Analysis of sex-specific lipid metabolism in *P. falciparum* gametocytes points to importance of sphingomyelin for gametocytogenesis

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Abstract

Male and female *Plasmodium falciparum* gametocytes are the parasite lifecycle stage responsible for transmission of malaria from the human host to mosquito vector. Not only are gametocytes able to survive in radically different host environments, but they are also precursors for male and female gametes that reproduce sexually soon after ingestion by the mosquito. Here we investigate the sex-specific lipid metabolism of gametocytes within their host red blood cell and poised for ingestion by the mosquito vector and subsequent sexual reproduction.

Comparison of the male and female lipidome identifies cholesteryl esters and dihydrosphingomyelin enrichment in female gametocytes. Chemical inhibition of each of these lipid types in mature gametocytes suggests dihydrosphingomyelin synthesis but not cholesteryl ester synthesis is important for sex-specific gametocyte viability. Genetic disruption of each of the two sphingomyelin synthase gene points towards sphingomyelin synthesis contributing to gametocytogenesis.

This study shows that gametocytes are not only distinct from asexual stages, but that the lipid composition is also vastly different between male and female gametocytes, reflecting the different cellular roles these stages play. Together our results highlight the sex-specific nature of gametocyte lipid metabolism that has the potential to be targeted to block malaria transmission.

Introduction

Transmission of *Plasmodium falciparum* from the human to mosquito host depends on male and female gametocytes. The development of transmissible gametocytes (gametocytogenesis) is by far the longest developmental sexual stage in *P. falciparum*. After 10-12 days of maturation in the human red blood cell (RBC), ingestion by a mosquito rapidly activates the gametocytes to form mature gametes capable of sexual reproduction (Bennink et al. 2016). Within 15 minutes of the blood meal, gametocytes egresses from their host RBC. While male gametocytes multiply into eight flagellated microgametes, female gametocytes form an immobile spherical macrogamete, poised for fertilisation. The zygote develops in the extracellular environment until invasion of the mosquito midgut wall 19-36 hours post blood meal (Aikawa et al. 1984; Sinden et al. 1985; Vlachou et al. 2004).

Such a rapid sex-specific metamorphosis is necessarily preceded by sex-specific preparations in the human host during the relatively slow gametocytogenesis. Surprisingly though, morphological gametocyte sex dimorphism is relatively subtle and mostly limited to the ultrastructural level. Recently the tagging of sex-specific molecular markers has revealed sex-specific gene and protein expression profiles (Lasonder et al. 2016; Miao et al. 2017). These studies identified differences in the expression of mRNA and protein involved in lipid metabolic pathways including phosphatidylcholine biosynthesis, fatty acid biosynthesis and ether lipid metabolism (Lasonder et al. 2016). Whether this results in differences in the lipid composition between male and female gametocytes remains unknown.

Lipids fulfil a vast range of functions in the cell. They not only provide building blocks in the form of fatty acids for the parasite's metabolism, but they also form the basis for membranes (mainly phospholipids and cholesterol), functionalise subdomains within membranes (e.g. sphingolipids), serve as energy storage (e.g. neutral lipids like cholesterylesters and triacylglycerol) and act as signalling molecules (e.g. diacylglycerol and ceramide). The nature of lipids in male and female gametocytes may reveal diverging cellular functions that precede sex-specific development in the mosquito.

69 Based on their resilience against many drugs that are active against asexual forms,
 70 gametocytes were generally thought to be relatively metabolically inactive (Canning
 71 & Sinden 1975; Sinden et al. 1978). However, the lipidome of combined male and
 72 female gametocytes changes significantly between young and mature gametocytes
 73 (Gulati et al. 2015; Tran et al. 2016), suggesting that lipid metabolism is active during
 74 gametocytogenesis. In particular, Gulati et al. (2015) identified intermediate products
 75 of sphingolipid metabolism in mature gametocytes, revealing that *de novo*
 76 sphingomyelin synthesis occurs during gametocytogenesis.

77

78 Sphingomyelin is synthesised *de novo* from serine and palmitoyl-CoA in five
 79 consecutive reactions. The final step of *de novo* sphingomyelin synthesis is mediated
 80 by sphingomyelin synthase (SMS). *P. falciparum* encodes two SMS (Gardner et al.
 81 2002), one of which is expressed throughout blood stages (SMS1, PF3D7_0625000),
 82 the other of which is gametocyte specific and female enriched (SMS2,
 83 PF3D7_0625100) (López-Barragán et al. 2011; Lasonder et al. 2016). In addition to
 84 generating sphingomyelin, SMS also regulates the levels of secondary messenger
 85 molecules such as ceramide and diacylglycerol (Fyrst & Saba 2010). The functional
 86 significance of SMS1 and SMS2 in *P. falciparum* has yet to be investigated.

87

88 Here we explore the sex-specific lipidome of mature *P. falciparum* gametocytes
 89 within their host RBC to determine the respective contribution of male and female
 90 gametocytes to the lipid profile of the infected RBC (iRBC). The functional
 91 significance of *de novo* synthesis of key sex-specific lipids is then investigated by
 92 pharmacological inhibition and reverse genetics.

93

94

95 **Materials and Methods**

96 **Culturing techniques**

97 Asexual 3D7 strain *P. falciparum* parasites were maintained in complete culture
98 medium (RPMI 1640-Hepes with Glutamax media supplemented with 10 mM D-
99 glucose, 480 μ M hypoxanthine, 20 μ g/mL gentamicin, 0.375% (w/v) Albumax II
100 and 2.5% v/v heat-inactivated human serum) as previously described (Maier & Rug
101 2013). Cultures were synchronised by sorbitol treatment as described previously
102 (Lambros & Vanderberg 1979) and isolated from their host cells by saponin treatment
103 (Dourmashkin et al. 1962).

104
105 Parasites were induced to form gametocytes as described by Fivelman et al. (2007)
106 with modifications to reduce asexual parasite proliferation (Ridgway et al. 2020).
107 Male and female gametocytes were collected by fluorescence activated cell sorting as
108 described by Ridgway et al. (2021). Briefly, magnet enriched 3D7 gametocytes with a
109 female-specific GFP tag (gABCG2-GFP, (Tran et al. 2014)) were stained with
110 Hoechst 33342 and collected by fluorescence activated cell sorting on day 9 post
111 commitment (mostly at stage IV). Hoechst staining identified gametocyte-infected
112 from uninfected RBC, while the female-specific GFP signal distinguished male and
113 female gametocytes.

