Plasmodium falciparum LipB mutants display altered redox and carbon metabolism in asexual stages and cannot complete sporogony in Anopheles mosquitoes

Marco Biddau a,b,⇑, T.R. Santha Kumar c, Philipp Henrich c, Larissa M. Laine b, Gavin J. Blackburn d, Achuthanunni Chokkathukalam d, Tao Li e, Kim Lee Sim e, Lewis King a, Stephen L. Hoffman e, Michael P. Barrett a,d, Graham H. Coombs f, Geoffrey I. McFadden g, David A. Fidock c,h,1, Sylke Müller b,1, Lilach Sheiner a,b,⇑

a Wellcome Centre for Integrative Parasitology, University of Glasgow, Glasgow, United Kingdom
b Department of Infection, Immunity and Inflammation, University of Glasgow, Glasgow, United Kingdom
c Department of Microbiology and Immunology, Columbia University Irving Medical Center, New York, NY 10032, USA
d Glasgow Polyomics, Wolfson Wohl Cancer Research Centre, University of Glasgow, Glasgow, United Kingdom
e Sanaria Inc., Rockville, MD 20850, USA
f Strathclyde Institute of Pharmacy and Biomedical Sciences, University of Strathclyde, Glasgow, United Kingdom
g School of Botany, University of Melbourne, Parkville, VIC 3010, Australia
h Division of Infectious Diseases, Department of Medicine, Columbia University Irving Medical Center, New York, NY 10032, USA

abstract
Malaria is still one of the most important global infectious diseases. Emergence of drug resistance and a shortage of new efficient antimalarials continue to hamper a malaria eradication agenda. Malaria parasites are highly sensitive to changes in the redox environment. Understanding the mechanisms regulating parasite redox could contribute to the design of new drugs. Malaria parasites have a complex network of redox regulatory systems housed in their cytosol, in their mitochondrion and in their plastid (apicoplast). While the roles of enzymes of the thioredoxin and glutathione pathways in parasite survival have been explored, the antioxidant role of α-lipoic acid (LA) produced in the apicoplast has not been tested. To take a first step in teasing a putative role of LA in redox regulation, we analysed a mutant Plasmodium falciparum (3D7 strain) lacking the apicoplast lipoic acid protein ligase B (lipB) known to be depleted of LA. Our results showed a change in expression of redox regulators in the apicoplast and the cytosol. We further detected a change in parasite central carbon metabolism, with lipB deletion resulting in changes to glycolysis and tricarboxylic acid cycle activity. Further, in another Plasmodium cell line (NF54), deletion of lipB impacted development in the mosquito, preventing the detection of infectious sporozoite stages. While it is not clear at this point if the observed phenotypes are linked, these findings flag LA biosynthesis as an important subject for further study in the context of redox regulation in asexual stages, and point to LipB as a potential target for the development of new transmission drugs.

1. Introduction
Malaria remains a tremendous threat to human health with 200 million infections resulting in 405,000 deaths in 2018 (World Health Organization, 2019), so it is imperative that we identify new antimalarial targets. One potential target is the parasite redox regulation system. Plasmodium falciparum is constitutively exposed in all stages of its complex life cycle to molecules that challenge its redox balance. Finding ways to disrupt this delicate balance hold promise for drug development. Indeed, in vitro experiments in which parasites were exposed to exogenous H2O2-generating systems proved lethal for intra-erythrocytic stages (Dockrell and Playfair, 1984). Similarly, mature gametocytes are sensitive to the oxidative stress generated by exposure to redox-cyclers...
in vitro (Siciliano et al., 2017). Finally, an animal diet that generates an environment rich in reactive oxygen species (ROS) in hepatocytes can reduce Plasmodium infection in vivo (Zuzarte-Luís et al., 2017). Despite this importance, and the fact that redox regulation is a fundamental aspect of cellular functions, our understanding of the parasite redox regulatory networks remains limited (Kehr et al., 2010; Müller, 2015).

The apicoplast, a non-photosynthetic plastid acquired via secondary endosymbiosis of a red algal cell, is an active metabolic hub in apicomplexan parasites including Plasmodium spp. (Sheiner et al., 2013; Mohring et al., 2014; Frohnecke et al., 2015; Kimata-Ariga et al., 2018; Biddau and Sheiner, 2019). In asexual red blood stages grown in culture, the apicoplast is essential only for the synthesis of isoprenoid precursors (Yeh and DeRisi, 2011). The apicoplast hosts components of the thio-redox and the glutathione systems, which represent the two best characterized cellular antioxidant systems. Apicoplast-based redox regulators include the peroxiredoxin antioxidant protein (AOP), the dually-targeted (cytosol and apicoplast) enzymes glutathione reductase (GR), and the glutathione peroxidase-like thioredoxin peroxidase (TPx2) (Kehr et al., 2010; Laine et al., 2015). Similarly, two glyoxalase system proteins are apicoplast targeted: glyoxalase-1-like protein (GILP), and glyoxalase 2 (gGloII) (Kehr et al., 2010; Urscher et al., 2010). Glyoxalase 2 is proposed to play a role in the detoxification of incomplete triosephosphate-isomerase reaction products, but is apparently dispensable during intraerythrocytic development (Wezena et al., 2017). Two additional apicoplast thio-redox-in-like proteins (Atrx1 and Atrx2) are found in the peripheral compartments where they play an essential role in the control of protein sorting and folding in response to organelle redox status (Biddau et al., 2018). The Plasmodium orthologue of Atrx2 ( PfAtrx2; PF3D7_0529100) also appears to be essential (Bushell et al., 2017; Zhang et al., 2018). Many other potential redox-active proteins are predicted to be localised in the apicoplast (Boucher et al., 2018), but their roles are uncharacterized.

An additional molecule proposed to take part in apicoplast redox regulation is α-lipoic acid (LA) (Günther et al., 2007; Frohnecke et al., 2015; Laine et al., 2015). Due to its reducing properties, LA is known as the ‘universal antioxidant’ (Kagan et al., 1992; Perham, 2000; Goraca et al., 2011; Moura et al., 2015; Tibullo et al., 2017). The proposed antioxidant role of LA in the apicoplast is based on a link between redox regulation and apicoplast pyruvate metabolism via the pyruvate dehydrogenase enzyme complex (PDC). The three enzymes in the PDC complex are pyruvate dehydrogenase (E1), dihydrolipoyl transacetylase (E2) and apicoplast dihydrolipoyl dehydrogenase (aE3). Through a series of reactions, PDC transfers an acetyl group from pyruvate to coenzyme A (CoA), then to CoA-SH and finally to acetyl-CoA for fatty acid biosynthesis (Mooney et al., 2002; Foth et al., 2005) (Fig. 1). This activity depends on LA bound to the E2 lipoyl domain, which is reduced to dihydrolipoyl acid (DHLA) during the process. The final reaction of Plasmodium PDC is catalysed by aE3, which re-oxidises DHLA back to LA to allow another cycle of PDC activity (Fig. 1). The activity of aE3 is coupled to the reduction of NAD⁺ to NADH + H⁺, which in turn takes part in apicoplast redox regulation (McMillan et al., 2005; Laine, L.M., 2014. Functional, biochemical and structural analyses of Plasmodium falciparum pyruvate dehydrogenase complex. PhD Thesis, University of Glasgow. University of Glasgow, UK). The DHLA/LA redox couple has a redox potential of −0.24 V, which is lower than the glutathione/glutathione disulphide (GSH/ GSSG) couple potential of −0.24 V, thus making glutathione a potential substrate for DHLA (Packer et al., 1995).

