Variation in *Plasmodium falciparum* sexual commitment rates and responses to environmental modification

Short title: Malaria parasite sexual commitment rate variation

Lindsay B. Stewart [1a], Aline Freville [1a], Till S. Voss [2], David A. Baker [1], Gordon A. Awandare [3], David J. Conway [1*]

[1] Department of Infection Biology, Faculty of Infectious and Tropical Diseases, London School of Hygiene and Tropical Medicine, London WC1E 7HT, UK

[2] Department of Medical Parasitology and Infection Biology, Swiss Tropical and Public Health Institute, and University of Basel, Switzerland

[3] West African Centre for Cell Biology of Infectious Pathogens (WACCBIP), Department of Biochemistry, Cell and Molecular Biology, University of Ghana, Accra, Ghana

[a] These authors contributed equally to the research

[*] Corresponding author: david.conway@lshtm.ac.uk

Abstract

Asexual blood stage malaria parasites must produce sexual progeny in order to infect mosquitoes. It is therefore important to understand the scope of variation in sexual commitment rates of the most important human parasite *Plasmodium falciparum*, and to assess responses to conditions that may affect this commitment. First, using a genetically modified line with inducible elevated sexual commitment we compared two different methods of measuring the rates, either staining the Pfs16 marker in very early gametocytes or counting gametocytes after further development. The methods showed correlated estimates in evaluation across multiple independent replicate assays, with higher sensitivity and precision being achieved by Pfs16 marker detection in early gametocytes, so this method was used to survey a wide range of *P. falciparum* lines. A panel of six recently culture-established clinical isolates from Ghana showed mean sexual commitment rates per cycle ranging from 3.3% to 12.2%, with some significant differences among isolates as well as variation among biological replicates of each. Comparing 13 long-term laboratory-adapted lines of diverse origins, eight had sexual commitment rates similar to those of the recent clinical isolates, with means across multiple biological replicates for each line ranging from 4.7% to 14.5%, while four lines had significantly lower rates with means ranging from 0.3 to 1.6%, and one line with a non-functional *ap2-g* gene always showed zero sexual commitment. Among a subset of parasite lines, adding choline to suppress commitment had quantitatively variable effects, although these were significant in most assays of lines that had relatively high rates. This study indicates the importance of multiple assay replicates and comparisons of diverse isolates to understand natural and culture-induced variation in this key reproductive trait, relevant to investigating potential effects of parasite adaptation on transmission.

Author Summary

Only sexual malaria parasites are transmitted from humans to mosquitoes, so it is vital to understand variation in sexual commitment rates of blood stage malaria parasites, and responses to conditions that affect this. We compared two different methods of measuring the rates in a *Plasmodium falciparum* line with engineered sexual commitment variation, demonstrating higher sensitivity and precision by detection of an early differentiation marker, and this method was then used to survey diverse *P. falciparum* lines in culture with extensive biological replicate measurements. Recent clinical isolates from Ghana showed mean sexual commitment rates per cycle ranging from 3% to 12%, with significant differences among isolates as well as variation among biological replicates. Long-term laboratory-adapted lines of diverse origins had a wide range, most having rates similar to those of the clinical isolates, while a minority consistently had much lower or zero rates. There was quantitative variation among lines in the effects of adding choline to suppress commitment, although most assays of lines that had relatively high rates showed significant effects. Performing multiple biological replicates and comparisons of recent parasite isolates is vital to understand intra-specific variation in this important reproductive trait, and move towards investigating direct effects on disease transmission.

62 **Introduction**

63 For malaria to be transmitted, asexual blood stage parasites must undergo differentiation
64 which leads to formation of male and female gametocytes that are required to infect
65 mosquitoes. This process involves an initial parasite commitment phase, when the switch to
66 sexual development is induced, followed by a longer developmental phase to produce the
67 mature gametocyte forms. From an evolutionary or ecological perspective, it is important to
68 understand what determines the rate of commitment to sexual forms [1], and how this may
69 vary within a species [2]. It is particularly vital for understanding *P. falciparum* transmission
70 as more effective control is needed to reduce the global malaria burden [3], which is
71 particularly challenging as chronic asymptomatic infections are often highly infective to
72 mosquitoes [4].

73 Research on malaria parasite sexual conversion has been long established [5], and progress
74 has been made in elucidating key aspects of the mechanism. An ApiAP2-family transcription
75 factor AP2-G is essential for sexual conversion, controlling expression of genes critical to
76 early gametocytogenesis [6-9]. The *ap2-g* locus is clonally variably expressed, with histone 2
77 lysine 9 trimethylation (H3K9me3) marks of heterochromatin silencing initially indicating
78 that its expression could be epigenetically regulated [10], and conditional depletion of
79 heterochromatin protein 1 (HP1) from the promoter was shown to increase *ap2-g*
80 transcription and sexual conversion [11]. Furthermore, in *P. falciparum* a gametocyte
81 development protein GDV1, that was previously linked with sexual commitment [12], is
82 involved in removal of HP1 from the *ap2-g* locus so that over-expression of *gdv1* causes
83 elevated sexual commitment rates [13].

84 The classic model is that sexual commitment occurs in the intra-erythrocytic cycle prior to
85 the cycle in which parasite sexual differentiation occurs, based on a finding that in static
86 culture individual schizonts of the 3D7 parasite clone yield spatially-clustered intra-
87 erythrocytic progeny that tend to differentiate similarly, either sexually or asexually [14].
88 However, there is recent evidence that some sexual commitment may also occur in the
89 same cycle as sexual differentiation, if *ap2-g* expression is induced to start very early after
90 erythrocyte invasion during the ring stage [15], although it is not known how commonly this
91 occurs in parasites under normal conditions of infection or laboratory culture. It is difficult to
92 study *P. falciparum* gametocyte conversion rates precisely in natural infections, as most
93 stages of developing gametocytes do not occur in the peripheral circulation and are

94 therefore not detected in blood samples, although circulating ring stage parasites that are
95 sexually committed may become detectable by developing assays for early markers such as
96 AP2-G or GEXP02 [16, 17]. Correlations between *P. falciparum* gametocyte numbers and
97 other measured variables in human hosts, such as age or hemoglobin levels, may not be
98 causally related to varying gametocyte conversion rates and are not well replicated across
99 different studies [18]. Experiments with other malaria parasite species in laboratory mice
100 indicate sexual commitment to be a plastic phenotype that can respond to the *in vivo*
101 environment [19]. There are few data relating to *P. falciparum* gametocyte conversion in
102 experimentally induced human infections, but modelling of results from human
103 experimental infections with the parasite clone 3D7 has indicated that the conversion rate
104 per cycle is usually less than 1% [20], and older data on induced malaria infections with
105 strains that no longer exist suggest broadly similar average rates [21].

106 Early studies of *P. falciparum* in culture indicated that gametocyte production varied among
107 parasite clones [22, 23], and also varied with culture conditions, although precise estimates
108 of conversion rates per cycle were not established. Subsequent studies estimated
109 gametocyte conversion rates of several laboratory-adapted *P. falciparum* clones to be
110 usually above 1% per cycle when grown at low parasitaemia [6, 24], with higher rates if
111 parasites were stressed at high parasitaemia or by antimalarial drug treatment [14, 24].
112 Parasites appear to respond to metabolic challenges during growth, although most cellular
113 mechanisms remain to be identified [25]. An increase in gametocytogenesis may be
114 triggered by cellular stress involving redox perturbation [26, 27], by adding low doses of the
115 antimalarial artemisinin at the early trophozoite growth stage [28], or by adding parasite-
116 conditioned culture medium [29] or extracellular vesicles from such medium [30]. Notably,
117 gametocyte conversion rates in cultured lines are suppressed by lysophosphatidylcholine
118 (LysoPC) within serum, or by adding choline as a supplement in serum-free medium [31, 32].
119 In a study of parasite development during the initial *ex vivo* cycle cultured from clinical
120 samples in Ghana, the proportions of parasites showing conversion to gametocytes
121 correlated positively with level of parasitaemia and negatively with concentration of LysoPC
122 in plasma of patients, supporting a hypothesis that developmental responses to metabolic
123 stress may occur during natural infections [33].