114
115 Gametocytemia was measured every second day from day 2 post commitment by flow
116 cytometry of an aliquot of culture stained with 50 μ g/mL Hoechst 33342 in PBS (10.6
117 mM KH₂PO₄, 1.55 M NaCl, 29.7 mM Na₂HPO₄, pH 7.4) for 15 min at 37°C, rinsed
118 twice in PBS with 2,000 $\times$ g, 1 min spins and resuspended in PBS. Samples were
119 analysed on a LSR II Flow Cytometer (BD Biosciences) detecting Hoechst 33342 in
120 the Pacific Blue channel (350 nm excitation/461 nm emission). Parasitemia was
121 calculated as the proportion of Hoechst 33342 positive cells in 200,000 whole, single
122 cells as gated in FACS Diva software. Statistical significance and graphing of results
123 was performed in GraphPad Prism 7.

124 **Sex-specific gametocyte viability assay**

125 Magnet enriched gametocytes were exposed to 10 μ M of each test compound, 200
126 μ M artemisinin (0% viable control) or 0.1% (v/v) DMSO (100% viable control) for

127 96 h from day 2 to 6 post commitment in complete culture medium with 50 mM N-
128 acetyl D-glucosamine in a 96 well plate at 37°C in hypoxic conditions (1% O₂, 5%
129 CO₂, 94% N₂).

130

131 Parasites were then stained in 50 µg/mL Hoechst 33342 and 500 nM MitoTracker
132 Deep Red in complete culture medium supplemented with 50 mM N-acetyl D-
133 glucosamine for 30 min at 37 °C in hypoxic conditions (1% O₂, 5% CO₂, 94% N₂).
134 Stained cells were rinsed twice in PBS with 1,000 ×g, 5 min spins and resuspended in
135 PBS. Single colour controls for flow cytometry consisted of i) asexual 3D7 WT
136 culture stained with 500 nM MitoTracker Deep Red or 50 µg/mL Hoechst 33342 for
137 30 min in complete culture medium, rinsed twice in PBS with 1,000 ×g, 1 min spins
138 and resuspended in PBS, ii) unstained 3D7 gABCG2-GFP gametocytes suspended in
139 PBS and iii) unstained asexual 3D7 WT parasites in PBS (for the unstained control).
140 Samples were analysed on a LSR II Flow Cytometer (BD Biosciences) detecting
141 Hoechst 33342 in the Pacific Blue channel (405 nm excitation/461 nm emission),
142 MitoTracker Deep Red in the APC-Cy7 channel (644 nm excitation/665 nm emission)
143 and GFP in the FITC channel (488 nm excitation/509 nm emission). A total of
144 500,000 events were recorded in each single colour control. In each sample data was
145 recorded until 500,000 female gametocytes were counted.

146

147 After applying the gating strategy to isolate male and female gametocytes (Ridgway
148 et al. 2020) the mean fluorescence intensity of MitoTracker Deep Red detected on the
149 APC-Cy7 channel was recorded for each of the gametocyte populations. Data was
150 analysed in FlowJo and statistical significance was determined by ANOVA (analysis
151 of variance) in GraphPad Prism. Cell viability is expressed as a percentage of the
152 mean fluorescence intensity of MitoTracker Deep Red in gametocytes treated with
153 0.1% DMSO (100% viable) and 200 µM artemisinin treated gametocytes (0% viable).

154 **Generation of transgenic *P. falciparum* cell lines**

155 Annotated sequence of *P. falciparum* 3D7 reference strain was sourced from the
156 *Plasmodium* Genomics Resource PlasmoDB (www.plasmodb.org). To disrupt SMS1
157 (PF3D7_0625000) by double recombination, a 5' fragment and a 3' fragment of
158 SMS1 locus were amplified using primers al398/al399 and al400/al401
159 (Supplementary Table 1) and cloned into a pCC-1 vector containing a human

160 dihydrofolate reductase (hDHFR) and 5-fluorocytosine drug selection cassette (Maier
161 et al. 2008) at SacII/SpeI and EcoRI/AvrII sites to generate pCC-1/SMS1 construct
162 (Supplementary Figure 1). SMS2 (PF3D7_0625100) was also disrupted by double
163 recombination by amplifying a 5' fragment with al406/al407 primers and a 3'
164 fragment with al408/409 primers (Supplementary Table 1), which were cloned into a
165 pCC-1 vector at SacII/SpeI and EcoRI/AvrII sites to generate pCC-1/SMS2 construct
166 (Supplementary Figure 2). Given that SMS1 and SMS2 are adjacent genes, the pCC-
167 1/SMS1 and 2 construct aiming to disrupt both SMS genes was generated by cloning
168 the 5' fragment of SMS1 (amplified with al398/al399 primers) and the 3' fragment of
169 SMS2 (amplified with al408/al409 primers) at SacII/SpeI and EcoRI/AvrII sites.
170 Inserted DNA regions were confirmed by analytical restriction enzyme digest and
171 sequenced to verify constructs. Plasmids were purified using Invitrogen Purelink
172 Maxiprep kit prior to transfection.

173

174 *P. falciparum* 3D7 reference strain parasites were transfected as described previously
175 (Rug & Maier 2013). Briefly, 400 μ L of 100 μ g of plasmid DNA in Cytomix (120
176 mM KCl, 0.15 mM CaCl₂, 10 mM K₂HPO₄/KH₂PO₄, 25 mM HEPES, 2 mM ethylene
177 glycol-bis(β -aminoethyl ether)-tetraacetic acid, 5 mM MgCl₂, pH 7.6) was
178 electroporated into a sorbitol synchronised ring stage culture at 5% parasitemia by
179 310 V, 950 μ F pulse delivered in a 0.2 cm electrode gap cuvette placed in a BioRad
180 Genepulser II with ∞ capacitor. Transformed cells were immediately resuspended in
181 complete culture medium with 3% haematocrit uninfected RBC and incubated at
182 37°C in hypoxic conditions (1% O₂, 5% CO₂, 94% N₂). Culture medium was
183 supplemented with 2 nM WR99210 from 4 h post transfection and was replaced daily
184 for 5 days post transfection, then three times a week until parasites were observed by
185 Giemsa-stained thin smears of the culture. Parasites having integrated the plasmid
186 were enriched by three 21-day off/on 2 nM WR99210 cycles then negative selection
187 was applied by adding 231 nM 5-fluorocytosine. The transgenic parasites were cloned
188 by limited dilution (Rug & Maier 2013).

189 DNA extraction

190 Genomic DNA was extracted from saponin-isolated trophozoite stage parasites using
191 DNeasy Blood and Tissue kit (Qiagen) as per the manufacturer's instructions. For
192 Southern blot analysis, genomic DNA was eluted twice in 200 μ L elution buffer each

193 then concentrated by adding 40 μ L 3 M sodium acetate and 880 μ L 100% ethanol at
194 4°C overnight. Samples were centrifuged at 17,000 $\times g$ for 30 min at 4°C then DNA
195 was washed twice in 70% ethanol with 17,000 $\times g$, 20 min spins. DNA was air dried
196 then re-suspended in TE buffer (10 mM Tris base, 1 mM EDTA, pH 8.0). DNA
197 concentration and purity was measured by NanoDrop spectrophotometer
198 (Thermofisher).