The apicoplast retains its exclusive LA biosynthesis pathway, catalysed by the enzymes octanoyl-ACP:protein N-octanoyltransferase (LipB) and lipoyl synthase (LipA) (Fig. 1), which operates independently from the mitochondrial LA salvage (Crawford et al., 2006; Günther et al., 2009). A lipB mutant was earlier generated and shown to have 90% LA depletion (Günther et al., 2007). As a first step to tackle the putative role of LA in redox regulation, we examined the changes in the expression of redox regulation enzymes in this P. falciparum mutant (named 3D7ΔlipB). We also describe the metabolic changes occurring upon lipB deletion. As a comparison, we also analysed an aE3 deletion mutant that has only a mild effect on redox balance in the parasite (Laine et al., 2015), potentially because aE3 function might be compensated by alternative apicoplast enzymatic systems coupled to NAD(P)⁺ reduction such as the NADP⁺-specific glutamate dehydrogenase (Zocher et al., 2012). Our results describe a correlation between LA availability and redox regulation. Additionally, lipB deletion led to changes in central carbon metabolism. Finally, lipB deletion, generated in a separate NF54 line, hampers the ability of the parasites to develop in the mosquito, suggesting that LipB is essential in this life stage.

2. Materials and methods

2.1. Parasite culture and assessment of growth

2.1.1. Plasmodium falciparum culturing and synchronisation

Plasmodium falciparum 3D7 parasites (isolated in the Netherlands) were cultured in RPMI 1640 (Invitrogen, UK) supplemented with 11 mM D-Glucose (Sigma Aldrich, UK), 0.5% w/v AlbuMAX II (Invitrogen, UK), 200 mM hyposmantine (Sigma Aldrich, UK), and 20 mg/ml of gentamycin (PAA, Germany) in human erythrocytes at 5% haematocrit (Trager and Jensen, 1976). Parasites were cultured, maintaining a reduced oxygen atmosphere (1% O₂, 3% CO₂ and 96% N₂) and a constant temperature of 37 °C (referred to as standard procedures). Parasitemias were determined by microscopy-based analysis of Giemsa-stained thin smears and synchronisations were performed following the sorbitol procedure (Lambros and Vanderberg, 1979). Tighter synchronisation was obtained by a combination of sorbitol treatment with magnetic-activated cell sorting (MACS) using LD columns (Miltenyi Biotech, Germany). Briefly, sorbitol-synchronised parasites were maintained in culture until they reached the early ring stage. Cultures were synchronised twice with sorbitol 6 h apart and then cultured until they reached the late schizont stage. Schizonts were purified over a MACS column once cultures had reached a schizont:ring ratio of 1:2. Schizonts were then placed in culture for 1 h with gentle shaking and cultures were synchronised again using the sorbitol method to obtain highly synchronous ring stages with 1 h synchrony. Highly synchronous parasites were used for all time point experiments and RNA extractions, with triplicate cultures for each condition.

2.1.2. Spent medium metabolite quantification

Analyses of D-glucose and L-lactate concentrations from spent culture medium samples were performed using the D-Glucose-HK and L-Lactic acid kits from Megazyme (Ireland) following the manufacturer’s protocol. Briefly, parasite cultures for each condition were synchronised using a double sorbitol treatment (Lambros and Vanderberg, 1979) with a 6 h window, and split into triplicate cultures at the same parasitemia. At each time point, an aliquot of the culture was collected from each condition and erythrocytes were pelleted by centrifuging at 1000g for 5 min. The resulting supernatant was then stored at −20 °C. For the enzymatic assay, parasite spent medium samples were diluted by 1:6 with double distilled water.
2.2. Evaluation of antioxidant gene expression

2.2.1. Reverse-Transcription quantitative PCR (RT-qPCR)

Highly synchronous parasites (see section 2.1.1) were cultured in triplicate for each condition until they reached 26, 30 and 34 h post-invasion (pi). Pellets of infected red blood cells (RBCs) at 6–8% parasitemias were then washed three times in PBS and kept at –80°C. Nucleic acids were extracted using the RNeasy kit (Qiagen). Contaminating DNA was removed using the Turbo DNA-free kit (Thermo Fisher Scientific). RNA samples were then reverse transcribed using the RETRO-script kit (Thermo Fisher Scientific). Quantitative PCR (qPCR) was performed using the Power SYBR Green Master Mix (Thermo Fisher Scientific). RNA samples were then reverse transcribed using the RETRO-script kit (Thermo Fisher Scientific). All reactions were run in a 7500 Real-Time PCR System (Applied Biosystems, UK). The calculation of relative gene expression was performed using the ΔΔCt method (Livak and Schmittgen, 2001).

2.2.2. Protein extraction and quantitative fluorescent western blot

For protein extraction, saponin-lysed parasite pellets were resuspended in 2D lysis buffer (100 mM Hepes pH 7.4, 5 mM MgCl2, 10 mM EDTA, 0.5% (v/v) Triton X-100, 5 μg/ml of RNase A, 1× complete protease inhibitor cocktail (Roche, Switzerland) in double distilled (dd)H2O). Samples were subjected to three rounds of freezing and thawing on dry ice, and incubated at 4°C for 5 min in a sonicated water bath. Samples were then centrifuged at 13,000g for 20 min at 4°C. Supernatants containing protein fractions were quantified using the Protein Assay kit (Bio-Rad, UK), with BSA used to generate a reference quantification curve.

Western blot analysis was performed by separating 20 μg of protein sample by SDS-PAGE with NuPage Novex 4–12% and 15% (w/v) Bis-Tris gels (Invitrogen, UK). Separated proteins were transferred to Protran nitrocellulose membranes (Schleicher & Schuell, Germany) using a Transblot semi-dry transfer system (Bio-Rad, UK). Membranes were blocked with 5% (w/v) dried skimmed milk in PBS for 1–18 h and incubated for 1 h with two or more primary antibodies. Primary antibodies used for relative quantification included P. falciparum rabbit anti-actin antibody (1:12,000, loading control), P. falciparum rabbit anti-1-CysPx (1-Cys peroxiredoxin) antibody 1:50,000; P. falciparum rabbit anti-2-CysPx (2-Cys peroxiredoxin) antibody at 1:70,000; P. falciparum rabbit anti-BCKDH E2 antibody at 1:5000; P. falciparum rabbit anti-iso-citrate dehydrogenase antibody at 1:10,000 and P. falciparum rabbit anti-PDC E2 lipoyl domain antibody at 1:250. Membranes were washed three times in PBS containing 0.2% (v/v) Tween 20 and 2.5% (w/v) dried skimmed milk. Blots were then probed with infrared dye-conjugated antibody (1:10,000, IRDye800CW goat anti-rabbit antibody; LI-COR Biosciences, USA) for 1 h and washed again twice. Membranes were loaded in an Odyssey SA scanner (LI-COR Biosciences, USA) and fluorescent signal intensities quantified with the Image Studio software (LI-COR Biosciences, USA). All antibodies were custom made by Eurogentec.