124 Despite these advances, the quantitative scope of sexual commitment rate variation in *P.*
125 *falciparum* has not yet been clearly defined. Estimates of commitment rates have previously

varied due to methodological differences or to biological differences between nominally similar parasite lines, and there are few controlled comparisons of different isolates employing biological replicate measurements. Performing multiple independent replicate assays is important, because some individual lines may have temporally variable phenotypes and because precision is generally increased with repeated measurements. In this study, we first compared different methods of measuring sexual commitment rates, and then employed the method with greater quantitative sensitivity and accuracy to conduct multiple biological replicate assays on recently adapted clinical isolates from Ghana as well as a broad selection of long-term laboratory adapted lines representing most of those that are commonly cultured globally. Although each clinical isolate showed variability among repeated assays, overall there are significant differences among isolates, indicating potentially adaptive variation in sexual commitment within this important parasite species. Separate assays of selected lines on the suppressive effect on sexual commitment by adding choline to serum-free culture media identified significant quantitative variation among lines, which further encourages research to test for relevance to disease transmission.

Results

Comparison of sensitivity and precision of two different assays to measure gametocyte conversion rates

Two different assay methods were compared to quantify sexual commitment rates in multiple independent experiments, using a genetically engineered *P. falciparum* clone (3D7/iGP_D9) to over-express GDV1 with a destabilising domain (DD) in the presence of the stabilising reagent Shield-1 which leads to elevated gametocyte conversion [34]. Method 1 involved differential counting of stage I gametocytes detected by anti-Pfs16 immunofluorescence staining as a proportion of all parasites developing beyond ring stages in the next cycle, while Method 2 involved Giemsa-stained slide microscopy to compare the next cycle ring stage parasitaemia (referred to as day 0 in this assay, D0) with the parasitaemia of morphologically-defined stage II gametocytes (at day 4, D4) (details given in the Materials and Methods section). In each of multiple biological replicate experiments, both methods clearly show that the gametocyte conversion rate is very significantly higher when GDV1 is overexpressed and stabilised with Shield-1 (GDV1-DD-ON) compared to when

157 GDV1 overexpression is disabled by the absence of Shield-1 (GDV1-DD-OFF) (Figure 1,
158 Supplementary Table S1).

159 In all biological replicate assays, Method 1 showed a marked difference in gametocyte
160 conversion rate between the GDV1-DD ON and OFF conditions, with a mean conversion rate
161 of 36.9 % under ON and 6.7 % under OFF conditions (Figure 1). In comparison, Method 2
162 yielded an estimated mean conversion rate of 15.9 % under ON and 4.1 % under OFF
163 conditions, with the rates for Method 2 being lower than for Method 1 in all replicates for
164 which both were tested in parallel. The precision of the estimates for each of the biological
165 replicates was higher for Method 1, with relatively narrow confidence intervals for each
166 experimental replicate as this method is based on direct counts of developmentally discrete
167 parasites. In comparison, Method 2 yielded wider confidence intervals as it is based on a
168 ratio-of-ratio proportion of two temporally sequential counts of parasitaemia which involves
169 more statistical sampling error (Supplementary Table S1), and this method also depends on
170 successful parasite development over a longer time in culture.

171

172 **Correlation between gametocyte conversion rate estimates from different assay methods**

173 To test for overall correlation between the estimates from the two assay methods, analysis
174 was performed on data from a broader range of paired assays. In addition to the eight
175 measurements from paired assays performed in the first comparison (Figure 1) nine
176 additional measurements of the 3D7/iGP_D9 clone were performed with different
177 concentrations of the Shield-1 reagent, to yield a total of 17 paired measurements with both
178 methods for analysis (Supplementary Table S2). Overall, this showed a high and significant
179 nonparametric rank correlation between the results from each assay method (Spearman's
180 rho = 0.70, P = 0.003), with Method 1 generally giving higher conversion rate estimates
181 (Figure 2) with narrower 95% confidence intervals (Supplementary Table S2). While this
182 supports both assays as separately giving informative relative estimates of gametocyte
183 conversion, Method 1 was chosen for subsequent quantitative analyses of additional lines
184 and culture conditions given its significantly higher sensitivity and tighter precision (Figures 1
185 and 2, Supplementary Tables S1 and S2).

186

187 **Variation in sexual commitment rates of recently culture-established Ghanaian clinical**
188 **isolates**

189 Recent culture-established clinical isolates from a highly endemic area in Ghana were
190 assayed to measure gametocyte conversion rates. Six isolates were selected from a larger
191 panel for analysis as they represent unrelated genetically unmixed infections [35], so that
192 biological replicate assays correspond to a single parasite genotype in each case, and after
193 culture for several months they did not have any known loss of function mutations detected
194 in their genome sequences that would affect sexual commitment [35]. These isolates show
195 mean gametocyte conversion rates that range from 3.3% to 12.2% (Figure 3, Supplementary
196 Table S3), and there were significant differences in pairwise comparisons (Mann-Whitney
197 tests on results of a minimum of six biological replicate assays performed for each isolate,
198 Supplementary Table S4). For example, isolate 296 had a significantly higher conversion rate
199 than three of the others (isolates 272, 289 and 292), and isolate 289 had a significantly lower
200 rate than three of the others (292, 293 and 296)(Supplementary Table S4).

201 There was also significant variation in gametocyte conversion rates among individual
202 biological replicate assays of each isolate, with non-overlapping 95% confidence intervals of
203 some replicates (Figure 3A). The variation among biological replicate assays for each line was
204 not correlated with the culture parasitaemia, which was always between 0.5 and 2.5%
205 (Figure 3B and Supplementary Table S3). Overall, there was no significant evidence of
206 reduction in gametocyte conversion rates over time in culture (Supplementary Table S3),
207 and variation among replicates did not correspond with the use of different erythrocyte
208 batches for culture (Supplementary Table S3).

209

210 **Range of gametocyte conversion rates among diverse long-term cultured laboratory lines**

211 To compare with these recent clinical isolates, a panel of 13 long-term culture-adapted *P.*
212 *falciparum* lines of diverse origins was tested, with multiple biological replicate assays
213 (Figure 4, Supplementary Table S5). The clone F12 (containing a nonsense mutation in *ap2-*
214 *g*)[6] here showed zero conversion in all replicate assays (Figure 4A, Supplementary Table
215 S5). Four of the other lines showed consistently low levels of gametocyte conversion, with
216 means ranging between 0.3% and 1.6%, significantly lower than the conversion rates of the
217 remaining lines (Mann-Whitney tests on pairwise comparisons, Supplementary Table S6).

218 The lines with the lowest conversion rates (D10 and T9/96) were previously described as
219 having multiple gene deletions in a sub-telomeric region of chromosome 9 including the
220 *gdv1* gene [36]. The 3D7 line may contain undetected mutants within bulk culture, as 3D7
221 has been elsewhere shown to be a source of parasite subclones with loss of function
222 mutations (including the *ap2-g* nonsense mutation in parasite subclone F12 [6], deletion of
223 *gdv1* in subclone 3D7.G_{def} [12] and truncation of *gdv1* in two other subclones [37]). The Palo
224 Alto line was previously shown to have undergone sub-telomeric gene deletions on different
225 chromosomes [36, 38] but not to have lost the *gdv1* locus on chromosome 9 [36, 39],
226 although the possibility of a loss-of-function mutation has not been excluded.

227 For the remaining eight long-term culture-adapted *P. falciparum* lines, the gametocyte
228 conversion rates for each showed means ranging from 4.7% (for D6) to 14.5% (for FCC2)
229 (Figure 4A). This range is similar to that seen for the recently culture-established clinical
230 isolates, with no significant differences between these groups (Mann-Whitney test, P =
231 0.47). Similar to the clinical isolates, the individual long-term culture-adapted parasite lines
232 also showed significant variation in gametocyte conversion rates among biological replicate
233 assays (Figure 4A), with non-overlapping 95% confidence intervals for some replicates
234 (Figure 4B). This shows that high and variable sexual commitment rates have been
235 maintained in a majority of the diverse cultured *P. falciparum* lines, in contrast to a minority
236 of lines that have markedly reduced rates or zero commitment.

