sporogony; whereas the lack of a phenotype during sporogony of the ELO-A deletion may suggest functional redundancy and/or a compensatory mechanism such as an overlapping specificity for condensation of malonyl-CoA by either ELO-B or ELO-C, as suggested in Trypanosoma brucei (Lee et al., 2006) and Toxoplasma gondii (Ramakrishnan et al., 2012).

In conclusion, our PbDEH analysis using various in-silico, in-vitro and in-vivo approaches provides important insights into the crucial role DEH plays during VLCFA synthesis, and how disruption of the gene can affect parasite development in the mosquito. Future studies will elucidate further how lipid metabolism in Plasmodium can be explored as a viable target for therapeutic intervention.
Figure legends:

**Figure 1: DEH-GFP protein expression in stages of the parasite life cycle**

Expression of DEH-GFP in rings, trophozoites, schizonts, gametocytes, zygotes, ookinetes, oocysts and sporozoites. P28, a cy3-conjugated antibody which recognizes P28 on the surface of zygotes, and ookinetes was used as a marker of the sexual stages. Note that the female gametocyte in this figure has not been activated, and is not expressing P28. Scale bar = 5 μm.

**Figure 2: Co-localization of DEH-GFP and ER tracker**

(A) Analysis of DEH-GFP localization using ER tracker in merozoites, zygotes, ookinetes and sporozoites. Scale bar = 5 μm. (B) Anti-GFP western blot for subcellular localization of DEH-GFP. PMF – Peripheral membrane protein fraction, IMF – Integral membrane protein fraction.

**Figure 3: Phenotypic analysis of Δdeh lines**

(A) Ookinetes conversion as a percentage in Δdeh and WT lines. Ookinetes were identified using the marker P28 and defined as those cells that successfully differentiated into elongated ‘banana shaped’ ookinetes. Bar is the mean ± SEM. n = 3 independent experiments. (B) Total number of GFP-positive oocysts per infected mosquito, including normal and smaller oocysts, at 7, 10, 14, and 21 days post-infection for Δdeh and WT parasite lines. Bar is the mean ± SEM. n = 3 independent experiments (20 mosquitoes for each). P <0.001 for all time points. (C) Oocysts (10x magnification) of Δdeh and WT lines at 7, 10, 14 and 21 days post-infection. Scale bar = 100 μm. (D) Individual Δdeh and WT oocyst diameters (μm) at 7, 10, 14 and 21 days post-infection. Horizontal line indicates the mean from 3 independent experiments (20 mosquitoes for each) of Δdeh and WT. *p <0.05, **p <0.01, ***p <0.001. (E) Total number of sporozoites per mosquito from 21 days post-infection salivary glands for Δdeh and WT lines. Three independent experiments, n = 20 mosquitoes for each replicate. *** p <0.001. (F) Oocysts (63x magnification) in Δdeh and WT lines. Images show DIC, GFP at 7, 10, 14 and 21 dpi. Scale bar = 20 μm. (G) Representative examples of Δdeh and
WT oocysts (63x magnification) at 21 dpi showing fragmented GFP and Hoechst staining. Scale bar = 20 μm.

**Figure 4: Ultrastructure analysis of oocyst development in Δdeh lines.**

Electron micrographs of Δdeh (a-f) and WT (g-l) parasites at 10 days (a, b, g, h), 14 days (c, d, i, j) and 21 days (e, f, k, l) post infection. Bars represent 10 μm (a, c, e, g, i, k) and 1 μm (b, d, f, h, j, l). (a) Low power image of an early oocyst showing lucent area made up numerous vacuoles (V). (b) Detail from a similar stage to that in (a) showing part of the cytoplasm containing a nucleus with a nuclear pole (NP). Note the lucent area due to the separation of the inner (inm) and outer (onm) nuclear membranes. (c) Low power image of a mid-stage oocyst showing nuclear swelling (N) and increased numbers of lucent cytoplasmic vacuoles (V). (d) Detail part of the cytoplasm showing a swollen nucleus (N), membrane bound lucent vacuoles (V) and mitochondria (Mi) with vesicles embedded in electron dense material. (e) Low power image of a late stage oocyst with abnormal nuclei (N) and the cytoplasm filled with electron lucent vacuoles. (f) Detail from the central region of (e) showing the peripheral location of electron dense chromatin (Ch) typical of apoptotic changes, while the cytoplasm consists of numerous vacuoles (V). (g) Low power image of an early oocyst (end of growth phase) in which the cytoplasm completely fills the oocyst and contains many nuclear profiles (N). (h) Detail of the peripheral cytoplasm limited by the plasmalemma (P) containing mitochondria (M) and nuclei (N). OW – oocyst wall. (i) Mid stage oocyst showing the surface formation of numerous sporozoites (Sp). N – nucleus. (j) Detail showing partially formed sporozoites (Sp) budding from the surface of the cytoplasmic mass. N – nucleus, R – rhoptry. (k) Mature oocysts containing large number of fully form sporozoites (Sp). (l) Detail of cross sections through mature sporozoites (Sp) containing rhoptries (R) and micronemes (MN).

**Figure S1: Phylogenetic analysis of DEH homologues across different species**

Phylogenetic analysis for PbDEH/PTPLA orthologs was performed using the neighbor joining method with MEGA6 software. Genome wide DEH/PTPLA analysis shows its presence in all organisms studied, including species from all major eukaryotic phyla.
The analysis shows the clustering of organisms based on their evolutionary relatedness. Blue – Chordata; Orange – Non-chordata; Purple – Nematoda; Pink - Ameobozoa; Red – Apicomplexa, Euglenozoa and Metamonada; Green – Plants and Algae; Yellow - Fungi and Black - Others.

**Figure S2: ClustalW alignment of *Plasmodium* DEH with human, mouse and other apicomplexan homologues**

ClustalW alignment of *Homo sapiens* (Hs), *Mus musculus* (Ms), *Plasmodium* (Pf, Pb), *Toxoplasma* (Tg) and *Cyclospora* (Cc) homologues of DEH. For apicomplexans, the proteins are currently annotated as PTPLA. Highlighted in red is the CXXGXXP motif that defines PTP-like proteins.

**Figure S3: Predicted interacting partners of PfDEH**

STRING analysis of PfDEH for potential interacting partners. The table on the right gives the annotation and combined score.

**Table S1:** Organisms and protein sequences used for phylogenetic analysis.

**Author contributions:** Conceived and designed the experiments: RT, RP and AAH. Performed the experiments: RT, RJW, RP, DJPF and DSG. Analyzed the data: RT, AAH, RP, RJW, DJPF and DSG. Contributed reagents/materials/analysis tools: DJPF, AAH and RT. Wrote the paper: RP, DSG, RJW, AAH and RT. Performed the functional and GFP tagging experiments: RP, RJW, DSG and RT. Phylogenetics analysis: RP and DSG. RP and DG performed database searches, sequence-based analysis, and other bioinformatics analysis. Electron microscopy experiment: DJPF.

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A  
\[ \text{Ookinite conversion (\%)} \]

B  
\[ \text{GFP-expressing oocysts per midgut} \]

C  
\[ \text{Day 7} \quad \text{Day 10} \quad \text{Day 14} \quad \text{Day 21} \]

D  
\[ \text{Oocyst Diameter (\text{nm})} \]

E  
\[ \text{Sporozoites per salivary gland} \]

F  
\[ \text{Day 7} \quad \text{Day 10} \quad \text{Day 14} \quad \text{Day 21} \]

G  
\[ \text{DIC} \quad \text{Hoechst} \quad \text{GFP} \quad \text{Merge} \]