2.3. Metabolomics experiment and analysis

2.3.1. 13C-U-D-glucose labelling experiment setup

Parasites were cultured until parasitemias attained 6–8%. Two sorbitol treatments were performed at approximately 8 and 14 h pi. After synchronisation, triplicate parasite cultures were initiated using a medium where D-glucose was replaced with 13C-U-D-glucose (99%, CK Gas Products Ltd, UK) and the haematocrit was set to 1%. A control culture of uninfected RBCs was prepared with the same conditions. All cultures were incubated for 20 h following standard procedures until late trophozoites. At this point, parasite culture metabolism was rapidly quenched at 4°C in a bath of dry ice and 70% ethanol (Vincent and Barrett, 2015). Erythrocyte pellets were then obtained and washed in ice-cold PBS by centrifugation at 800g for 5 min at 4°C. Infected RBCs in the pellets were enriched using MACS LD column purification (Miltenyi Biotech, Germany) and a QuadroMACS magnet (Miltenyi Biotech, Germany) with all steps performed at 4°C. Enriched samples were quantified using a Neubauer cell counting chamber and a Scepter 2.0 Handheld Automated Cell Counter (Millipore) to have 2.0 × 10⁸ infected RBCs per sample. The same number of uninfected RBCs was collected as a control. All the samples were then added to a solution...
of HPLC-grade chloroform:methanol:water (1:3:1; v/v/v), at a concentration of $2 \times 10^6$ parasites per 0.5 mL solution, incubated in ice in a sonicating water bath for 2 min and extracted for 1 h at 4 °C and 1500 rpm on an orbital shaker. After extraction, samples were centrifuged at 13,000g for 20 min at 4 °C, and supernatants transferred into glass mass spectrometry vials (Thermo Fisher Scientific, UK) and stored at −80 °C until LC-MS analysis.

2.3.2. LC-MS analyses

Metabolomics analyses were performed by LC-MS using an Ultimate 3000 LC system ( Dionex, UK) connected to a Q Exactive HF Hybrid Quadrupole-Orbitrap mass spectrometer, (Thermo Fisher Scientific). The system was controlled by the software Chromeleon (Dionex, UK) and Xcalibur (Thermo Scientific), acquiring both positive and negative ionisation modes. Chromatographic separation was performed with a ZIC-pHILIC chromatography column (150 mm (length) $\times$ 4.6 mm (diameter) 5um ( bead size ) : SeQuant, Uemå, Sweden) using a two solvent system consisting of solvent A: 20 mM ammonium carbonate and solvent B: acetonitrile (Table 1).

2.3.3. Metabolomic data analysis

Vendor-specific raw data were initially centroided and converted into the open format mzXML for subsequent processing. PeakML files (Scheltema et al., 2011) were then generated by extracting the chromatographic peaks contained in the mzXML files using the detection algorithm from XCMS (Tautenhahn et al, 2008). The data processing pipeline mzMatch.R (Jankевичs et al., 2012) was used to sort and combine all PeakML files corresponding to replicates and to exclude all non-reproducible data. Further steps of noise-filtering, gap-filling, and metabolite identification were performed on PeakML files utilising data obtained from metabolic standards run in parallel. For each metabolite of interest, the proportions of each isotopologue and its relative abundance in the sample were determined. The PeakMLIsotope.TargetedIsotopes function of mzMatch-ISO (Chokkathukalam et al., 2013) was used to scan the PeakML files for labelled metabolite quality and quantity. All metabolites of interest in this study were reliably identified by comparison of the chromatographic retention times and the m/z values with an authentic metabolic standard processed in parallel. These should be considered as “identified compounds” or level 1 according to the Metabolomic Standard Initiative (Sumner et al., 2007). All metabolomics data was corrected for natural carbon isotope abundance and reagent impurity using the software IsoCor (Millard et al., 2019).

2.4. Double cross-over deletion of pflipB in the NF54 strain by the Cre-loxP system

Plasmodium falciparum NF54 parasites were cultured in complete medium containing RPMI 1640 salts and 10% heat-inactivated human serum (Graves et al., 1984). The strategy for deleting pflipB using Cre-loxP is depicted in Supplementary Fig. S1. Briefly, we employed a double crossover recombination strategy to generate parasite lines lacking a functional pflipB locus (PlasmoDB ID: PF3D7_0823600). A 0.5 kb fragment of pflipB, PCR-amplified from NF54 genomic DNA using primers p1/p2 (Supplementary Table S1), was cloned into PCC1-clup-hdhfr-DXO between the Sacl and AflI sites and served as the homology region for the first cross-over (O'Neill et al., 2011). The second homologous fragment (0.5 kb) was PCR-amplified using primers p3/p4 and cloned between EcoRI and AvRII sites to give rise to an 8.5 kb PCC1-clup-hdhfr-AflI plasmid. Fifty µg of this plasmid was used to transfected NF54 parasites by electroporation and transformed parasites were selected with 1.5 mM WR99210. Drug pressure was maintained for 7 days and parasites were then cultured in drug-free medium up to day 27 when WR99210-resistant parasites appeared. Resistant parasites were then treated with 1.0 µM 5-Fluorocytosine (in DMSO) to remove single crossover-integrated plasmid and episomal forms, both of which contain the cdup negative selection marker (Maier et al., 2006). PTET-BSD-Cre (a kind gift from Alan Cowman, Walter and Eliza Hall Institute, Australia) was transfected into recombinant NF54 parasites and cultured in 2.5 µM blasticidin S-hydrochloride for 7 days, after which single-cell cloning was performed (Goodyer and Taraschi, 1997).

2.5. Generation of PfAlip8 gametocytes and production of salivary gland sporozoites

Gametocytes were induced from PfAlip8 and wild-type (WT) NF54 parasite lines by multiple rounds of sub-culturing for 14–18 days with nutrient deprivation, as described (Ponnudurai et al., 1986). Gametocyte induction and maturation was monitored microscopically by Giemsa-stained thin blood smears from culture samples. Mature gametocyte pellets were mixed with fresh O-type Rh- blood and human serum to produce an artificial blood meal at approximately 50% hematocrit for mosquito feeds. The final concentration of stage V gametocytes in artificial blood meals was 1.0%±0.1% (mean ± S.E.M. across seven experiments), which was equivalent to the gametocyte numbers observed with NF54 parasites (Supplementary Table S1). Anopheles stephensi mosquito larvae were fed 3–5 days after their emergence from pupae. Fed mosquitoes were maintained for 13–16 days before recovery of salivary gland sporozoites (SPZ) by hand dissection.

2.6. Ultrastructural analysis of midgut oocysts

The infected midgut oocysts were fixed in 4%/0.1% formaldehyde/glutaraldehyde and immersed in a 50:50 glycerol/water solution for Differential Interference Contrast (DIC) imaging. Using a Nikon Ti Eclipse inverted microscope, cut midgut sections were pre-screened for the presence of oocysts at 10× magnification using an automated tiling feature of the Nikon NIS Elements 3.2 software. Individual oocysts were imaged at high magnification using a 60 × NA 1.4 oil immersion objective with a matching DIC slider. Images were compiled using the National Institute of Health (USA) ImageJ program (https://imagej.nih.gov/ij).

Morphology of developing oocysts was examined by fixing dissected mosquito midguts in 2.5% glutaraldehyde in PBS, and then in 0.5% OsO₄, dehydration in an ethanol series, embedding in London Resin White, and semi-thin sections (400 nm) were mounted on glass slides and stained with 0.5% toluidine blue (w/v): 0.1% Na₂CO₃ (w/v) for 10 s, and then imaged on an Olympus BH-2 light microscope.