237

238 **Choline in culture medium suppresses sexual commitment rate with varying levels of**
239 **effect among *P. falciparum* lines**

240 As it has been previously shown that parasite sexual commitment responds to the presence
241 or absence of choline in culture medium [13, 28, 31], we investigated five long-term culture-
242 adapted lines (T9/96, 3D7, Dd2, NF54 and HB3) for quantitative effects of 2 mM choline
243 compared to no added choline, performing between six and eight biological replicate assay
244 comparisons for each parasite line. Significant suppression of sexual commitment in the
245 presence of choline occurred in most lines (Figure 5, Supplementary Table S7). No significant
246 effect was seen in the *P. falciparum* clone T9/96 which has a deletion of *gdv1* and several
247 adjacent genes deleted on Chromosome 9 [36], and which has a very low rate of sexual
248 commitment either in the presence or absence of choline, consistent with the importance of

249 *gdv1* in responding to environmental induction [13]. The responses of the other lines to
250 choline varied, with statistically significant suppression in sexual commitment in most of the
251 replicate assays for NF54 and Dd2, in half of the assays for HB3, and in a quarter of the
252 assays for 3D7 (Figure 5). Some individual assay replicates showed more than 10 fold
253 suppression of sexual commitment rate in the presence of choline, but most showed more
254 moderate rates (Supplementary Table S7).

255 The mean response of each parasite line to choline was estimated by the Mantel-Haenszel
256 rate ratio (RR_{M-H}), which analyses data across replicates under a simple fixed-effects model.
257 With the exception of T9/96 which showed no response as noted above, the remaining four
258 lines show overall increases in gametocyte conversion rates, but the mean responses varied
259 among the different lines (Supplementary Table S7). The greatest mean response to choline
260 was shown by line Dd2 (3.5 fold, 95% CI 3.0-4.1 fold), while 3D7 showed the lowest level of
261 effect (1.5 fold, 95% CI 1.2-1.9 fold), and the other lines showed intermediate levels (for
262 NF54 = 2.4 fold, 95% CI 2.0-3.0 fold; for HB3 = 2.0 fold, 95% CI 1.6-2.5 fold).

263

264 **Discussion**

265 Analyses of multiple biological replicate assays are informative to characterise the range of
266 sexual commitment rates in *P. falciparum*. A controlled comparison of two alternative
267 methods showed that phenotyping was more sensitive and precise with an assay that
268 measures proportions of sexually differentiating parasites by immunofluorescence staining
269 of the Pfs16 marker expressed in early stage I gametocytes [15]. Although the sensitivity and
270 precision of the methods differ, they give correlated measurements, and the lower
271 proportions measured using a method that does not employ Pfs16 staining may be
272 expected, given the requirement for parasites to successfully develop to stage II
273 gametocytes over four days in culture before they are counted by morphological inspection
274 of Giemsa-stained slides [33].

275 The recently culture-established Ghanaian clinical isolates analysed here had gametocyte
276 conversion rates of between 3.3% and 12.2% per cycle, measured as means of results from
277 at least six biological replicate assays for each isolate. These rates are higher than those
278 previously estimated for clinical isolates in the first cycle *ex vivo* in Ghana with an assay

279 which involves counting of stage II gametocytes, which indicated median rates less than 1%
280 and only approximately a quarter of isolates having rates above 3% [33]. The lower *ex vivo*
281 rates may be partly due to lower assay sensitivity compared to the Pfs16 staining method, as
282 well as effects of plasma components such as variable lysophosphatidylcholine levels in
283 some individuals inhibiting parasite sexual commitment [33]. It is notable that the isolates in
284 the present study were cultured consistently in serum-free medium supplemented by
285 Albumax-II for several months, demonstrating that use of serum in culture is not required to
286 preserve the intrinsic capacity of parasites to show robust rates of sexual commitment over
287 this period. Nevertheless, parasites with very high rates of sexual commitment might have
288 been selected against, while eventual loss of sexual commitment in some cultures may occur
289 over longer periods of culture.

290 Most of the diverse long-term laboratory-adapted lines had mean gametocyte conversion
291 rates between 4.7% and 14.5% per cycle, and showed variation in conversion rate among
292 different biological replicates. The only line that always had zero sexual conversion was F12
293 with a previously described non-functional *ap2-g* gene due to a nonsense mutation [6]. All
294 other lines showed some detectable sexual conversion, although four lines had much lower
295 rates (means of 0.3 to 1.6%) than the rest, likely due to loss-of-function mutations acquired
296 in culture including deletion of the *gdv1* gene in lines T9/96 and D10 [36] which showed the
297 lowest conversion rates. Loss of *gdv1* in two other laboratory lines was reportedly
298 associated with no detectable gametocytes [12, 37], and it is possible that the low level of
299 conversion in T9/96 and D10 reflects a small amount of commitment to gametocyte
300 development post-invasion due to stochastic activation of *ap2-g*, independent of the main
301 pathway involving *gdv1* [15].

302 The multiple replicate measurements of each *P. falciparum* line enable the identification of
303 significant variation among recently derived clinical isolates from the same local endemic
304 population, as well as among long-term laboratory-adapted lines that have diverse
305 geographical origins. Given that modelling of data from experimental infections has
306 indicated much lower rates of approximately 1% or less per cycle [20, 21], there may be a
307 significant loss *in vivo* during the long period of development to circulating late stage
308 gametocytes, or suppression of gametocyte conversion in these infections compared to
309 culture.

310 Variation in gametocyte conversion rates among biological replicate assays was similar for
311 recently established clinical isolates and most of the long-term laboratory adapted lines.
312 Measured differences among replicates may reflect intrinsic parasite variation, stochastic or
313 due to determinants yet unidentified, or could be due to assay conditions. Previous studies
314 on a several different culture-adapted *P. falciparum* clones did not show as much variation
315 between experimental replicates [6, 13, 28, 31], which may be due to tighter parasite
316 synchronisation in those studies, whereas a broad window of synchronisation in the present
317 study was intended to be similar to what may be sampled in peripheral blood (in which ring
318 stage parasites may range in ages up to approximately 24 hours). The inter-replicate assay
319 variation in this study might be partly due to the possibility that a proportion of parasites in
320 some replicates were too young to stain with the Pf16 marker, although this was aimed to
321 be minimised by inspecting parasite development to determine the exact time of assay
322 completion. Although most parasites assayed would be mature enough for Pfs16 staining,
323 some replicates may have parasites at least 36 hours post invasion when all stage I
324 gametocytes would be Pfs16-positive [15, 17, 40], while other replicates may have parasites
325 24 hours post invasion when a majority but not all stage I gametocytes may already be
326 Pfs16-positive [15]. Exact standardisation of parasite ages in culture of diverse isolates is not
327 feasible, as isolates intrinsically vary in developmental cycle times [41, 42], but it is likely
328 that tighter synchronisation of parasite cultures would reduce variation among replicates for
329 any individual isolate. Assay precision may also be increased if detection assays are
330 developed to enable antibody staining of sexually committed parasites before they express
331 Pfs16, which may become possible as particular gene transcripts are expressed in committed
332 ring stages [43], and at least one protein (GEXP02) is expressed earlier than Pfs16 [17].

333 Although a majority of the diverse laboratory-adapted parasite lines tested in this study
334 retain an intact mechanism for gametocyte production, there were differences in responses
335 to choline. The presence or absence of choline had no effect in any of eight replicate
336 experiments on the T9/96 clone that has a culture-acquired deletion of *gdv1*, consistent
337 with the requirement of this locus for mediating a response to induce enhanced levels of
338 sexual commitment [12, 13]. In contrast, choline had a significant but variable effect on
339 sexual commitment in each of the other parasite lines tested, each of which also showed
340 substantial variation in responsiveness among biological replicates. Although a few
341 individual replicate assays showed greater than 10 fold effects, mean suppressive effects of

342 choline in each parasite line ranged from 1.5 fold (for 3D7) to 3.5 fold (for Dd2), more
343 moderate than described in previous studies of parasite clones maintained in medium
344 containing choline or serum before being switched to choline-free medium [13, 17, 31].
345 Gametocyte conversion rates of >30% have occasionally been reported elsewhere [28],
346 which were not seen in the non-genetically engineered lines here, but only with
347 overexpression of GDV1 in an engineered line. As all parasite lines including clinical isolates
348 in this study were maintained in a standard medium with Albumax without choline
349 supplementation, it is possible that higher gametocyte conversion rates were selected
350 against, as they might not be sustained in continuous culture without using conditions to
351 suppress conversion during long-term maintenance [31].