199 **Quantitative PCR**

200 The abundance of WT and knock out (KO) genotypes in co-cultures was monitored
201 by quantitative PCR (qPCR) of regions of DNA specific to each genome. SMS1 KO
202 and SMS2 KO parasites were each quantified by primers specific to the sequence of
203 the inserted hDHFR drug resistance cassette. WT parasites were distinguished from
204 SMS1 KO parasites by primers specific to the region of SMS1 disrupted by
205 homologous recombination in the SMS1 KO parasites. WT parasites in the SMS2
206 KO/WT co-cultures were similarly quantified by primers specific to the disrupted
207 section of SMS2 in the SMS2 KO parasites. The primers for this experiment are
208 described in Supplementary Table 2.

209
210 The qPCR were performed using a Light Cycler 480 SYBR Green I Master mix
211 (Roche) as per the manufacturer's instructions for 10 μ L reactions in a 384 well plate.
212 Thermocycling was performed with a 10 min, 95°C pre-incubation; 45 cycles of 15 s
213 denaturation at 95°C, 15 s annealing at 52°C and 20 s elongation at 72°C; followed by
214 a melt curve established by denaturing at 95°C for 30 s, annealing at 60°C for 30 s
215 and then slowly denaturing by increasing the temperature to 95°C at 0.11°C/s. Melt
216 curves were observed using Light Cycler 480 software. The exported text file was
217 converted using Light Cycler 480 converter (Roche) and Cq and PCR efficiencies
218 were determined using the LinReg program (Ruijter et al. 2009). Relative
219 quantification of transcripts was expressed as described previously (Pfaffl 2001).

220

221 For the quantification of WT and KO parasite genotypes in co-cultures:

$$222 \text{ ratio} = (E_{\text{target}}^{\Delta C_{\text{Ptarget}}(\text{clonal-competition})}) / (E_{\text{ref}}^{\Delta C_{\text{Pref}}(\text{clonal-competition})})$$

223 with: E_{target} : primer efficiency of the target gene

224 E_{ref} : primer efficiency of the reference gene

225 $\Delta CP_{\text{target}}$ (clonal-competition): difference in crossing points of target gene
 226 amplification in a clonal population and the fitness competition co-culture
 227 ΔCP_{ref} (clonal-competition): difference in crossing points of reference gene
 228 amplification in a clonal population and the fitness competition co-culture
 229

230 **Southern Blot**

231 Disruption of a gene locus by homologous recombination was confirmed by
 232 diagnostic digest with restriction enzymes followed by Southern blot probing for both
 233 the 5' and 3' homologous regions (Rug & Maier 2013). DNA was digested with either
 234 AflIII and PacI (for SMS1 KO screening) or Xmn I and Hind III- HF (for SMS2 KO
 235 screening) as per the manufacturer's instructions then run on a 0.8% agarose gel at 90
 236 V for 15 min followed by a run at 20 V for 18 h. The gel was then rinsed as follows:
 237 15 min in depurination solution (0.25 M HCl); 3 min in milliQ water; 2× 15 min in
 238 denaturation solution (0.5 M NaOH, 1.5 M NaCl); 3 min in milliQ water; 2× 15 min
 239 in neutralisation solution (0.5 M Tris base, 1.5 M NaCl, pH 7.5); 3 min in milliQ
 240 water then 5 min in 20× SCC (3 M NaCl, 0.3 M citric acid trisodium dihydrate, pH
 241 7.0). DNA was transferred from the gel to the membrane overnight in a capillary
 242 transfer setup.

243
 244 Alkali-labile digoxigenin labelled deoxyuridine triphosphate (DIG-dUTP) was
 245 incorporated in Southern blot probes by PCR using PCR DIG Probe Synthesis kit
 246 (Roche) with the following specifications (refer to sequences in Supplementary table
 247 3). Each probe was amplified from the corresponding plasmid using either OneTaq
 248 (NEB) or Taq polymerase (Roche). SMS1 probes were purified by gel extraction
 249 (Qiagen kit). All probes were synthesised in the presence of 12 μM DIG-dUTPs
 250 except the SMS2 3' probe synthesised from 6 μM DIG-dUTPs. Thermocycling
 251 conditions consisted of initial denaturation for 30 s at 94°C; 30 cycles of 30 s at 94°C,
 252 1 min at 45°C (SMS2 3') or 50°C (SMS2 5') or 52°C (SMS1 5') or 55°C (SMS1 3'),
 253 1 min at 68°C; final elongation for 7 min at 68°C.

254
 255 Following transfer, the membrane was rinsed in 2× SCC for 5 min then DNA was
 256 crosslinked to the membrane in a UV cross linker set to deliver 70 mJ/cm^2 . The
 257 membrane was incubated with gentle rocking in DIG Easy Hyb solution (Roche Cat.

No. 11 796 895 001) at the respective probe hybridisation temperature (T_{hyb}) for 30 min. The probe was denatured in 50 μL water at 95°C for 5 min, cooled quickly on ice then added to DIG Easy Hyb solution at 0.15% (v/v) to prepare the hybridisation solution. The membrane was incubated overnight in hybridisation solution at T_{hyb} with gentle rocking.

263

The membrane was prepared for detection by rinsing in 2 $\times$ SCC for 5 min; equilibrating in washing buffer for 1 min (0.1 M maleic acid, 0.15 M NaCl, pH 7.5 with 0.3% (v/v) Tween-20); incubating in 1% blocking solution (0.1 M maleic acid, 0.15 M NaCl, pH 7.5 with 1% (w/v) skim milk powder) for 30 min; incubating in 1% blocking solution with 1:10,000 α -Digoxigenin-AP Fab fragments (Roche) for 30 min; washing membrane in washing buffer for 2 $\times$ 15 min then equilibrating membrane in detection buffer (100 mM Tris-hydrochloride, 100 mM NaCl, pH 9.5) for 2 min. The membrane was incubated in the dark with CSPD (Roche) diluted 1:100 in detection buffer for 5 min at room temperature then 10 min at 37°C. The membrane was exposed to Fuji Super RX-N Medical X-Ray film in an Amersham pharmacia biotech hypercassette and developed film in an AGFA CP1000 photo developer.

275 **Lipidomic analysis**

276

Uninfected human RBC from six donors were pooled in three independent biological replicates each from two donors and incubated at 4% haematocrit in complete culture medium for at least 48 h at 37°C in hypoxic conditions (1% O₂, 5% CO₂, 94% N₂). Cells were pelleted at 524 $\times g$ over 5 min and counted on an Improved Neubauer haemocytometer (Hirschmann). Aliquots of 10⁷ cells were resuspended in 300 μL of methanol in 2 mL tough tubes (Geneworks) and stored at -80°C. For each biological replicate parasites of different stages were grown in the same donor batch of RBC to reduce host specific influences.