3. Results

3.1. Deletion of lipB results in accelerated differentiation and changes in apicoplast and cytosol antioxidant levels

Pronounced LA deficiency was previously detected in the trophozoite stage in lipB knockout (KO) line 3D7<sup>ΔlipB</sup> parasites (Günther et al., 2007). The LA depletion seen in this mutant
provided an opportunity to take the first steps to investigate whether this deficiency affected the apicoplast antioxidant composition. We monitored the relative transcription levels of genes encoding apicoplast antioxidants at 26, 30 and 34 h pi by qPCR for both 3D7^PflipB and the parental (3D7WT) parasites. We used primer sets to specifically quantify the transcripts of the apicoplast redox-active proteins TpxD (PF3D7_1212000), Atrx2 (PF3D7_0529100) and AOP (PF3D7_0729200) (Fig. 2A), which take part in the thioredoxin redox system, and the apicoplast glyoxalase system proteins GlpL (PF3D7_0604700) and tGlo (PF3D7_1205700) (Supplementary Fig. S2). Among these, TpxD had the largest change in expression levels, with relative transcription levels displaying three to four-fold increases at 30 h pi and 34 h pi compared with 3D7WT. Similarly, Atrx2 relative expression showed a four-fold increase compared with the 3D7WT parasites at 26 h pi followed by a two-fold decrease at 34 h pi, when AOP also showed a two-fold decrease (Fig. 2A). In contrast, the apicoplast glyoxalase system enzymes presented no significant differences in relative expression (Supplementary Fig. S2).

In light of these observed changes in apicoplast antioxidant expression, we wanted to test whether the cytosolic antioxidant composition was also affected. Therefore, we used quantitative fluorescent western blotting to monitor the relative levels of GST (Laine et al., 2015), which makes 3D7^PflipB mild (Laine et al., 2015), which makes 3D7WT upon deletion at the time points examined. It remains to be determined whether the redox changes observed occur as a result of the shorter differentiation window or are the cause for it. The detection of some changes in redox regulators starting at 26 h pi, 4 h prior to the observed accelerated differentiation, suggests the latter.

3.2. Deletion of lipB affects parasite carbon metabolism

In eukaryotic cells, the compartmental redox state and the availability of redox conducting and regulating molecules in different cellular compartments are intertwined with the activity of metabolic pathways. The redox conditions in a cellular compartment affect the function of its metabolic enzymes, while in turn, the metabolic reactions in a compartment generate metabolites that impact its redox state. Thus, we proceeded to examine whether the observed redox changes in 3D7^PflipB mutants coincide with changes in central carbon metabolism. We chose to make this analysis alongside a P. falciparum apicoplast dihydrolipoamide dehydrogenase (aE3) knock-out mutant (3D7^PflipE3) (Laine et al., 2015). Unlike 3D7^PflipB, the deletion of a PDC component in 3D7^PflipE3 does not result in disruption of LA biosynthesis nor of PDC activity, and its effect on the expression of redox regulators is only mild (Laine et al., 2015), which makes 3D7^PflipB a relevant negative control.

The levels of D-glucose and L-lactate in spent medium were monitored using commercial enzymatic assays in two independent experiments. As glycolytic activity in P. falciparum typically peaks during intra-erythrocytic trophozoite development (Shivapurkar et al., 2018), we collected samples at 30, 34, 38 and 42 h pi to cover this developmental stage. Results showed that spent medium samples from 3D7^PflipB contained significantly less D-glucose (Fig. 3A) and more L-lactate (Fig. 3B) than 3D7WT at 42 h pi. Conversely, 3D7^AopE3 mutants did not present this trend and the concentrations for these metabolites in spent medium were comparable to the WT controls (Supplementary Fig. S4).

We further hypothesised that the up-regulation of glycolysis and the change to antioxidant expression described above might affect downstream metabolism and especially the tricarboxylic acid (TCA) cycle. Therefore, we proceeded to set up two steady-state targeted metabolomics experiments in technical triplicates, using the isotope-labelled nutrient ^13C-U-D-glucose. 3D7^PflipB and 3D7WT parasites were synchronised and metabolically labelled for 28 h. Parasites at the late trophozoite stage were then rapidly chilled, and the extracted metabolites analysed by LC-MS to follow ^13C labelling. In agreement with the observation from the spent medium, 3D7^PflipB mutants displayed a two-fold increase in the relative levels of the glycolytic metabolite pyruvate and of glycerol-3-phosphate, which derive from a glycolytic intermediate (Fig. 4A, B). A similar trend was also displayed by the metabolites 2-oxoglutarate and succinate associated with the TCA cycle, as well as the amino acid aspartate, whereas alanine showed a reduction in relative intracellular level (Fig. 4A, B). The analysis of the labelled fraction for these metabolites showed an immediate conversion of glucose into glycolytic intermediates (Supplementary Fig. S5), in line with previous analyses (Storm et al., 2014). Conversely, the analysis of 3D7^AopE3 mutants showed no significant differences in the relative abundances for these metabolites (Fig. 4C).

Only a small fraction of ^13C-labelled triose phosphates was fed into the TCA cycle intermediates in all three parasite lines (Supplementary Fig. S5), as has been well established in the literature (MacRae et al., 2013; Storm et al., 2014; Ke et al., 2015). Interestingly, whereas the 3D7WT control had M + 4, M + 5 and M + 6 citrate labelling, indicative of a complete TCA cycle activity, this was not the case for 3D7^PflipB. Rather, in 3D7^PflipB mutants compared with 3D7WT, however, with high variability between the two experiments (Fig. 4D). In both the experiments an increment of M + 2 citrate was detected for 3D7^AopE3 mutants compared with 3D7WT, but with high variability between the two experiments (Fig. 4D). These changes were not observed for 3D7^AopE3 mutants (Fig. 4E). In the 3D7^PflipB mutant, the presence of M + 2 label in citrate suggests an unchanged flux of glycolytic pyruvate into the TCA cycle. Conversely, the absence of M + 4 and M + 6 citrate isotopologues might lead to a non-cyclic activity of the cycle, unlike that seen in 3D7WT and 3D7^AopE3. These observations are similar to the phenotype performed quantitative western blot analysis of the enzymes operating the first steps of the TCA cycle. Interestingly, the 3D7^PflipB mutant up-regulated the branched-chain α-keto acid dehydrogenase (BCKDH) component E2, the first enzyme converting pyruvate to acetyl-CoA (Oppenheim et al., 2014), while its apicoplast parallel PDC E2 displayed no significant difference in abundance (Fig. 5A,
Additionally, isocitrate dehydrogenase (ICDH), which operates three steps downstream in the TCA cycle, displayed no differences in abundance compared with 3D7 WT (Fig. 5A, Supplementary Fig. S6). These variations in enzyme expression require further analysis. In light of the described increased glycolytic activity, an up-regulation of BCKDH might be required to sustain the flux of acetyl-CoA in the TCA cycle. The unaltered levels of ICDH contrast with the possible reduced conversion of isocitrate to 2-oxoglutarate in 3D7/DpflipB, suggested by the observed variations in citrate labelling (Fig. 4D).
Lastly, we tested cofactors involved in carbon and energy metabolism, where we observed a decrease in relative intracellular levels of ATP in 3D7-ΔlipB parasites, while all other cofactors tested were unchanged (Fig. 5B). In addition, 3D7-ΔlipB mutants had an increase in the ADP M + 5 fraction (Fig. 5C). These results might suggest an increased ATP demand in the mutants, possibly linked to the accelerated differentiation (Fig. 2C), although this explanation remains to be demonstrated directly. In summary, the analysis of 3D7-ΔlipB mutant metabolism reveals an effect on the activity of both glycolysis and the TCA cycle. The specificity of this phenotype compared with 3D7-ΔlipB metabolism (Fig. 4B, Ch) provide indirect support to the possibility that there is a link between LA depletion and the observed changes.