352 The results support the utility of choline in medium to suppress parasite sexual commitment
353 under controlled environmental conditions (10, 20, 21), and notably show significant
354 variation in the responsiveness of different parasite lines. It will be important to understand
355 if there is intraspecific variation in natural regulation of GDV1 expression, particularly the
356 control of gene-silencing antisense transcripts [13], and investigate the potential influence
357 of naturally-occurring cis-variation at the sub-telomeric locus on chromosome 9 [33, 39, 44]
358 distinct from the deletions disrupting function in some cultured lines [12, 36, 37, 45]. An
359 important new insight into the effect of available choline has just been reported, indicating
360 that sexual commitment is affected by competition between histone methyltransferases
361 (involved in *ap2-g* gene silencing) and phosphoethanolamine methyltransferase (involved in
362 *de novo* synthesis of phosphatidylcholine when choline availability is low) for the methyl
363 donor S-adenosylmethionine, which could have implications for future studies on inter-
364 isolate variation in rates of sexual commitment [32]. Investigation of parasites in natural
365 populations requires assay precision and experimental replication to be considered where
366 possible alongside statistical power from increased numbers of clinical samples, whether for
367 candidate gene investigations or larger scale scanning for associations with unlinked
368 genomic variation in parasite populations [46].

369

370 **Materials and Methods**

371 ***Ethics Approval***

372 Approval for sampling of clinical isolates for culture experimental analysis was granted by
373 the Ethics committees of the Ghana Health Service, the Noguchi Memorial Institute for
374 Medical Research at the University of Ghana, the Navrongo Health Research Centre and the
375 London School of Hygiene and Tropical Medicine, as previously described for the collection
376 [35] from which specific isolates were selected in this analysis.
377
378 ***Plasmodium falciparum laboratory-adapted clones and clinical isolates***
379 The genetically modified *P. falciparum* cloned line 3D7/iGP_D9 was used to compare the
380 sensitivity and precision of gametocyte conversion rate assays, as artificially elevated sexual
381 commitment is selectively inducible in this line by overexpression of GDV1 fused to a
382 destabilization domain (DD) which maintains activity in the presence of Shield-1 reagent
383 [34].

384 Thirteen different *P. falciparum* long-term laboratory-adapted lines that have not
385 undergone targeted genetic modification were studied. Each of these was either previously
386 derived by cloning or is a line that has an apparently monoclonal single-genome sequence,
387 and a putative origin based on the geographical location of the original isolation: NF54 (a
388 strain of 'airport malaria' from The Netherlands with genome sequence similarity to African
389 isolates), 3D7 (the *P. falciparum* reference genome strain originally cloned from NF54 and
390 maintained in culture for many years) [47], F12 (derived by cloning from cultured 3D7 and
391 having a nonsense mutant *ap2-g* gene) [6], D10 (Papua New Guinea), D6 (Sierra Leone), Dd2
392 (Southeast Asia), FCC2 (China), HB3 (Honduras), R033 (Ghana), Palo Alto (Uganda), 7G8
393 (Brazil), GB4 (Ghana) and T9/96 (Thailand). All lines were thawed from pre-existing frozen
394 stocks with distinct identities verified by previous targeted genotyping and sequencing [39,
395 48] and were not re-cloned prior to this study, and each line was cultured for at least one
396 week after thawing prior to any assay, all culture being conducted using a commercially-
397 prepared serum-free medium supplemented with lipid-rich bovine serum albumen (Gibco
398 RPMI with 0.5% AlbuMAX™ II lot number 2177881). This consistent medium preparation had
399 very minimal choline (less than 0.02 mM choline chloride) which would not suppress
400 parasite sexual commitment, unlike high concentrations of choline (2 mM as used for some
401 assays described in a separate section below) or serum which has variable components
402 between batches.

403 Six *P. falciparum* clinical isolates from Ghana with single-genome unmixed infections were
404 selected from a larger panel of infections sampled from children with clinical malaria in
405 Navrongo (in the Upper East Region of the country), in which parasite asexual multiplication
406 rate variation was previously studied [35]. These isolates were cultured with the same batch
407 of commercially-prepared serum-free medium with 0.5% AlbuMAX™ II as for the laboratory-
408 adapted parasite lines, ensuring the same batch was used throughout all experiments so
409 that no biological variation should be due to media components, and these clinical isolates
410 were not cultured with added serum at any time as they had been cultured with 0.5%
411 AlbuMAX™ II since isolation [35]. Clinical isolates with mixed genome-infections were
412 purposefully not selected for inclusion in this study, as their sexual commitment rates would
413 be complex to interpret due to potential changes in mixed genomic composition over time
414 [35].

415 ***Quantification of sexual commitment rates by measuring gametocyte conversion***

416 Multiple biological replicate preparations of *P. falciparum* long-term laboratory-adapted
417 lines and more recently culture-established clinical isolates were each cultured in human
418 erythrocytes at 3% hematocrit in RPMI 1640 media supplemented with AlbuMAX™ II and
419 incubated at 37°C in atmospheric air with 5% CO_2. Prior to assay, each asynchronous
420 parasite culture was synchronized to within a 24-hour window of intra-erythrocytic asexual
421 stage development, intended to correspond approximately to the parasite age range that
422 would be typically sampled in peripheral blood of patients, using the following two-step
423 technique. Parasites containing haemozoin, predominantly schizont and late trophozoite
424 stages, were first positively selected on MACS® LD magnetic columns [49], and after culture
425 for 24 hours a second MACS purification step was performed from which the flow through
426 erythrocytes (containing predominantly ring stage parasites) were collected and put back
427 into culture, which then contained exclusively ring stage parasites as any gametocytes or
428 residual mature asexual parasites were removed on the column. This broad synchronization
429 scheme is illustrated in Supplementary Figure S1. For the 3D7/iGP parasite line the effect of
430 GDV1-DD overexpression was tested by adding Shield-1 reagent to this preparation of ring
431 stages (1 µM compared to none, with intermediate concentration treatments of 0.05, 0.15
432 and 0.5 µM Shield-1 also being tested in some assays), with Shield-1 being retained in the
433 culture throughout the cycle.

434 Two methods were used for measuring the gametocyte conversion rate:

435 *Method 1. Counting of the proportion of stage I gametocytes compared to all parasites*
436 *developing in the first cycle post-invasion using anti-Pfs16 staining.*

437 This method involves distinguishing very early gametocytes from asexual parasites by
438 detection of the gametocyte marker Pfs16 protein [15, 40, 50]. After two rounds of MACS
439 column purification to obtain ring stages followed by a further 24 hours of growth, when a
440 culture with a broad synchronicity containing schizonts was obtained, parasites were
441 allowed to invade erythrocytes and were cultured for a period of between 38 and 46 hours,
442 the exact time of harvesting of each culture being decided on the basis of microscopical
443 examination of Giemsa stained slides indicating that most post-invasion parasites were
444 clearly beyond the ring stage of development and therefore should be either trophozoites or
445 stage I gametocytes. The harvested post-invasion parasites were examined by fluorescence
446 microscopy by Pfs16 staining of early gametocytes and DAPI staining of all parasites. Stage I
447 gametocytes were counted as those staining positive for Pfs16 and showing typical round
448 morphology similar to trophozoites (whereas any Pfs16-positive parasites with
449 differentiated morphology of stage II gametocytes potentially residual from induction prior
450 to the previous cycle were not counted). The assay enables most sexually-developing
451 parasites to be counted in comparison to the total of post-invasion parasites, on the basis
452 that a majority of early stage I gametocytes express Pfs16 after 24 hours post invasion [15]
453 as a proportion of all gametocytes which eventually express [40], with only a minority of
454 sexual parasites likely not to be counted due to being too young. Parasite cultures harvested
455 for this purpose were washed in phosphate buffered saline (PBS) with 3% bovine serum
456 albumen (BSA), resuspended to 2% hematocrit, spotted onto multiwell slides (Hendley,
457 Essex, UK), air-dried and stored at -80°C. Prior to immunofluorescence staining, slides were
458 fixed with 4% paraformaldehyde in PBS for 30 min and permeabilized with 0.1% Triton X-100
459 in PBS for 10 min. The slides were incubated for 30 minutes at room temperature with a
460 monoclonal α-Pfs16 murine antibody 93A3A2 [50], diluted 1:2000 in PBS with 3% BSA. Alexa
461 Fluor® 594-conjugated anti-mouse IgG (Invitrogen, A11032) was used as secondary
462 antibody, diluted 1:1000 in PBS with 3% BSA and incubated for 30 minutes at room
463 temperature. Slides were mounted with coverslips using DAPI-containing Prolong™
464 Diamond antifade mountant (ThermoFisher Scientific), and parasite counting was performed
465 using a Zeiss CCD fluorescence microscope, with identical settings used for each experiment.