For collection of asexual parasite infected RBC, three independent asexual *P. falciparum* cultures were sorbitol synchronised then magnet purified to more than 98% iRBC, as determined by Giemsa-stained thin smear, and counted on an Improved Neubauer haemocytometer (Hirschmann). Aliquots of 10⁷ iRBC at 26-42 h post invasion were resuspended in 300 μL of methanol in 2 mL tough tubes (Geneworks) and stored at -80°C.

291 Male and female gametocytes were sorted live by FACS as described above, pelleted
292 at 754 $\times g$ for 10 min and resuspended in 300 μL of methanol per 10^7 cells (as counted
293 during FACS) in 2 mL tough tubes (Geneworks). Each biological replicate of 10^7
294 cells is pooled from 2-5 independent gametocyte cultures.

295

296 Lipids were extracted as described previously (Matyash et al. 2008) with
297 modifications. Internal standards (Tran et al. 2016) in 50 μL of methanol with 0.01%
298 butylated hydroxytoluene were aliquoted among tubes. Internal standards and 1.4 mm
299 beads (Geneworks) were added to tough tubes containing samples in 300 μL methanol
300 and samples were homogenized with a bead homogenizer (FastPrep-24, MP
301 Biomedical) at 6 m/s for 40 s and transferred to new tubes. Beads were rinsed in 100
302 μL of methanol, which was added to the sample in new tubes. Samples were vortexed
303 with 1 mL of methyl-tert butyl ether at 4°C for 1 h. Phase separation was induced by
304 adding 300 μL of 150 mM ammonium acetate (liquid chromatography–MS grade,
305 Fluka), vortexing for 5 min and centrifuging at 2,000 $\times g$ for 5 min. The upper organic
306 layer (representing around 800 μL) was transferred to a 2 mL glass vial and stored at
307 –20°C. Prior to MS analysis, each sample was diluted into methanol:chloroform (2:1
308 v/v) with 5 mM ammonium acetate.

309 An aliquot of each extract was hydrolyzed to remove acyl-linked lipids and re-
310 extracted to improve mass spectrometric analysis of sphingolipids. Two hundred μL
311 of extract was added to 60 μL of methanol containing 0.01% butylated
312 hydroxytoluene, 22 μL of 10 M NaOH was added (final concentration 0.7 M), and
313 vortexed at 800 rounds per minute on an Eppendorf Mixmate for 2 h at room
314 temperature. Sixty μL of 150 mM aqueous ammonium acetate was added to induce
315 phase separation. Tubes were vortexed and spun at 20,000 $\times g$ for 5 min to complete
316 phase separation. The upper organic layer was removed to a new 2 mL glass vial, and
317 diluted into methanol:chloroform (2:1 v/v) containing 5 mM ammonium acetate prior
318 to mass spectrometric analysis.

319 Mass spectra were obtained with a chip-based nanoESI source (TriVersa Nanomate,
320 Advion) and a hybrid linear ion-trap-triple quadrupole MS (QTRAP 5500,
321 ABSCIEX). Ten μL of each sample extract in a sealed Eppendorf Twin-Tec 96 well
322 plate was aspirated and delivered to the MS through a nanoESI chip. Positive ion and

323 negative ion acquisition was obtained as described previously (Tran et al. 2016).

324 Data smoothing, lipid identification, removal of isotope contribution from lower mass
 325 species and correction for isotope distribution was performed in LipidView
 326 (ABSCIEX) software version 1.2. A signal to noise ratio threshold of 20 was applied
 327 for inclusion of ionized lipids. Extraction and solvent blanks were analyzed in each
 328 data acquisition batch to exclude chemical or solvent impurities. Lipids were
 329 quantified in LipidView by comparing peak area of each lipid to its class-specific
 330 internal standard after isotope correction. Where odd-chain fatty acid phospholipids or
 331 ether-linked phospholipids could not be distinguished, phospholipids were assumed to
 332 be ether linked. A correction factor of 3.45 was applied to all ether-PE species to
 333 account for the ~29% difference in efficiency in the neutral loss of the 141 Da
 334 fragment from plasmalogen and diacyl PE respectively (Mitchell et al. 2007; Abbott et
 335 al. 2013). Lipid species are annotated as per Liebisch et al. (2013) shorthand, except
 336 for DAG and TAG.

337 LipidView data was exported to Excel then imported to Markerview v1.2.1.1
 338 (Applied Biosystems, MDS Sciex) with lipid annotation and quantification for
 339 statistical analysis of all individual lipid species. Pareto scaling without weighting was
 340 applied for unsupervised mode principal component analysis. Multiple t-tests for
 341 volcano plots were also performed in MarkerView (MDS Sciex). Grouping into lipid
 342 classes and calculation of lipid proportions were performed in Microsoft Excel.
 343 GraphPad Prism 7 was used to prepare graphs and perform ANOVA with multiple
 344 comparisons on lipid class abundance.

345

346

Results

The lipid composition of infected RBC is characteristic of the parasite's sex and lifecycle stage

The lipidomic analysis was performed on uninfected RBC and RBC infected with male gametocytes, female gametocytes or mature asexual blood stage parasites. Over 230 lipid species were identified in the samples (for a complete list refer to Supplementary Table 4). We performed a principle component analysis to determine the relative contribution of each of these lipid species to the variation between samples (Figure 1). The principal component analysis plot (Figure 1A) groups together samples of similar lipid composition and the corresponding loading plot (Figure 1B) illustrates which lipid species distinguish sample groups.

In considering all measured lipids by principal component analysis, the biggest difference in lipid composition distinguishes infected from uninfected RBC regardless of parasite lifecycle stage (principal component (PC) 1, Figure 1A). A third of the lipid variation between samples nevertheless separates asexual parasite-infected RBC, male gametocyte-infected RBC and female gametocyte-infected RBC (principal component (PC) 2, Figure 1A). Parasite stages mainly cluster based on cholesteryl ester (CE) species, free cholesterol (FC), diacylglycerol (DAG) and phosphatidylcholine (PC) (Figure 1B).

Female gametocyte infected RBC contain more neutral lipids than other stages

Upon infection, total lipid abundance in infected RBC increases 3-5 fold and in sexual stages, female gametocyte-infected RBC accumulate significantly more lipids than male gametocyte-infected RBC (Figure 1C). However, this increase is not reflected across all lipids: of the four main lipid categories (phospholipids, sphingolipids, free cholesterol and neutral lipids), only neutral lipids are significantly more abundant in female gametocyte-infected RBC compared to male gametocyte-infected RBC (Figure 1C). This is also reflected in the relative proportions of lipid groups in each stage: neutral lipids account for 31% of lipids in female gametocyte-infected RBC compared to only 13% in male gametocyte-infected RBC (Figure 1D). Sphingolipid and free cholesterol contents are similar in abundance but decreases in relative terms in RBCs upon infection or in sexual stages. Phospholipids on the other hand are more

380 abundant and represent a larger proportion to the relative lipid contribution of infected
381 RBCs.