3.3. A LipB mutant cannot complete development in a mosquito

We further investigated the development of lipB-deleted parasites in mosquitoes. For this analysis, a second lipB deletion line was generated by double-crossover gene deletion (Supplementary Fig. S1) in the NF54 background (NF54-ΔlipB), which is superior to 3D7 parasites in its ability to infect mosquitoes. Parasites were maintained in media containing 10% human serum, with the aim to examine the morphology of the midgut oocysts, and found that the NF54-ΔlipB mutants and 3D7 WT parasites, while all other cofactors correspond to metabolites from 3D7 parasites, while dashed bars correspond to metabolites from 3D7 D parasites, where we observed a decrease in relative intracellular levels of ATP in 3D7-ΔlipB parasites, while all other cofactors tested were unchanged (Fig. 5B). In addition, 3D7-ΔlipB mutants had an increase in the ADP M + 5 fraction (Fig. 5C). These results might suggest an increased ATP demand in the mutants, possibly linked to the accelerated differentiation (Fig. 2C), although this explanation remains to be demonstrated directly. In summary, the analysis of 3D7-ΔlipB mutant metabolism reveals an effect on the activity of both glycolysis and the TCA cycle. The specificity of this phenotype compared with 3D7-ΔlipB metabolism (Fig. 4B, Ch) provide indirect support to the possibility that there is a link between LA depletion and the observed changes.

3.3. A LipB mutant cannot complete development in a mosquito

We further investigated the development of lipB-deleted parasites in mosquitoes. For this analysis, a second lipB deletion line was generated by double-crossover gene deletion (Supplementary Fig. S1) in the NF54 background (NF54-ΔlipB), which is superior to 3D7 parasites in its ability to infect mosquitoes. Parasites were maintained in media containing 10% human serum, with the aim to examine the morphology of the midgut oocysts, and found that the NF54-ΔlipB mutants and 3D7 WT parasites, while all other cofactors correspond to metabolites from 3D7 parasites, while dashed bars correspond to metabolites from 3D7 D parasites, where we observed a decrease in relative intracellular levels of ATP in 3D7-ΔlipB parasites, while all other cofactors tested were unchanged (Fig. 5B). In addition, 3D7-ΔlipB mutants had an increase in the ADP M + 5 fraction (Fig. 5C). These results might suggest an increased ATP demand in the mutants, possibly linked to the accelerated differentiation (Fig. 2C), although this explanation remains to be demonstrated directly. In summary, the analysis of 3D7-ΔlipB mutant metabolism reveals an effect on the activity of both glycolysis and the TCA cycle. The specificity of this phenotype compared with 3D7-ΔlipB metabolism (Fig. 4B, Ch) provide indirect support to the possibility that there is a link between LA depletion and the observed changes.

3.3. A LipB mutant cannot complete development in a mosquito

We further investigated the development of lipB-deleted parasites in mosquitoes. For this analysis, a second lipB deletion line was generated by double-crossover gene deletion (Supplementary Fig. S1) in the NF54 background (NF54-ΔlipB), which is superior to 3D7 parasites in its ability to infect mosquitoes. Parasites were maintained in media containing 10% human serum, with the aim to examine the morphology of the midgut oocysts, and found that the NF54-ΔlipB mutants and 3D7 WT parasites, while all other cofactors correspond to metabolites from 3D7 parasites, while dashed bars correspond to metabolites from 3D7 D parasites, where we observed a decrease in relative intracellular levels of ATP in 3D7-ΔlipB parasites, while all other cofactors tested were unchanged (Fig. 5B). In addition, 3D7-ΔlipB mutants had an increase in the ADP M + 5 fraction (Fig. 5C). These results might suggest an increased ATP demand in the mutants, possibly linked to the accelerated differentiation (Fig. 2C), although this explanation remains to be demonstrated directly. In summary, the analysis of 3D7-ΔlipB mutant metabolism reveals an effect on the activity of both glycolysis and the TCA cycle. The specificity of this phenotype compared with 3D7-ΔlipB metabolism (Fig. 4B, Ch) provide indirect support to the possibility that there is a link between LA depletion and the observed changes.

Lastly, we tested cofactors involved in carbon and energy metabolism, where we observed a decrease in relative intracellular levels of ATP in 3D7-ΔlipB parasites, while all other cofactors tested were unchanged (Fig. 5B). In addition, 3D7-ΔlipB mutants had an increase in the ADP M + 5 fraction (Fig. 5C). These results might suggest an increased ATP demand in the mutants, possibly linked to the accelerated differentiation (Fig. 2C), although this explanation remains to be demonstrated directly. In summary, the analysis of 3D7-ΔlipB mutant metabolism reveals an effect on the activity of both glycolysis and the TCA cycle. The specificity of this phenotype compared with 3D7-ΔlipB metabolism (Fig. 4B, Ch) provide indirect support to the possibility that there is a link between LA depletion and the observed changes.

Table 2, no sporozoites were detected in any of the infected mosquitoes in any of the seven experiments, while an average of 136,922 ± 17,717 sporozoites were detected in four mosquito infection experiments performed with the parental NF54 (Table 2, Fig. 6). These data indicated a major defect in development in the mosquito in the NF54-ΔlipB parasites, while dashed bars correspond to metabolites from 3D7 D parasites, where we observed a decrease in relative intracellular levels of ATP in 3D7-ΔlipB parasites, while all other cofactors tested were unchanged (Fig. 5B). In addition, 3D7-ΔlipB mutants had an increase in the ADP M + 5 fraction (Fig. 5C). These results might suggest an increased ATP demand in the mutants, possibly linked to the accelerated differentiation (Fig. 2C), although this explanation remains to be demonstrated directly. In summary, the analysis of 3D7-ΔlipB mutant metabolism reveals an effect on the activity of both glycolysis and the TCA cycle. The specificity of this phenotype compared with 3D7-ΔlipB metabolism (Fig. 4B, Ch) provide indirect support to the possibility that there is a link between LA depletion and the observed changes.

4. Discussion

The cellular redox balance of Plasmodium parasites is constantly under threat of oxidative stress generated by the metabolic functions of the parasite and by the metabolic activities and defence mechanisms of the host (Becker et al., 2004; Nepveu and Turrini, 2013; Patzewitz et al., 2013; Müller, 2015). Apicoplast-specific redox balance is an integral part of the overall cellular redox steady-state (Kehr et al., 2010; Mohring et al., 2017; Biddau et al., 2018). While fragmented information is available about the different apicoplast redox control pathways, their importance is evident in the series of specific antioxidant systems it hosts (Kehr et al., 2010) and in the redox regulators controlling its bio- genesis (Biddau et al., 2018). Similarly, apicoplast-hosted pathways are coupled to redox reactions, including the biosynthesis of isoprenoid precursors, which is coupled to the reduction of NADP+ to NADPH plus H+ (Seeber et al., 2005; Seeber and Soldati-Favre,
LA is proposed to contribute to redox regulation in other systems (Tibullo et al., 2017). The results presented here describing the phenotypes of lipB deletion provides preliminary evidence in support of this role within the apicoplast of Plasmodium, albeit indirect.