466 An average of approximately 1000 parasites were counted to determine proportions
467 expressing Pfs16 in each measurement, and where the density of parasites did not allow this
468 a quality control was performed to exclude any assay data that did not have a minimum of
469 at least 300 parasites counted.

470 *Method 2. Counting of developing gametocytemia compared to all parasites at the ring*
471 *stage culture.*

472 This method estimates the proportion of parasites that produce gametocytes developing to
473 morphological stage II, by comparing with the ring parasitaemia counted 4 days previously
474 at the beginning of the parasite developmental cycle. The technique is based on a protocol
475 that uses Giemsa staining and does not require immunofluorescence staining of an early
476 gametocyte marker [33]. To initiate this assay, after two MACS column purifications and
477 when a broadly synchronous culture containing a majority of schizonts were obtained,
478 parasites were allowed to re-invade fresh erythrocytes and cultured until ring stage
479 development was observed. Then, ring stage parasitaemia was assessed by visual
480 examination of thin blood smears of each culture to determine the proportion of
481 erythrocytes containing parasites (referred to as D0 parasitaemia), ensuring that at least
482 1,000 erythrocytes were counted. The cultures were then treated with 50 mM N-acetyl-D-
483 glucosamine (GlcNAc) to prevent further development of asexual parasites [51]. After 4
484 days, the stage II gametocytemia was assessed by counting of Giemsa-stained thin blood
485 smears (referred to as D4 gametocytemia), ensuring that at least 10,000 erythrocytes were
486 counted. The gametocyte conversion rate was calculated by dividing D4 stage II
487 gametocytemia (as a proportion of erythrocytes infected) by D0 ring parasitaemia (as a
488 proportion of erythrocytes infected), yielding a 'ratio-of-ratio' proportion with 95%
489 confidence intervals.

490 For both methods, all assays were set up at 3% haematocrit in a culture volume of 5 ml
491 (static in 6-well culture plates), with parasitaemia levels at the start of each replicate assay
492 being between 0.5 - 2.5% erythrocytes infected, to allow sufficient numbers of parasites for
493 high precision counting while minimising stress on parasites that may be induced if cultures
494 have higher parasitaemia.

495 ***Quantification of gametocyte conversion rate using culture media without choline***

496 Parasites were prepared for assays in the same manner as described in the previous section,
497 except that they were split after the second MACS column purification (at ring stage in the
498 cycle before gametocyte counting) into separate wells with culture media (RPMI 1640 with
499 25 mM HEPES, 100 µM Hypoxanthine, 1 mM L-Glutamine, 0.39% fatty acid-free BSA, 30 mM
500 Palmitic acid, 30 mM Oleic acid) either supplemented with 2 mM choline or lacking choline,
501 based on a previously described protocol [31]. Parasites were allowed to grow until schizont
502 stage and to invade fresh erythrocytes, without changing the culture medium. After 38-46
503 hours post invasion, parasites were collected and fixed for analysis of gametocyte
504 conversion by differential counting using Pfs16 staining by immunofluorescence microscopy
505 as well as DAPI staining as described above for Method 1, with an average of approximately
506 1000 parasites being counted for each individual measurement. Between six and eight
507 replicate assays were performed for each parasite line, the effect within each replicate
508 calculated as the rate ratio (with 95% confidence intervals) of the gametocyte conversion
509 rate in choline-free medium compared with the conversion rate in medium containing 2 mM
510 choline.

511 ***Statistical analyses***

512 Spearman's ρ (rho) coefficient was used to evaluate the strength and significance of the rank
513 correlation between results from the two different gametocyte conversion rate assays
514 performed in parallel. Statistical comparisons of gametocyte conversion rate variation
515 between different *P. falciparum* lines were performed using the Mann-Whitney test on the
516 multiple replicate assay measurements of each line. Each different parasite line was assayed
517 with a mean of more than six biological replicate cultures, most individual lines having at
518 least six and some up to nine biological replicates performed. Each individual biological
519 replicate data point had 95% confidence intervals calculated for the proportions of parasites
520 showing sexual commitment on the basis of the raw numerical counts performed, enabling
521 accurate estimation of within-assay sampling variation, and all count data are provided for
522 verification and to enable any future meta-analyses. For each parasite line, the mean effect
523 of modified culture media conditions was estimated by calculating the Mantel-Haenszel rate
524 ratio (RR_{M-H}) across biological replicate assays, based on a simple fixed-effects model.
525 Statistical analyses were performed using Prism version 9 and Epi-Info version 7 software.

526

Acknowledgements

This study was funded by Project Grant MR/S009760/1 from the UK Medical Research Council to DJC and DAB. Original sampling of clinical isolates was supported by a Royal Society Africa Award AA110050 to GAA and DJC. TSV received funding from the Swiss National Science Foundation grant BSCGI0_157729. We are grateful to all colleagues who supported the research environment, including sharing of laboratory facilities and equipment, enabling continuous parasite culture and experimentation as well as storage and shipment of samples and reagents.

535 **Figure Legends**

536

537 **Figure 1.** Comparison of the sensitivity and precision of two different methods for measuring
538 *P. falciparum* sexual commitment in terms of gametocyte conversion rates. Multiple paired
539 replicate assays were performed with the parasite clone 3D7/iGP_D9 [34] which allows
540 inducible overexpression of GDV1 fused to a destabilization domain (DD) stabilized in the
541 presence of Shield-1 reagent to provide controlled elevation of sexual commitment (closed
542 circles indicate GDV1–ON, open circles GDV1-OFF). Both methods show the expected
543 significant elevation in gametocyte conversion in the presence of Shield-1 reagent in all
544 individual assays (asterisks indicate P<0.0001 for each of the individual replicate assays). In
545 each panel, the green dotted line denotes the mean for GDV1-ON and the red dotted line
546 for GDV1-OFF. **A.** The gametocyte conversion rate (with 95% CI) for each of seven
547 independent experiments using Method 1 involving staining of the gametocyte marker
548 Pfs16, yielding a mean conversion rate of 38.3 % under GDV1-ON and 6.3% under GDV1-OFF
549 conditions **B.** The gametocyte conversion rate (with 95% CI) for each of four experiments
550 using Method 2 which involves a comparison of Day 4 (D4) gametocyte parasitaemia with D0
551 ring-stage parasitaemia (assays paired with the first four independent experiments shown
552 for Method 1), yielding a mean conversion rate of 15.9 % under GDV1-ON and 4.1 % under
553 GDV1-OFF conditions. Exact parasite and erythrocyte counts and statistical analyses for all
554 assays are detailed in Supplementary Table S1.

555 **Figure 2.** Significant correlation in the estimates of gametocyte conversion rate obtained by
556 two different assay methods. Paired assays were performed using Method 1 (involving Pfs16
557 staining to discriminate sexually from asexually-developing parasites) and Method 2 (D0D4,
558 involving counts of parasites 4 days apart) on multiple biological replicates of 3D7/iGP_D9
559 with a range of concentrations of Shield-1 reagent (N=17 paired assays in total, details in
560 Supplementary Table S2). This highly significant correlation confirms that either method
561 gives valid measurements if used alone, whereas Method 1 is more sensitive (as indicted
562 here and in Figure 1). Method 1 is also more precise as it involves calculation of a single
563 numerical ratio, rather than a ratio-of-ratios based on two different counts performed 4
564 days apart which is highly vulnerable to effects of small numbers in either count. For visual
565 clarity, the individual assay 95% confidence intervals are not shown on the plot, but these
566 and numerical data of all assays are given in Supplementary Table S2.