382

383 In general, neutral lipids are a cellular means of storing energy and contribute to
384 intracellular signalling pathways. The nature of neutral lipids in asexual parasite-
385 infected RBC contrasts to that of gametocyte-infected RBC (Figure 2). Of the three
386 neutral lipids groups (cholesteryl ester (CE), diacylglycerol (DAG) and triacylglycerol
387 (TAG)), DAG and TAG are the predominant neutral lipids in asexual parasite-
388 infected RBC, whereas CE dominates the neutral lipid profile of gametocyte-infected
389 RBC and uninfected RBC (Figure 2A and B). The proportions of neutral lipid
390 categories are highly dynamic upon infection with asexual or sexual stage *P.*
391 *falciparum*. In addition, accumulation of neutral lipids is sex-specific: female
392 gametocyte-infected RBC accumulate significantly more CE than male gametocyte-
393 infected RBC (Figure 2A). These trends are also observed at the individual lipid
394 species level (Figure 2C and D). CE species characterise female gametocyte-infected
395 RBC while asexual parasite-infected RBC are distinguished by DAG and TAG
396 species (Figure 2C and D).

397

398 **Phospholipids of gametocyte-infected RBC differ from those of asexual stage** 399 **parasite infected RBC but are not sex-specific**

400 Phospholipids are the main component of membranes and amongst others are required
401 for organelle biogenesis. Hence it is not surprising that most lipids in iRBC belong to
402 this group (Figure 1A). Although male gametocyte-infected RBC have a higher
403 proportion of phospholipids (61% phospholipids in males compared to 44% in
404 females, Figure 1D), phospholipid abundance is similar in male and female
405 gametocyte-infected RBC (Figure 1C). There were no significant differences in major
406 phospholipid groups phosphatidyl choline (PC), phosphatidyl ethanolamine (PE),
407 phosphatidyl serine (PS) or phosphatidyl glycerol (PG) between sexes (Figure 3A).
408 PG is only detected in iRBC. Male gametocyte-infected RBC contain slightly more
409 PC but slightly less PE than female gametocyte-infected RBC, both in terms of
410 abundance and proportion of phospholipids (Figure 3A and B).

411

412 Principal component analysis based only on phospholipids does not distinguish
413 between male and female gametocyte-infected RBC (Figure 3C). However,

gametocyte-infected RBC cluster apart from uninfected RBC and asexual parasite-infected RBC mostly due to PE species (Figure 3C and D). Overall, male and female gametocyte-infected RBC phospholipids are similar to each other, but distinct from host cell and asexual parasite-infected RBC phospholipids.

418

The sphingolipid dihydrosphingomyelin is a key characteristic of female gametocytes

Sphingolipids are also components of cell membranes and form detergent-resistant lipid domains that are platforms for intracellular signalling. Like phospholipids, overall sphingolipid abundance is not significantly different between male and female gametocyte-infected RBC (Figure 1C). However, breaking down sphingolipids into its three main groups (sphingomyelin (SM), dihydrosphingomyelin (DHSM) and ceramide) reveals gametocyte and sex-specific sphingolipid groups (Figure 4). Unlike the abundance of the major sphingolipid SM that is similar in all samples (Figure 4A), gametocyte-infected RBC (especially females) accumulate DHSM (Figure 4A and B).

429

Principal component analysis of sphingolipids alone does not distinguish uninfected RBC from asexual parasite-infected RBC. However, male and female gametocyte-infected RBC cluster separately (Figure 4C). DHSM 20:0, 16:0 and 18:0 especially distinguish gametocyte-infected RBC from the other samples (Figure 4D). Overall, accumulation of DHSM marks gametocytogenesis and is even more pronounced in females.

436

Of the lipid species with more than a two-fold difference between female and male gametocyte-infected RBC, six are significantly more abundant in female gametocyte-infected RBC (Figure 5A). Five of these are saturated DHSM species (between C16-C20 in size), the other is SM 22:2, identifying sphingolipids as the major sex-specific lipid group. On average several neutral lipid species also appear more abundant in female gametocytes, however this difference lacks statistical significance due to variation between biological replicates. No lipid species are significantly more abundant in male gametocyte-infected RBC, although some phospholipid species are more abundant on average. Overall, the sex-specific lipid profile of gametocyte-infected RBC points towards DHSM as a key characteristic of female gametocytes.

447

448 Compared to asexual stage parasite-infected RBC, both sexes of gametocyte-infected
449 RBC contain more DHSM 20:0, 18:0 and 16:0 (Figure 5B & C). Asexual parasite-
450 infected RBC on the other hand contain significantly more of some PC species
451 compared to both sexes of gametocyte-infected RBC (PC32:0, PC32:1, PC34:0,
452 PC36:0 [in males only]). CE 16:1 and CE 16:0 are the only lipid species that are more
453 abundant in female gametocyte-infected RBC but not in males when compared to
454 asexual parasite-infected RBC, which might point to specialised functions of these
455 lipid species in female parasites. Several phospholipid and sphingolipid species are
456 significantly more abundant in each sex of gametocyte-infected RBC compared to
457 uninfected RBC (Figure 5D & E). Overall, gametocytes significantly modify the lipid
458 profile of the host RBC during maturation in a manner that distinguishes them from
459 asexual parasites.

460

461 **Sphingolipid synthesis is functionally important in both asexual and sexual blood** 462 **stages**

463 To investigate the functional significance of the sex-specific lipid composition,
464 gametocytes were exposed to three compounds that block the synthesis of the most
465 sex specific lipids (CE and sphingolipids) from day 2 to 6 post commitment (Figure
466 6). Thereafter the sex-specific viability of gametocytes was measured by flow
467 cytometry. GT11 is an analogue of dihydroceramide that cannot be metabolised by
468 dihydroceramide desaturase in mammalian cells, thereby blocking the enzyme
469 responsible for ceramide synthesis (Bedia et al. 2005). At concentrations greater than 5
470 μ M, GT11 more generally decreased *de novo* sphingolipid synthesis in cultured
471 mammalian cells (Triola et al. 2004). Glibenclamide and Sandoz 58-035 directly
472 inhibit cholesterol esterification by acyl-coenzyme A: cholesterol acyltransferase in
473 mammalian cells (Ross et al. 1984). At 10 μ M only the sphingolipid synthesis
474 inhibitor GT11 appears to impact gametocyte viability in a male-specific manner
475 (Figure 6).