LA is a powerful antioxidant with low redox potential (Packer et al., 1995; Bilska and Włodek, 2005), which prompted us to test how interfering with LA biosynthesis affected apicoplast redox regulation. As a means to address this question, and in the absence of a method to directly and specifically deplete LA from the

![Fig. 5](image-url) Analysis of the expression of metabolic enzymes and the levels of cofactors involved in glycolysis and in the tricarboxylic acid (TCA) cycle of Plasmodium falciparum. (A) Relative protein levels for the mitochondrial enzymes branched-chain ketoacid dehydrogenase (BCKDH E2), isocitrate dehydrogenase (ICDH) and of the apicoplast enzyme dihydrodioldeoxynate transacylase (PDC E2). Data from three experiments performed in biological triplicates (n = 3) are shown as the 3D7WT/3D7PflipB ratio of the actin-normalised mean fluorescent signals for each protein. Error bars represent S.D. The variance was analysed with the Student’s t-test using GraphPad Prism 5. *P < 0.05. (B) Relative intracellular levels of metabolic cofactors are represented as Log2 of the 3D7WT/3D7PflipB ratio of the means from two experiments in biological triplicates. The relative mean levels are the sum of all peak areas relative to each isotopologue of each metabolite (n = 2). Error bars represent S.D. (C) (a) Bar graph summing the percentage of isotope incorporation in the identified cofactors AMP, ADP, ATP and NAD+. Data correspond to two experiments performed in biological triplicates (n = 2). The bars are divided to the mean contribution of each isotopologue to the total labelled fraction and displays error bars correspond to S.D. Empty bars correspond to metabolites identified in 3D7WT parasites, while dashed bars refer to metabolites from 3D7PflipB mutants. The variance of each isotopologue fraction between 3D7WT and 3D7PflipB (n = 2) is indicated by the asterisk symbol (coloured according to legend) and was analysed with the Student’s t-test using GraphPad Prism 8; *P < 0.05. (b) the same graph as (a) is shown with data points indicated for M + 5 (orange) and M + 10 (yellow).

**Table 2** Summary of mosquito infection attempts with Plasmodium falciparum cell line NF54PflipB.

| Stage V gametocytemia in cultures | Final concentration of stage V gametocytemia in the infectious blood meals | Wet mount exflagellation of infectious blood meal per field (x40 phase contrast) | Oocysts/mosquito | Sporozoites/mosquito |
|-----------------------------------|-------------------------------------------------|-------------------------------------------------|-----------------|---------------------|
| Parental (NF54)                   | Knockout (NF54 LipB KO)                         | Parental (NF54)                                 | Knockout (NF54 LipB KO) |
| 0.40%                            | 1.62%                                           | 0.11%                                           | 0.11%           | 5.2 11.7            |
| 1.37%                            | 0.63%                                           | 0.12%                                           | 0.12%           | 8.1 6.3             |
| 1.45%                            | 0.75%                                           | 0.11%                                           | 0.11%           | 9.3 4.9             |
| 0.92%                            | 0.93%                                           | 0.10%                                           | 0.10%           | 1.9 2.7             |
| 0.87%                            | 1.10%                                           | 0.12%                                           | 0.12%           | 9.4 5               |
| 1.10%                            | 1.03%                                           | 0.12%                                           | 0.12%           | 9.4 5               |
| 1.04% ± 0.24%                    | 0.99% ± 0.12%                                   | 0.11% ± 0.004%                                 | 0.11% ± 0.003% | 6.1 ± 1.7 6.5 ± 1.2|

Each row represents a separate experiment, with parental NF54 included in the first four. Despite equivalent numbers of mature gametocytes and rates of exflagellation of male gametes in comparison with parental NF54 parasites, the LipB knockout (KO) parasites failed to produce any salivary gland sporozoites, as determined from dissection and counting of 20 infected Anopheles mosquitoes per parasite line per experiment. The bottom row shows composite mean ± S.E.M. data.

![Fig. 3](image-url) Analysis of the expression of metabolic enzymes and the levels of cofactors involved in glycolysis and in the tricarboxylic acid (TCA) cycle of Plasmodium falciparum. (A) Relative protein levels for the mitochondrial enzymes branched-chain ketoacid dehydrogenase (BCKDH E2), isocitrate dehydrogenase (ICDH) and of the apicoplast enzyme dihydrodioldeoxynate transacylase (PDC E2). Data from three experiments performed in biological triplicates (n = 3) are shown as the 3D7WT/3D7PflipB ratio of the actin-normalised mean fluorescent signals for each protein. Error bars represent S.D. The variance was analysed with the Student’s t-test using GraphPad Prism 5. *P < 0.05. (B) Relative intracellular levels of metabolic cofactors are represented as Log2 of the 3D7WT/3D7PflipB ratio of the means from two experiments in biological triplicates. The relative mean levels are the sum of all peak areas relative to each isotopologue of each metabolite (n = 2). Error bars represent S.D. (C) (a) Bar graph summing the percentage of isotope incorporation in the identified cofactors AMP, ADP, ATP and NAD+. Data correspond to two experiments performed in biological triplicates (n = 2). The bars are divided to the mean contribution of each isotopologue to the total labelled fraction and displays error bars correspond to S.D. Empty bars correspond to metabolites identified in 3D7WT parasites, while dashed bars refer to metabolites from 3D7PflipB mutants. The variance of each isotopologue fraction between 3D7WT and 3D7PflipB (n = 2) is indicated by the asterisk symbol (coloured according to legend) and was analysed with the Student’s t-test using GraphPad Prism 8; *P < 0.05. (b) the same graph as (a) is shown with data points indicated for M + 5 (orange) and M + 10 (yellow).
apicoplast, we examined the phenotypes of a lipB deletion mutant (3D7ΔlipB) where 90% reduction of LA was earlier reported (Günther et al., 2007). We detected transcriptional changes of the apicoplast redox enzymes peroxidase-like enzyme TPxG, thioredoxin ATx2, and the peroxiredoxin AOP (Fig. 2A). We cannot exclude that these observations are a result of other outcomes of lipB deletion not related to LA depletion. Nevertheless, we propose the hypothesis that the observed redox changes might promote apicoplast redox homeostasis in response to an oxidative stress that may be caused by the depletion of the LA antioxidant function. In support of this hypothesis, up-regulation of TPxG also occurs in response to other oxidative stresses in P. falciparum (Foth et al., 2005), despite the dispensability of fatty acid biosynthesis in this organism (Packer et al., 1995). DHLA bound to PDC-E2 and to α-ketoglutarate dehydrogenase (KGDH) acts as an electron donor to glutaredoxins in an Escherichia coli mutant where both the thioredoxin and the GSH systems were disrupted (Feeney et al., 2011). Similarly, Mycobacterium tuberculosis KGDH E2 uses DHLA to transfer electrons received from E3 to peroxiredoxins and contributes to antioxidant defence (Bryk et al., 2002). Additionally, KADH E2-mediated reduction of thioredoxins was observed in mammals (Bunik and Follmann, 1993). A similar redox regulatory role of the apicoplast PDC E2 may explain why it is expressed during the intra-erythrocytic stages (Foth et al., 2005; McMillan et al., 2005), despite the dispensability of fatty acid biosynthesis in this stage (Vaughan et al., 2009). Our attempts to delete PDC E2 in P. falciparum using gene replacement were unsuccessful (data not shown), raising the possibility that its role during intra-erythrocytic development is essential. In agreement with this observation, Pfpdce2 was proposed to be essential during intra-erythrocytic development in a recent whole-genome random mutagenesis screen for P. falciparum (Zhang et al., 2018), however, Pfpdce2, was not found to be essential in a genome-wide screen performed for Plasmodium berghei (Bushell et al., 2017). This hypothetical role for lipB, and potentially LA, in redox regulation raises questions about the sources of electrons for LA/DHLA recycling. This might not be attributed exclusively to the flux of glycolytic pyruvate to PDC as this was suggested to contribute to a build-up of acetyl-CoA in the organelle (Lim and McFadden, 2010). A possible alternative candidate for LA/DHLA recycling might be the apicoplast-targeted GSH reductase (GR) (Müller, 2015). The GR-mediated reduction of LA was demonstrated in rats and, in particular, the use of NADPH + H+ as an electron donor was described in mitochondrial fractions (Pick et al., 1995; Haramaki et al., 1997).