567 **Figure 3.** Gametocyte conversion rates of six recently culture-established Ghanaian clinical
568 isolates. These were selected for study as they are unrelated single genotype infections,
569 chosen from a larger number of samples from a highly endemic population that contained
570 many multiple genotype infections [35]. **A.** For each isolate, a minimum of six biological
571 replicate assays was performed, and the estimate from each replicate with 95% confidence
572 intervals (based on numbers of parasites counted) is plotted, while the red dotted line
573 represents the mean across replicate assays. There were significant differences in pairwise
574 comparisons (Mann-Whitney tests, Supplementary Table S4), isolate 296 having a
575 significantly higher conversion rate than three others (isolates 272, 289 and 292), and isolate
576 289 having a significantly lower rate than three others (292, 293 and 296)(Supplementary
577 Table S4). There were no significant correlations with length of time in culture, or batches of
578 erythrocytes used for the different replicate assays (these details and all numerical data
579 values are given in Supplementary Table S3). **B.** For each isolate the gametocyte conversion
580 rate of individual replicates is plotted in relation to the culture parasitaemia in the previous
581 cycle (between 0.5 and 2.5% for all experimental replicates). 95% confidence intervals
582 individual conversion rate points are not shown as they are the same as in panel A. There
583 was no systematic trend towards positive or negative correlation, except a marginally
584 significant positive correlation for isolate 289 (P = 0.03) which may indicate slight
585 enhancement at higher parasitaemia or a chance finding due to multiple comparisons.

586 **Figure 4.** Gametocyte conversion rates of 13 long-term culture adapted *P. falciparum* lines
587 of diverse geographical origins. Each different parasite line was tested with multiple
588 independent biological replicate assays, most individual lines having at least six replicates.
589 All counts and calculated values for all replicates are given in full in Supplementary Table S5.
590 **A.** For each parasite line, the conversion rates are shown for each of the biological replicates
591 and the horizontal line shows the mean across all biological replicates, with a spectrum of
592 variation among the different lines, means ranging from 0% to 14.5%. The background with
593 different shades of grey show that the conversion rates separate into three different groups,
594 based on pairwise comparisons of all replicates between the parasite lines (Mann-Whitney
595 tests, Supplementary Table S6): One line (F12) showed no conversion, four lines (D10, T9/96,
596 Palo Alto, and 3D7) showed consistently low conversion (means between 0.3% and 1.6%),
597 and eight lines (D6, GB4, Dd2, HB3, NF54, RO33, 7G8, and FCC2) showed higher levels of
598 conversion (means between 4.7% and 14.5%). **B.** Variation in the gametocyte conversion

599 rates among independent replicate assays for each line is shown in more detail by including
600 95% CI of the counts for each assay of each line, except F12 for which there was zero
601 conversion in all assays. Identical methods were used in assaying these parasite lines as for
602 the clinical isolates shown in Figure 3, assays being conducted in parallel under the same
603 conditions in the same laboratory. Erythrocytes from different erythrocyte donors used at
604 various times throughout the series of experiments did not determine the variation between
605 assay replicates (Supplementary Table S5).

606 **Figure 5.** Significant variation in sensitivity of different parasite lines to choline in media to
607 suppress sexual commitment. Closed circles represent biological replicates cultured in the
608 presence of 2 mM choline, open circles represent biological replicates grown in the absence
609 of choline. T9/96 shows no response to choline in any of seven biological replicate assays,
610 whereas each of the other cultured lines show responses that vary among biological
611 replicates. Significant differences between paired measurements with and without choline
612 in individual replicate assays for 3D7, Dd2, HB3 and NF54 are highlighted with asterisks
613 (Fisher's Exact Test * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$). Across all replicates, estimated by
614 Mantel-Haenszel rate ratios the mean effects of choline were greatest for Dd2 (3.5 fold, 95%
615 CI 3.0-4.1 fold), intermediate for NF54 (2.4 fold, 95% CI 2.0-3.0 fold) and HB3 (2.0 fold, 95%
616 CI 1.6-2.5 fold), and least for 3D7 (1.5 fold, 95% CI 1.2-1.9 fold). All individual counts and
617 assay values for all biological replicates are given in Supplementary Table S7.

618 **References**

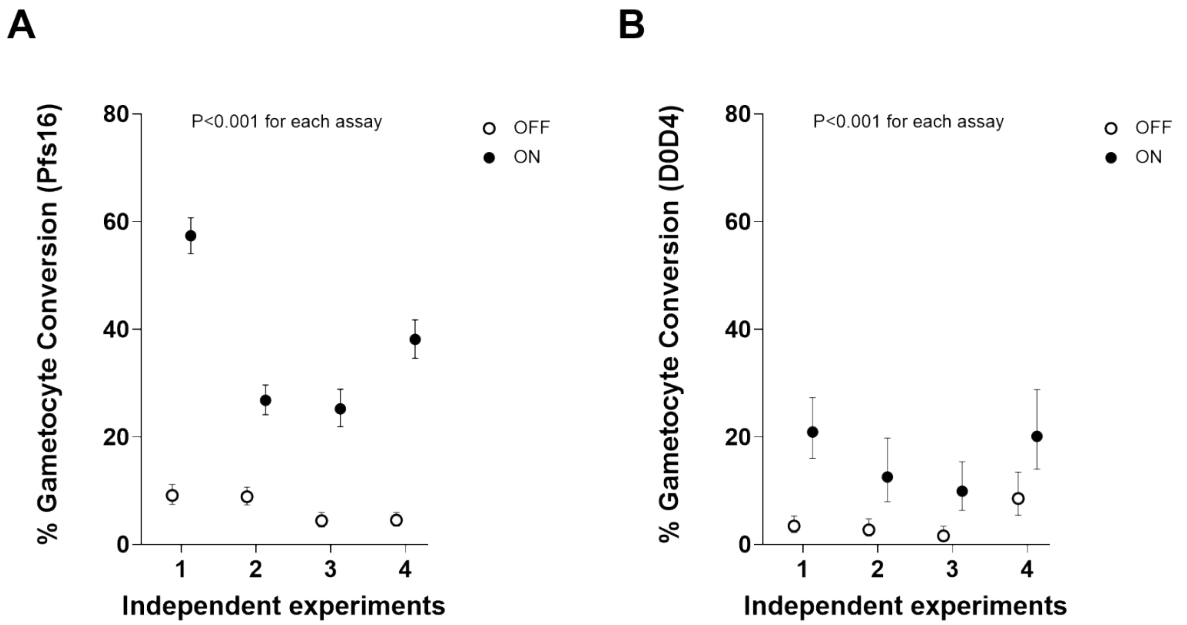
619 1. McKenzie FE, Bossert WH. The optimal production of gametocytes by *Plasmodium*
620 *falciparum*. J Theor Biol. 1998;193:419-28. Epub 1998/09/15. doi: 10.1006/jtbi.1998.0710. PMID:
621 9735270.
622 2. Schneider P, Greischar MA, Birget PLG, Repton C, Mideo N, Reece SE. Adaptive plasticity in
623 the gametocyte conversion rate of malaria parasites. PLoS Pathog. 2018;14:e1007371. Epub
624 2018/11/15. doi: 10.1371/journal.ppat.1007371. PMID: 30427935.
625 3. WHO. World Malaria Report 2020. Geneva: 2020.
626 4. Barry A, Bradley J, Stone W, Guelbeogo MW, Lanke K, Ouedraogo A, et al. Higher gametocyte
627 production and mosquito infectivity in chronic compared to incident Plasmodium falciparum
628 infections. Nat Commun. 2021;12:2443. Epub 2021/04/28. doi: 10.1038/s41467-021-22573-7. PMID:
629 33903595.
630 5. Alano P, Carter R. Sexual differentiation in malaria parasites. Annu Rev Microbiol.
631 1990;44:429-49. doi: 10.1146/annurev.mi.44.100190.002241. PMID: 2252389.
632 6. Kafsack BF, Rovira-Graells N, Clark TG, Bancells C, Crowley VM, Campino SG, et al. A
633 transcriptional switch underlies commitment to sexual development in malaria parasites. Nature.
634 2014;507:248-52. doi: 10.1038/nature12920. PMID: 24572369.
635 7. Kent RS, Modrzynska KK, Cameron R, Philip N, Billker O, Waters AP. Inducible developmental
636 reprogramming redefines commitment to sexual development in the malaria parasite Plasmodium
637 berghei. Nat Microbiol. 2018;3:1206-13. Epub 2018/09/05. doi: 10.1038/s41564-018-0223-6. PMID:
638 30177743.
639 8. Sinha A, Hughes KR, Modrzynska KK, Otto TD, Pfander C, Dickens NJ, et al. A cascade of DNA-
640 binding proteins for sexual commitment and development in *Plasmodium*. Nature. 2014;507:253-7.
641 doi: 10.1038/nature12970. PMID: 24572359.
642 9. Josling GA, Russell TJ, Venezia J, Orchard L, van Biljon R, Painter HJ, et al. Dissecting the role
643 of PfAP2-G in malaria gametocytogenesis. Nat Commun. 2020;11:1503. Epub 2020/03/22. doi:
644 10.1038/s41467-020-15026-0. PMID: 32198457.
645 10. Rovira-Graells N, Gupta AP, Planet E, Crowley VM, Mok S, Ribas de Pouplana L, et al.
646 Transcriptional variation in the malaria parasite *Plasmodium falciparum*. Genome Res. 2012;22:925-
647 38. Epub 2012/03/15. doi: 10.1101/gr.129692.111. PMID: 22415456.
648 11. Brancucci NMB, Bertschi NL, Zhu L, Niederwieser I, Chin WH, Wampfler R, et al.
649 Heterochromatin protein 1 secures survival and transmission of malaria parasites. Cell Host Microbe.
650 2014;16:165-76. doi: 10.1016/j.chom.2014.07.004. PMID: 25121746.
651 12. Eksi S, Morahan BJ, Haile Y, Furuya T, Jiang H, Ali O, et al. *Plasmodium falciparum*
652 gametocyte development 1 (Pfgdv1) and gametocytogenesis early gene identification and
653 commitment to sexual development. PLoS Pathog. 2012;8:e1002964. Epub 2012/10/25. doi:
654 10.1371/journal.ppat.1002964. PMID: 23093935.
655 13. Filarsky M, Fraschka SA, Niederwieser I, Brancucci NMB, Carrington E, Carrio E, et al. GDV1
656 induces sexual commitment of malaria parasites by antagonizing HP1-dependent gene silencing.
657 Science. 2018;359:1259-63. doi: 10.1126/science.aan6042. PMID: 29590075.
658 14. Bruce MC, Alano P, Duthie S, Carter R. Commitment of the malaria parasite *Plasmodium*
659 *falciparum* to sexual and asexual development. Parasitology. 1990;100 Pt 2:191-200. Epub
660 1990/04/01. doi: 10.1017/s0031182000061199. PMID: 2189114.
661 15. Bancells C, Llora-Batlle O, Poran A, Notzel C, Rovira-Graells N, Elemento O, et al. Revisiting
662 the initial steps of sexual development in the malaria parasite *Plasmodium falciparum*. Nat
663 Microbiol. 2019;4:144-54. Epub 2018/11/28. doi: 10.1038/s41564-018-0291-7. PMID: 30478286.
664 16. Llora-Batlle O, Michel-Todo L, Witmer K, Toda H, Fernandez-Becerra C, Baum J, et al.
665 Conditional expression of PfAP2-G for controlled massive sexual conversion in *Plasmodium*
666 *falciparum*. Sci Adv. 2020;6:eaaz5057. Epub 20200610. doi: 10.1126/sciadv.aaz5057. PMID:
667 32577509.
668 17. Portugaliza HP, Llora-Batlle O, Rosanas-Urgell A, Cortes A. Reporter lines based on the
669 gexp02 promoter enable early quantification of sexual conversion rates in the malaria parasite