476

477 The apparent sex-specific function of sphingolipid synthesis in gametocytes was
478 further investigated by reverse genetics. DHSM and SM are synthesised by
479 sphingomyelin synthase (SMS) from dihydroceramide and ceramide respectively. *P.*
480 *falciparum* encodes two such enzymes (SMS1 and SMS2) that are expressed in a

481 parasite stage- and sex-specific manner. SMS activity contributes to the synthesis of
482 SM, a component of detergent-resistant lipid domains in cell membranes, but also
483 catabolises the intracellular signalling molecule ceramide.

484

485 Three independent attempts to disrupt both SMS1 and SMS2 genes together did not
486 yield any parasites after transfection. However, disruption of each SMS gene
487 individually produced asexual blood stage parasites that could be analysed further
488 (Supplementary Figures 1 and 2). We first tested the relative fitness of these knock
489 out (KO) parasites. In 28-day co-cultures wild type parasites quickly outgrew each of
490 the KO parasites (Figure 7), suggesting that SMS1 and SMS2 are each required for
491 optimal asexual parasite proliferation. Depleting the culture medium lipids by 75%
492 did not exacerbate the growth defect observed in each KO cell line (Figure 7). In other
493 words, SMS disruption is not readily compensated by an increase in extra-cellular
494 lipid scavenging. Treatment with the sphingolipid synthesis inhibitors GT11 had the
495 same effect on WT and each KO cell line (Figure 8). This suggests that GT11, an
496 analogue of dihydroceramide, does not block SMS in asexual blood stage parasites.

497 Given that sphingolipid composition is distinctive in gametocytes, the impact of
498 disrupting each SMS was next investigated in sexual blood-stage parasites. The
499 absence of either SMS1 or SMS2 increased gametocyte commitment compared to WT
500 parasites (Figure 8A and B). This may result from asexual blood-stage parasite stress
501 given that gametocyte commitment is a generic parasite stress response (Dixon et al.
502 2008). When treated with GT11 however, gametocytes lacking a SMS were
503 hypersensitive compared to WT gametocytes (Figure 8C). This suggests that SMS and
504 dihydroceramide desaturase function is additive in gametocytes, especially in
505 conditions where SMS function is limited either through disruption of one of the SMS
506 genes (Figure 8C) or by native reduction of SMS gene expression in males relative to
507 females (Figure 6).

508

509

Discussion

In summary the lipid composition and abundance of lipids in *P. falciparum* gametocytes is sex-specific. Male/female gametocyte lipid dimorphism precedes gametocyte activation and reflects their sex-specific functions.

Female gametocyte-infected RBC stockpile the neutral lipid CE but were resistant to CE synthesis inhibitors Sandoz 58-035 and Glibenclamide. This suggests that acyl-coenzyme A: cholesterol acyltransferase -mediated CE synthesis is not essential in mature gametocytes. CE could instead be synthesised by lecithin-cholesterol acyltransferase, or could be scavenged from the host. Alternatively, CE may only be required for later developmental stages. Indeed the lipids in the fertilised zygote are exclusively contributed by the female gametocyte. While the zygote develops in the midgut lumen, neutral lipids are diverted from the blood meal to the eggs of the mosquito *via* lipophorin (Costa et al. 2018). The zygote is therefore vulnerable to lipid depletion in the mosquito midgut lumen, and may depend on maternal CE reserves at this lifecycle stage. In particular, given that mosquitoes are incapable of cholesterol synthesis (Clayton 1964), CE could be a source of cholesterol for the parasite in mosquito stages. *P. falciparum* encodes esterases that could potentially release cholesterol from CE stores (Butler et al. 2020). Neutral lipid staining of the female gametocyte previously highlighted a large neutral lipid body in the cytoplasm (Tran et al. 2014). Potentially the female specific CE store is amassed by the putative lipid transporter gABCG2. Hence, it would be interesting to test the effect of CE synthesis inhibitors on the mosquito stages in future studies.

Male gametocyte-infected RBC do not contain more phospholipids than female gametocyte-infected RBC. Phospholipids are the main component of membranes and are likely required for the rapid cell divisions during male gametocyte activation when eight microgametes are formed within the male gametocyte. While additional plasma membranes may not be required in females, female gametocytes contain an elaborate mitochondrial network bound by a double membrane rich in phospholipids, as well as abundant membrane bound osmiophilic bodies (Langreth et al. 1978; Jensen 1979; Sinden 1982; Ponnudurai et al. 1986). Although the total phospholipid content of male and female gametocyte-infected RBC is similar, the allocation of phospholipids between organelles may be sex-specific.

For example, PG serves as the precursor for cardiolipin found exclusively in the mitochondria (Gebert et al. 2009; de Kroon et al. 1999; Daum 1985). Consistent with this, PG is absent in uninfected RBC, which lack mitochondria. Surprisingly however, there is no difference in PG between male and female gametocytes despite female gametocytes relying more on their mitochondria than males. Indeed mitochondrial proteins have been shown to be more abundant in female than in male gametocytes (Miao et al., 2017). Female gametes in the mosquito have an increased energy demand (probably in anticipation of the post-fertilisation stage (MacRae et al. 2013), whereas male gametocytes lose their mitochondria completely during the development into microgametes (Okamoto et al. 2009). The presence of comparable amounts of PG both in male and female iRBC argues for a similar number or size of mitochondria at least at the analysed stage of development (stage IV gametocytes). However, less PG is detected in the RBC infected with sexual stages compared to the RBC infected with asexual stages despite the upregulation of tricarboxylic acid cycle function in gametocytes (MacRae 2013). This cautions against the simple correlation between PG and mitochondrial function. Nonetheless, the specificity and accumulation of PG in the parasite could be targeted by antimalarial compounds effective against both asexual and sexual blood stage parasites.

Phospholipid composition distinguished both male and female gametocyte-infected RBC from asexual parasite-infected RBC and uninfected RBC. Less phospholipids were present in gametocyte-infected RBC compared to asexual parasite-infected RBC consistent with asexual replication requiring phospholipid-rich membrane biogenesis (e.g. for the plasma membrane of a growing parasites, for merozoite formation and for RBC modifications such as Maurer's clefts). The increase in PS between trophozoites and gametocytes reported by Gulati et al. (2015) was not observed in this study, perhaps due to technical differences in sample preparation.

570

In asexual blood stages, reduction of sphingolipid metabolism by disruption of either SMS1 or SMS2 was not lethal, but incurred a growth defect. Disruption of both SMS genes on the other hand appears to be lethal for asexual blood stage parasites, despite SMS2 being expressed at very low levels in this lifecycle stage. This suggests that *de novo* sphingomyelin synthesis is essential in asexual blood stages and indicates that each SMS is – to a certain degree – functionally redundant. Surprisingly this defect was not exacerbated by depleting the culture medium lipids by 75%, or by inhibiting

578 dihydroceramide desaturase with GT11. This supports the hypothesis that SMS
579 activity regulates intracellular ceramide concentration rather than sphingomyelin itself
580 being essential for asexual blood stage proliferation. Indeed, ceramide might
581 otherwise build up to cytotoxic levels due to sphingomyelinase activity, which has
582 previously been described in asexual stages (Hanada et al. 2002). Inhibition of
583 ceramide production by sphingomyelinase with GW4869 is also lethal for asexual *P.*
584 *falciparum* (Gulati et al. 2015), further illustrating the importance of regulating
585 ceramide abundance.