Our data identified changes in cytosolic antioxidant expression in 3D7ΔlipB parasites. Both enzymes for which we observed upregulation, GST and 1-CysPx (Fig. 2B), are highly abundant in the P. falciparum cytosol (Liebau et al., 2002), and both have demonstrated antioxidant activity in this compartment (Krajski et al., 2001; Harwaldt et al., 2002; Liebau et al., 2002; Deponte and Becker, 2005). One potential explanation for this phenotype might be that a plastid-to-cytosol redox signalling, such as the signalling described in plant chloroplasts and cytosols (reviewed in Dietz et al., 2016) might also exist in Plasmodium. Testing this possibility will require further experimentation. In Arabidopsis thaliana, chloroplast-originated H2O2 signal induces upregulation of the expression of genes encoding different GSTs as well as enzymes involved in glycolysis and the pentose phosphate pathway (PPP) (Sewelam et al., 2014). Moreover, chloroplast-originated ROS signalling induces changes in the cell cycle progression (Bode et al., 2016).

The hypothesis that plastid-to-cytosol redox signalling occurs in Plasmodium is an exciting one which we think the field should consider in future work. Nevertheless, it is important to notice that while generating lipB deletion mutants, the parasites were forced through an intense selection bottleneck, and it is not possible to exclude that the observed redox changes may have resulted from adaptation that could have occurred during this process. In the future, it would be interesting to explore this hypothesis further using conditional mutants that would circumvent this complication and that may allow a temporal control of LA depletion.

Because we wanted to examine metabolic changes that might be linked to redox changes, we compared the metabolites between two mutants of the apicoplast PDH complex that show strong (lipB our study) or mild (aE3, Laine et al., 2015) changes in the expression of redox regulators. Metabolic analysis of 3D7ΔlipB mutants highlighted increased glycolytic activity, which is the main energy releasing pathway in blood stage P. falciparum (Salcedo-Sora et al., 2014). Increased glycolytic activity in the 3D7ΔlipB mutant was mostly evident through increased glucose demand and increased lactate production via spent medium analysis (Fig. 3) and was not seen in 3D7Δnear3 parasite (Supplementary Fig. S4).
Fig. 7. *Plasmodium falciparum* oocyst morphology on days 7 and 13 post-feeding of gametocytes to *Anopheles* mosquitoes shows a defect in sporozoite development for lipoic acid protein ligase B (LipB) knockout parasites. (A) Two representative light microscopy images of NF54ΔlipB and of NF54WT oocysts on day 7 post-feeding. Scale bars = 25 microns. (B) A representative differential interference contrast (DIC) microscopy image of NF54ΔlipB and of NF54WT oocysts on day 13. The insets are shown in white squares. Scale bar = 25 μm. The NF54ΔlipB oocysts show malformation in both methods and time points.
As it stands, these observations and the changes in redox regulators are linked to the deletion of lipB. However, we propose one possible model that could be considered for tying together the phenotypes of lipB deletion in the asexual stages: lipB deletion leads to LA depletion; changes to apicoplast redox balance due to this depletion of LA might induce changes in cytosolic redox through plastid-to-cytosol signalling as seen with other plastids. This putative signal leads to the temporary accelerated differentiation observed (Fig. 2C), which may be linked to the observed metabolic changes during the time window examined. Some uncertainty with this model stems from the potential dual roles of LA in (i) potentially regulating redox and (ii) supporting PDC-dependent fatty acid synthesis. However, accumulating evidence suggests that the latter is not essential during intra-erythrocytic development (Yeh and DeRisi, 2011; Cobbold et al., 2013) unless fatty acid starvation occurs (Botté et al., 2013). We thus propose that the changes in carbon metabolism observed upon lipB deletion are not the consequence of disruption of fatty acid metabolism. That said, we cannot rule out that the changes in cytosolic redox regulator expression, asexual cycle progression and the metabolic changes taking place in this mutant are not the cause of other outcomes of the lipB deletion not yet identified.

*Plasmodium falciparum* asexual blood stages are characterised by an oxidative TCA cycle, where the main carbon source is provided by 2-oxoglutarate that originates from glutamine. The majority of these glutamine-derived carbons exit the cycle as malate, while only a small fraction is further oxidised to generate oxaloacetate and citrate that then can take part again in the cycle (Cobbold et al., 2013; MacRae et al., 2013; Ke et al., 2015). Comparing the TCA cycle metabolism of 3D7*ΔlipB* mutants with 3D7WT parasites revealed differences in the glucose-derived carbon input. In 3D7*ΔlipB* mutants, citrate presented mainly as M + 2 labelling and showed a significant decrease in M + 5 while all other isotopologues for this metabolite were below detection levels (Fig. 4D). These results suggest that citrate is likely mainly generated by unlabelled oxaloacetate that originated from anaplerotic unlabelled 2-oxoglutarate entering the TCA cycle and fully labelled pyruvate providing M + 2 acetyl-CoA (Fig. 4A). This observation and the apparent absence of M + 4 and M + 6 citrate isotopologues could suggest that the small fraction of glucose-derived carbon is not taking part in the full cycle of 3D7*ΔlipB* mutants, unlike what happens in 3D7WT parasites (Fig. 4D).

The link between lipB deletion and the changes seen in the TCA cycle requires further study, and the lack of change in ICDH protein levels (Fig. 5A, Supplementary Fig. S6) is puzzling. One possible explanation is that the enzymatic activity of ICDH, or indeed citrate synthase and aconitate, may be inhibited in response to the observed cellular redox changes. Studies on plant mitochondria highlight the role of thiol redox switches in adjusting mitochondrial function in light of external stresses (reviewed in Nietzel et al., 2017), and citrate synthase, aconitate and ICDH are all substrates for thiol based redox regulation (Yoshida et al., 2013; Schmidtmann et al., 2014; Yoshida and Hisabori, 2014).

Interestingly, alongside the altered levels of citrate labelling, we observed an increase in relative levels of BCKDH E2 (Fig. 5A) and no change in the total relative intracellular levels of citrate (Fig. 4B). Previous studies suggested that citrate could take part in a malate shuttle, whereby cytosolic citrate is used to generate oxaloacetate and acetyl-CoA, which in turn take part in carbon fixation and protein acetylation (Cobbold et al., 2013; Storm et al., 2014). The apparent absence of M + 4 and M + 6 citrate in 3D7*ΔlipB* mutants is in line with this scenario and could suggest that the citrate fraction that is not cycling in the TCA cycle but may instead be channelled towards this citrate shuttle. If true, this would result in increased availability of cytosolic acetyl-CoA, which in turn may affect histone acetylation and thus gene expression patterns (Cobbold et al., 2016). Similarly, in macrophages activated by LPS and IFNγ, variations in the TCA cycle activity was observed along with reduced ICDH enzyme with consequent accumulation of citrate and succinate, generation of ROS signalling, and modulation of gene expression by alternative acetylation (Seim et al., 2019), lending precedence to our proposed explanation. This scenario remains, nonetheless, a speculative model that, if true, would explain the altered progression through the *P. falciparum* cell cycle, which typically depends on very tight regulation of gene expression (Bzdch et al., 2003).