670 Plasmodium falciparum. Sci Rep. 2019;9:14595. Epub 2019/10/12. doi: 10.1038/s41598-019-50768-
671 y. PMID: 31601834.
672 18. Koepfli C, Yan G. *Plasmodium* gametocytes in field studies: Do we measure commitment to
673 transmission or detectability? Trends Parasitol. 2018;34:378-87. Epub 2018/03/17. doi:
674 10.1016/j.pt.2018.02.009. PMID: 29544966.
675 19. Carter LM, Kafsack BF, Llinas M, Mideo N, Pollitt LC, Reece SE. Stress and sex in malaria
676 parasites: Why does commitment vary? Evolution, Medicine, and Public Health. 2013;2013:135-47.
677 doi: 10.1093/emph/eot011. PMID: 24481194.
678 20. Cao P, Collins KA, Zaloumis S, Wattanakul T, Tarning J, Simpson JA, et al. Modeling the
679 dynamics of *Plasmodium falciparum* gametocytes in humans during malaria infection. Elife. 2019;8.
680 Epub 2019/10/30. doi: 10.7554/eLife.49058. PMID: 31658944.
681 21. Eichner M, Diebner HH, Molineaux L, Collins WE, Jeffery GM, Dietz K. Genesis, sequestration
682 and survival of *Plasmodium falciparum* gametocytes: parameter estimates from fitting a model to
683 malariatherapy data. Trans R Soc Trop Med Hyg. 2001;95:497-501. Epub 2001/11/15. doi:
684 10.1016/s0035-9203(01)90016-1. PMID: 11706658.
685 22. Carter R, Miller LH. Evidence for environmental modulation of gametocytogenesis in
686 *Plasmodium falciparum* in continuous culture. Bull World Health Organ. 1979;57 Suppl 1:37-52. Epub
687 1979/01/01. PMID: 397008.
688 23. Graves PM, Carter R, McNeill KM. Gametocyte production in cloned lines of *Plasmodium*
689 *falciparum*. Am J Trop Med Hyg. 1984;33:1045-50. doi: 10.4269/ajtmh.1984.33.1045. PMID:
690 6391217.
691 24. Buckling A, Ranford-Cartwright LC, Miles A, Read AF. Chloroquine increases *Plasmodium*
692 *falciparum* gametocytogenesis in vitro. Parasitology. 1999;118:339-46. Epub 1999/05/26. doi:
693 10.1017/s0031182099003960. PMID: 10340323.
694 25. Neveu G, Beri D, Kafsack BF. Metabolic regulation of sexual commitment in *Plasmodium*
695 *falciparum*. Curr Opin Microbiol. 2020;58:93-8. Epub 2020/10/15. doi: 10.1016/j.mib.2020.09.004.
696 PMID: 33053503.
697 26. Beri D, Balan B, Chaubey S, Subramaniam S, Surendra B, Tatu U. A disrupted
698 transsulphuration pathway results in accumulation of redox metabolites and induction of
699 gametocytogenesis in malaria. Sci Rep. 2017;7:40213. Epub 2017/01/17. doi: 10.1038/srep40213.
700 PMID: 28091526.
701 27. Chaubey S, Grover M, Tatu U. Endoplasmic reticulum stress triggers gametocytogenesis in
702 the malaria parasite. J Biol Chem. 2014;289:16662-74. Epub 2014/04/24. doi:
703 10.1074/jbc.M114.551549. PMID: 24755215.
704 28. Portugaliza HP, Miyazaki S, Geurten FJ, Pell C, Rosanas-Urgell A, Janse CJ, et al. Artemisinin
705 exposure at the ring or trophozoite stage impacts *Plasmodium falciparum* sexual conversion
706 differently. Elife. 2020;9. Epub 2020/10/22. doi: 10.7554/eLife.60058. PMID: 33084568.
707 29. Fivelman QL, McRobert L, Sharp S, Taylor CJ, Saeed M, Swales CA, et al. Improved
708 synchronous production of *Plasmodium falciparum* gametocytes in vitro. Mol Biochem Parasitol.
709 2007;154:119-23. Epub 20070420. doi: 10.1016/j.molbiopara.2007.04.008. PMID: 17521751.
710 30. Mantel PY, Hoang AN, Goldowitz I, Potashnikova D, Hamza B, Vorobjev I, et al. Malaria-
711 infected erythrocyte-derived microvesicles mediate cellular communication within the parasite
712 population and with the host immune system. Cell Host Microbe. 2013;13:521-34. Epub 2013/05/21.
713 doi: 10.1016/j.chom.2013.04.009. PMID: 23684304.
714 31. Brancucci NMB, Gerdt JP, Wang C, De Niz M, Philip N, Adapa SR, et al.
715 Lysophosphatidylcholine regulates sexual stage differentiation in the human malaria parasite
716 *Plasmodium falciparum*. Cell. 2017;171:1532-44 e15. Epub 2017/11/14. doi:
717 10.1016/j.cell.2017.10.020. PMID: 29129376.
718 32. Harris CT, Tong X, Campelo R, Vanheer LN, Marreiros IM, Nahiyaan N, et al. Metabolic
719 competition between lipid metabolism and histone methylation regulates sexual differentiation in
720 human malaria parasites. BioRxiv. 2022. doi: 10.1101/2022.01.18.476397.
721 33. Usui M, Prajapati SK, Ayanful-Torgby R, Acquah FK, Cudjoe E, Kakaney C, et al. *Plasmodium*
722 *falciparum* sexual differentiation in malaria patients is associated with host factors and GDV1-