586

587 The most distinctive feature of gametocyte-infected RBC, particularly female
588 gametocyte-infected RBC, is the presence of DHSM species. This is consistent with
589 other studies that suggest *de novo* sphingolipid synthesis, rather than host SM
590 hydrolysis by neutral sphingomyelinase, is active in gametocytes (Gulati et al. 2015;
591 Tran et al. 2016). Here we sought to test whether DHSM accumulation in
592 gametocytes results from decreased catabolism by dihydroceramide desaturase and/or
593 from increased synthesis by SMS. Inhibition of dihydroceramide desaturase with 10
594 μ M GT11 selectively killed male gametocytes (Figure 6) suggesting that
595 dihydroceramide desaturase activity is sex-specific, which may contribute to the
596 difference in DHSM abundance. The male-specific effect of GT11 was not observed
597 in combined male and female gametocytes (Figure 8), presumably due to the
598 gametocyte population being female biased. However, gametocytes that lack an SMS
599 were hypersensitive to GT11 (Figure 8). In other words, reduced SMS activity (due to
600 endogenous differential SMS gene expression between male and female gametocytes
601 or through SMS gene disruption) appears to make parasites more susceptible to
602 dihydroceramide desaturase inhibition by GT11. Given that dihydroceramide
603 desaturase and SMS share dihydroceramide as a substrate, hypersensitivity to GT11 in
604 gametocytes with reduced SMS activity may result from an accumulation of
605 dihydroceramide. Conversely, accumulation of DHSM in gametocytes may be a
606 means of reducing intracellular dihydroceramide levels.

607 Similarly, the observed increased gametocyte commitment in SMS KO cell lines
608 could be a stress response to elevated dihydroceramide concentrations. Indeed it has
609 recently been observed that dihydroceramides are biologically active lipids involved
610 in diverse mammalian cell functions (Siddique et al. 2015). Lipids have previously
611 been implicated in inducing gametocytogenesis in *P. falciparum* (Brancucci et al.

2017). In particular, depletion of serum lysophosphatidylcholine, a precursor of PC synthesis, is known to trigger gametocytogenesis. We observed no significant difference in lysophosphatidylcholine abundance between male and female gametocytes (supplementary figure S3). PC is a substrate of SMS-mediated SM and DHSM synthesis. As such, reducing parasite SMS activity may have mimicked the downstream effect of PC depletion and triggered gametocytogenesis.

Overall, this study has shown that lipid metabolism is not only variable between parasite lifecycle stages, but is also sex-specific. These differences have implications for the effectiveness of drugs targeting metabolic pathways, and should be considered in the design of future transmission blocking antimalarial treatments. In terms of the fundamental biology of the parasite, the lipid profile of gametocytes is a testament to the parasite's ability to thrive in two radically different host environments. The lipidome presented here provides a reference for unravelling the complex sex-specific function of lipids in the *P. falciparum*.

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Author contributions:

M.C.R. and A.G.M. conceived and designed the study; M.C.R., P.T., S.B. and D.C. performed the experiments; M.C.R., S.B., D.C., T.M. and A.G.M. analysed the data; and M.C.R. and A.G.M. wrote the paper. All authors contributed to the editing of the paper.

642

643 **Conflict of Interest Disclosure:**

644 The authors declare that they have no conflict of interest.

645

646 **Submission of data set to database:**

647 The mass spectrometry raw data of the lipidomics experiments have been deposited in
648 the MetaboLights database (<http://www.ebi.ac.uk/metabolights>).

649

650

651 **Figure legends:**

652 **Figure 1:** A-B: Principal component analysis (A) and loading plot (B) of the lipidome
653 of uninfected red blood cells (uRBC- red) and red blood cells infected with asexual
654 stage parasites (Asexual- green), female gametocytes (Female - pink) and male
655 gametocytes (Male - blue). Black circles in A indicate sample groups. The individual
656 lipid species are colour-coded to indicate the group they belong to (Neutral lipids –
657 blue; Phospholipids – red, Sphingolipids – green, Free Cholesterol – indigo). C-D:
658 Mean lipid abundance with standard deviation (C) and proportions (D) of lipid classes
659 in uninfected red blood cells (uRBC) and red blood cells infected with asexual stage
660 parasites (Asexual), male gametocytes (Male) and female gametocytes (Female).
661 Results averaged from three independent biological replicates of 10^7 cells each.
662 Significance calculated by ANOVA. ***: $p < 0.001$, *: $p < 0.1$, ns: not significant, $p >$
663 0.1. PC1 and 2: first and second principal component; CE: cholesteryl ester; FC: free
664 cholesterol; PC: phosphatidylcholine; PE: phosphatidylethanolamine; DAG:
665 diacylglycerol.

666

667

668 **Figure 2:** A-B: Mean neutral lipid abundance with standard deviation (A) and mean
669 proportions (B) of uninfected red blood cells (uRBC) and red blood cells infected
670 with asexual stage parasites (Asexual), male gametocytes (Male) or female
671 gametocytes (Female). Results averaged from three independent biological replicates
672 of 10^7 cells. Significance calculated by ANOVA. *****: $p < 0.0001$, ns: not significant,
673 $p > 0.1$. CE: cholesteryl ester; DAG: diacylglycerol; TAG: triacylglycerol. C-D:
674 Principal component analysis (C) and loading plot (D) of neutral lipid composition of
675 uninfected red blood cells (uRBC) and red blood cells infected with asexual stage
676 parasites (Asexual), male gametocytes (Male) or female gametocytes (Female). PC1
677 and 2: first and second principal component; CE: cholesteryl ester; DAG:
678 diacylglycerol; TAG: triacylglycerol.

679

680

681 **Figure 3:** A-B: Mean phospholipid composition with standard deviation (A) and
682 mean proportions (B) of uninfected red blood cells (uRBC) and red blood cells
683 infected with asexual stage parasites (Asexual), male gametocytes (Male) or female
684 gametocytes (Female). Results averaged from three independent biological replicates
685 of 10^7 cells. Significance calculated by two-way ANOVA. ns: not significant, $p > 0.1$.
686 C-D: Principal component analysis (C) and loading plot (D) of phospholipid
687 composition of uninfected red blood cells (uRBC) and red blood cells infected with
688 asexual stage parasites (Asexual), male gametocytes (Male) or female gametocytes
689 (Female). PC1 and 2: first and second principal component; PC: phosphatidylcholine;
690 PE: phosphatidylethanolamine; PS: phosphatidylserine; PG phosphatidylglycerol.