We were unable to detect sporozoites in the salivary glands of mosquitoes infected with the NF54*ΔlipB* mutant, despite the fact that normal numbers of oocysts were produced (Table 2, Fig. 6). This observation suggests that lipB is essential for successful sporogony. This finding is surprising considering the evidence from the rodent parasite *P. berghei*, where deletion of lipB caused no defect to the production of salivary gland sporozoites but showed a moderate defect in liver-stage development (Falkard et al., 2013). This is not the first example of such a discrepancy. PDC E1α is dispensable for mosquito development in the rodent malaria parasite *P. yoelii* but was necessary for sporozoite maturation in *P. falciparum* (Cobbold et al., 2013). These findings point to different dependencies of human and rodent malaria on PDC enzymes for development in the mosquito. A possible reason for this difference may be the increased number of sporozoites produced per oocyst, which is four-fold higher in the human malaria parasite *P. falciparum* (Rungsiwongse and Rosenberg, 1991) than in rodent malaria parasites (Lindner et al., 2013; Shimizu et al., 2010). High sporozoite numbers in rodent malaria oocysts may require enhanced metabolism, which would depend on both PDC activity and a functional redox regulation network. Future examination of the asexual stages of the NF54*ΔlipB* mutant, such as the work presented here for the 3D7*ΔlipB* mutant, is merited to address the possibility that LA synthesis in the apicoplast is essential for completion of *P. falciparum* development in the mosquito vector.

**Acknowledgements**

We would like to thank Dr Sujaan Das, Dr Mahmood Alam and Dr Sonal Sethia for their help and advice. The research was supported by the European Community’s Seventh Framework Programme [grant number FP7/2007-2013] under grant agreements No 242095 and No ParaMet 290080 (MB), by Medical Research Council, UK, grant number MR/S024573/1 (LS), and by the National Institute of Health (USA) (RO1 AI085584) (DAF). The Wellcome Centre for Integrative Parasitology, UK, is supported by core funding from the Wellcome Trust, UK [104111]. DAF gratefully acknowledges earlier funding from the NIH (RO1 AI085584). Finally, GIM was awarded a Laureate Fellowship from the Australian Research Council and LS is a Royal Society of Edinburgh Personal Research Fellow, UK.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijpara.2020.10.011.

**References**

Akiode-Ndunge, O., Tambini, E., Gribaldi, G., McMillan, P.J., Müller, S., Aresse, P., Turrini, F., 2009. Co-ordinated stage-dependent enhancement of *Plasmodium falciparum* antioxidant enzymes and heat shock protein expression in parasites growing in oxidatively stressed or GDPD-deficient red blood cells. Malar. J. 8, 113. https://doi.org/10.1186/1475-2875-8-113.

Becker, K., Tilley, L., Vennerstrom, J.L., Roberts, D., Rogerson, S., Ginsburg, H., 2004. Oxidative stress in malaria parasite-infected erythrocytes: Host-parasite interactions. Int. J. Parasitol. doi:10.1016/j.ijpara.2003.09.011.
Dockrell, H.M., Playfair, J.H., 1984. Killing of Plasmodium yoelii
Biddau, M., Sheiner, L., 2019. Targeting the apicoplast in malaria. Biochem. Soc.
Bode, R., Ivanov, A.G., Hüner, N.P.A., 2016. Global transcriptome analyses provide
Falkard, B., Kumar, T.R.S., Hecht, L.-S., Matthews, K.A., Henrich, P.P., Gulati, S., Lewis,
Feeney, M.A., Veeravalli, K., Boyd, D., Gon, S., Faulkner, M.J., Georgiou, G., Beckwith,
Dietz, K.-J., Turkan, I., Krieger-Liszkay, A., 2016. Redox- and reactive oxygen species-
Trans. 47, 973–983. https://doi.org/10.1042/BST20170563.
McMillan, P.J., Stimmler, L.M., Foth, B.J., McFadden, G.I., Müller, S., 2005. The human
Plasmodium falciparum. PLoS Biol. 1, E5. doi:10.1371/journal.pbio.0000005.
Lambros, C., Vanderberg, J.P., 1979. Synchronization of Plasmodium falciparum erythrocytic stages in culture. J. Parasitol. 65, 418–420. https://doi.org/10.2307/
Lim, I., Foth, B.J., McFadden, G.I., 2010. The evolution, metabolism and functions of the apicoplast. Philos. Trans. R. Soc. Lond. B. Biol. Sci. 365, 749–763. https://doi.org/10.1098/rstb.2009.0273.
Lindner, S.E., Mikolajczak, S.A., Vaughn, A.M., Moon, W., Joyce, B.R., Sullivan, W.J., Kappe, S.H.I., 2013. Perturbation of Plasmodium falciparum Pfε2- deficient sporozoites reveal a critical role in maintaining RNA homeostasis and parasite transmissibility. Cell. Microbiol. 15, 1266–1283. https://doi.org/10.1111/j.1462-5822.2012.01603.x.
Liva, K.A., Schüttgen, T.E., 2018. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25, 402–408. https://doi.org/10.1016/j.meth.2001.12.062.
MacRae, J.J., Dixon, M.W.A., Deanley, M.K., Chua, H.H., Chambers, J.M., Keny, S., Bottova, I., Tilley, L., McCrineville, M.J., 2013. Mitochondrial metabolism of sexual and asexual blood stages of the malaria parasite Plasmodium falciparum. BMC Biol. 11, 67. https://doi.org/10.1186/1741-7007-11-67.
Maier, A.G., Braks, J.A.M., Waters, A.P., Cowman, A.F., 2006. Negative selection using yeast cytosine deaminase/uracil phosphoribosyl transferase in Plasmodium falciparum for targeted gene deletion by double crossover recombination. Mol. Biochem. Parasitol. 150, 118–121. https://doi.org/10.1016/j.molbiopara.2006.04.011.
McMillan, P.J., Stimmmer, L.M., Foth, B.J., McFadden, G.I., Müller, S., 2005. The human malaria parasite Plasmodium falciparum possesses two distinct dihydrolipoamide dehydrogenases. Mol. Microbiol. 55, 27–38. https://doi.org/10.1111/j.1365-2958.2004.04047.x.
Millard, P., Delpeigne, B., Guionnet, M., Heuillet, M., Bellvert, F., Létsise, F., Wren, J., 2019. IsoCor: Isootope correction for high-resolution MS labeling experiments. Bioinformatics 35, 4844–4847. https://doi.org/10.1093/bioinformatics/btz150.
Mohr, K., Kretzel, J., Jortner, E., Becker, K., 2014. The redox systems of Plasmodium falciparum and Plasmodium vivax: vision, in silico analyses and investigator studies. Curr. Med. Chem. 21, 1728–1756.
Mohring, F., Rahbari, M., Zechmann, B., Rahbari, S., Przyborski, J.M., Meyer, A., Beck, D., 2017. Determination of glutathione redox potential and pH value in subcellular compartments of malaria parasites. Free Radic. Biol. Med. 104, 104–117. https://doi.org/10.1016/j.freeradbiomed.2017.01.001.
Mooney, B.P., Miernyk, J.A., Randall, D.D., 2002. The complex fate of alpha-ketocarboxyls. Annu. Rev. Plant Biol. 53, 357–375. https://doi.org/10.1146/annurev.arplant.53.1.3575.