691

692

Figure 4: A-B: Mean sphingolipid abundance with standard deviation (A) and mean proportion (B) in uninfected red blood cells (uRBC) and red blood cells infected with asexual stage parasites (Asexual), male gametocytes (Male) or female gametocytes (Female). Results averaged from three independent biological replicates of 10^7 cells. Significance calculated by two-way ANOVA. **: $p < 0.01$, ns: not significant, $p > 0.1$. C-D: Principal component analysis (C) and loading plot (D) of sphingolipid composition of uninfected red blood cells (uRBC) and red blood cells infected with asexual stage parasites (Asexual), male gametocytes (Male) or female gametocytes (Female). PC1 and 2: first and second principal component; DHSM: dihydrosphingomyelin; SM: sphingomyelin; Cer: ceramide.

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Figure 5: Volcano plot of lipid species in male and female gametocyte-infected red blood cells. A, C and E: Comparison of female gametocyte-infected red blood cell lipids to male gametocyte-infected red blood cells (A), asexual-stage infected red blood cells (C) and uninfected red blood cells (E). B and D: Comparison of male gametocyte-infected red blood cell lipids to asexual-stage infected red blood cells (B) and uninfected red blood cell (RBC) (D). Fold change averaged from three biological repeats of 10^7 cells each. P values calculated by student t tests. Grey area represents changes that are less than two-fold different between samples and/or have a significance of $p > 0.01$. Circles in A highlight groups of lipids. DAG: diacylglycerol; DHSM: dihydrosphingomyelin; CE: cholesteryl ester; PC: phosphatidylcholine; PE: phosphatidylethanolamine; PS: phosphatidylserine; SM: sphingomyelin; TAG: triacylglycerol.

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719 **Figure 6:** A: Gametocyte viability following chemical inhibition of sphingolipid or
 720 CE synthesis. Sphingolipid synthesis was disrupted with 10 μ M GT11 and CE
 721 synthesis was inhibited with 10 μ M Sandoz 58-035 or 10 μ M glibenclamide from day
 722 2 to 6 post-commitment. For each gametocyte sex, results are normalised to viability
 723 following exposure to the DMSO solvent control and 100 μ M artemisinin. Average
 724 result and standard deviation presented from three biological replicates, each
 725 performed in technical triplicates. B: diagram of GT11 inhibition of sphingolipid
 726 synthesis. DHCer: dihydroceramide; DHSM: dihydrosphingomyelin; SMS:
 727 sphingomyelin synthase; Cer: ceramide; SM: sphingomyelin.

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729

730 **Figure 7:** Fitness competition between 3D7 WT and SMS1 KO (A) or SMS2 KO
 731 clones (B) in complete (CCM, continuous line) and lipid-depleted culture medium
 732 (ICM, dashed line) over 28 days. On day 0 equal amounts of 3D7 WT and SMS1 KO
 733 clone E6 (blue, A), SMS1 KO clone E10 (red, A), SMS2 KO clone C9 (green, B) or
 734 SMS2 KO clone D4 (black, B) were combined in a co-culture. The proportion of each
 735 genotype in the culture was monitored on day 0, 12 and 28 by qPCR of the hDHFR
 736 resistance cassette (to detect KO genotype) and the disrupted section of the SMS1 or 2
 737 genes (to detect WT genotype). At some time points technical problems inhibited
 738 monitoring the culture so measurements were performed 4 days afterwards instead.
 739 Results from 2-4 independent biological repeats are shown as mean and standard
 740 deviation. Statistical significance between day 0 and day 28 was calculated by two-
 741 way ANOVA. In B results in ICM were not significant (ns), whereas $p < 0.001$ (***)

742 for WT vs SMS1 clone E10 in ICM in A and $p < 0.0001$ (****) for all other results in
743 A and B.

744

745

746 **Figure 8:** Gametocytaemia in 3D7 WT and SMS1 KO (A) or SMS2 KO clones (B)
747 was monitored every second day from day 2 to day 8 post commitment and compared
748 to WT gametocytaemia on day 2. (C) Effect of 10 μ M GT11 on the commitment to
749 gametocytaemia in 3D7 WT, SMS1 KO and SMS2 KO clones. The cultures were
750 treated with GT11 (10 μ M) or 0.1% DMSO (control) for 72 hours from day 3 to day 0
751 pre-commitment. Gametocytaemia was determined on day 8 and compared to
752 gametocytaemia of the untreated WT culture. Gametocytaemia is presented as mean
753 and standard error of mean from three biological replicates, each performed in
754 technical triplicates. Significance calculated by unpaired t-test. ns: not significant,
755 $p > 0.1$, *: $p < 0.05$, **: $p < 0.01$.

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758 **Figure 9:** Overview of lipid changes in *P. falciparum* during asexual and sexual
759 blood stages. Grey background: graphic representation of uninfected RBC, RBC
760 infected with trophozoite parasites and male and female infected RBCs. Dashed arrow
761 indicate that there is no direct development from trophozoites to sexual stages and the
762 intermediate stages are not shown. Blue background: neutral lipids; red background:
763 phospholipids; green background: sphingolipids; yellow background: free cholesterol.
764 The left column shows graphic representations of the lipid groups and their
765 abbreviation. The red arrows indicate changes relative to the previous life-cycle stage.
766 Upward arrow means >3-fold increase; horizontal arrow means moderate or no

767 changes (changes that are more than 0.5 -fold and less than 2-fold); a declining arrow
768 indicates decrease of less than half-fold and a downward arrow signifies a decrease of
769 more than 0.3 of the previous value. The male and female infected RBCs are relative
770 to the trophozoite-infected RBCs. Stars indicate significant changes between male and
771 females. CE: cholesteryl ester; DAG: diacylglycerol; TAG: triacylglycerol; PC:
772 phosphatidylcholine; PE: phosphatidylethanolamine; PS: phosphatidylserine; PG:
773 phosphatidylglycerol; DHSM: dihydrosphingomyelin; SM: sphingomyelin. Cer:
774 ceramide; FC: free cholesterol.
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882

A **B** **C** **D**

A **B**

A

- WT vs SMS1 KO clone E6 in ICM
- WT vs SMS1 KO clone E6 in CCM
- ◆ WT vs SMS1 KO clone E10 in ICM
- WT vs SMS1 KO clone E10 in CCM

B

- WT vs SMS2 KO clone D4 in ICM
- WT vs SMS2 KO clone D4 in CCM
- ◆ WT vs SMS2 KO clone C9 in ICM
- WT vs SMS2 KO clone C9 in CCM

A **B** **C**

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> 3x

> 0.5x < 2x

< 0.5x

< 0.3x

* diff.♀/♂