723 dependent genes. Nat Commun. 2019;10:2140. Epub 2019/05/16. doi: 10.1038/s41467-019-10172-
724 6. PMID: 31086187.
725 34. Boltryk SD, Passecker A, Alder A, Carrington E, van de Vegte-Bolmer M, van Gemert GJ, et al.
726 CRISPR/Cas9-engineered inducible gametocyte producer lines as a valuable tool for Plasmodium
727 falciparum malaria transmission research. Nat Commun. 2021;12:4806. Epub 2021/08/12. doi:
728 10.1038/s41467-021-24954-4. PMID: 34376675.
729 35. Stewart LB, Diaz-Ingelmo O, Claessens A, Abugri J, Pearson RD, Goncalves S, et al. Intrinsic
730 multiplication rate variation and plasticity of human blood stage malaria parasites. Commun Biol.
731 2020;3:624. Epub 2020/10/30. doi: 10.1038/s42003-020-01349-7. PMID: 33116247.
732 36. Cheeseman IH, Gomez-Escobar N, Carret CK, Ivens A, Stewart LB, Tetteh KK, et al. Gene copy
733 number variation throughout the *Plasmodium falciparum* genome. BMC Genomics. 2009;10:353.
734 PMID: 19653891.
735 37. Tiburcio M, Hitz E, Niederwieser I, Kelly G, Davies H, Doerig C, et al. A 39-amino-acid C-
736 Terminal truncation of GDV1 disrupts sexual commitment in *Plasmodium falciparum*. Msphere.
737 2021;6. doi: 10.1128/mSphere.01093-20. PMID: WOS:000692590000015.
738 38. Scherf A, Mattei D. Cloning and characterization of chromosome breakpoints of *Plasmodium*
739 *falciparum*: breakage and new telomere formation occurs frequently and randomly in subtelomeric
740 genes. Nucleic Acids Res. 1992;20:1491-6. Epub 1992/04/11. doi: 10.1093/nar/20.7.1491. PMID:
741 1579440.
742 39. Duffy CW, Amambua-Ngwa A, Ahouidi AD, Diakite M, Awandare GA, Ba H, et al. Multi-
743 population genomic analysis of malaria parasites indicates local selection and differentiation at the
744 *gdv1* locus regulating sexual development. Sci Rep. 2018;8:15763. Epub 2018/10/27. doi:
745 10.1038/s41598-018-34078-3. PMID: 30361631.
746 40. Bruce MC, Carter RN, Nakamura K, Aikawa M, Carter R. Cellular location and temporal
747 expression of the *Plasmodium falciparum* sexual stage antigen Pfs16. Mol Biochem Parasitol.
748 1994;65:11-22. Epub 1994/05/01. doi: 10.1016/0166-6851(94)90111-2. PMID: 7935618.
749 41. Reilly Ayala HB, Wacker MA, Siwo G, Ferdig MT. Quantitative trait loci mapping reveals
750 candidate pathways regulating cell cycle duration in *Plasmodium falciparum*. BMC Genomics.
751 2010;11:577. doi: 10.1186/1471-2164-11-577. PMID: 20955606.
752 42. Smith LM, Motta FC, Chopra G, Moch JK, Nerem RR, Cummins B, et al. An intrinsic oscillator
753 drives the blood stage cycle of the malaria parasite Plasmodium falciparum. Science. 2020;368:754-
754 9. Epub 2020/05/16. doi: 10.1126/science.aba4357. PMID: 32409472.
755 43. Prajapati SK, Ayanful-Torgby R, Pava Z, Barbeau MC, Acquah FK, Cudjoe E, et al. The
756 transcriptome of circulating sexually committed *Plasmodium falciparum* ring stage parasites
757 forecasts malaria transmission potential. Nat Commun. 2020;11:6159. Epub 2020/12/04. doi:
758 10.1038/s41467-020-19988-z. PMID: 33268801.
759 44. Rono MK, Nyonda MA, Simam JJ, Ngoi JM, Mok S, Kortok MM, et al. Adaptation of
760 *Plasmodium falciparum* to its transmission environment. Nat Ecol Evol. 2018;2:377-87. doi:
761 10.1038/s41559-017-0419-9. PMID: 29255304.
762 45. Day KP, Karamalis F, Thompson J, Barnes DA, Peterson C, Brown H, et al. Genes necessary for
763 expression of a virulence determinant and for transmission of *Plasmodium falciparum* are located on
764 a 0.3-megabase region of chromosome 9. Proc Natl Acad Sci U S A. 1993;90:8292-6. PMID: 8367496.
765 46. MalariaGen, Ahouidi A, Ali M, Almagro-Garcia J, Amambua-Ngwa A, Amaratunga C, et al. An
766 open dataset of *Plasmodium falciparum* genome variation in 7,000 worldwide samples. Wellcome
767 Open Res. 2021;6:42. Epub 2021/07/30. doi: 10.12688/wellcomeopenres.16168.2. PMID: 33824913.
768 47. Bohme U, Otto TD, Sanders M, Newbold CI, Berriman M. Progression of the canonical
769 reference malaria parasite genome from 2002-2019. Wellcome Open Res. 2019;4:58. Epub
770 2019/06/06. doi: 10.12688/wellcomeopenres.15194.2. PMID: 31080894.
771 48. Tetteh KK, Stewart LB, Ochola LI, Amambua-Ngwa A, Thomas AW, Marsh K, et al. Prospective
772 identification of malaria parasite genes under balancing selection. PLoS One. 2009;4:e5568. PMID:
773 19440377.

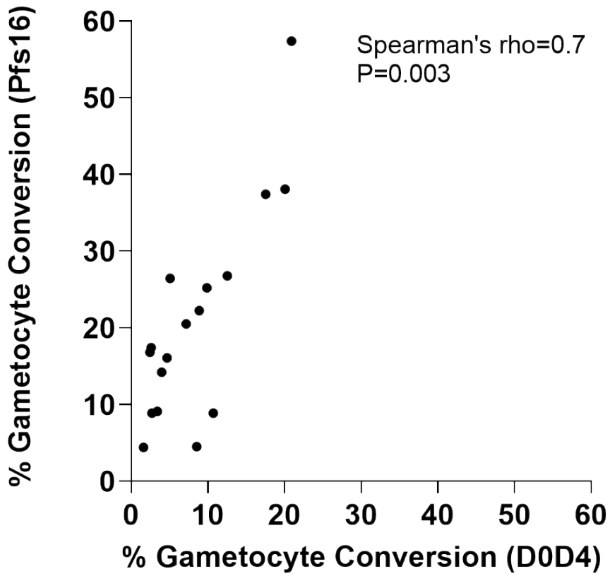
774 49. Ribaut C, Berry A, Chevalley S, Reybier K, Morlais I, Parzy D, et al. Concentration and
775 purification by magnetic separation of the erythrocytic stages of all human *Plasmodium* species.
776 Malar J. 2008;7:45. PMID: 18321384.
777 50. Baker DA, Daramola O, McCrossan MV, Harmer J, Targett GA. Subcellular localization of
778 Pfs16, a *Plasmodium falciparum* gametocyte antigen. Parasitology. 1994;108 129-37. Epub
779 1994/02/01. doi: 10.1017/s0031182000068219. PMID: 8159458.
780 51. Gupta SK, Schulman S, Vanderberg JP. Stage-dependent toxicity of N-acetyl-glucosamine to
781 *Plasmodium falciparum*. J Protozool. 1985;32:91-5. Epub 1985/02/01. doi: 10.1111/j.1550-
782 7408.1985.tb03020.x. PMID: 3886901.

783 **Figure 1.**



784

785 **Figure 2.**



786

Figure 3.

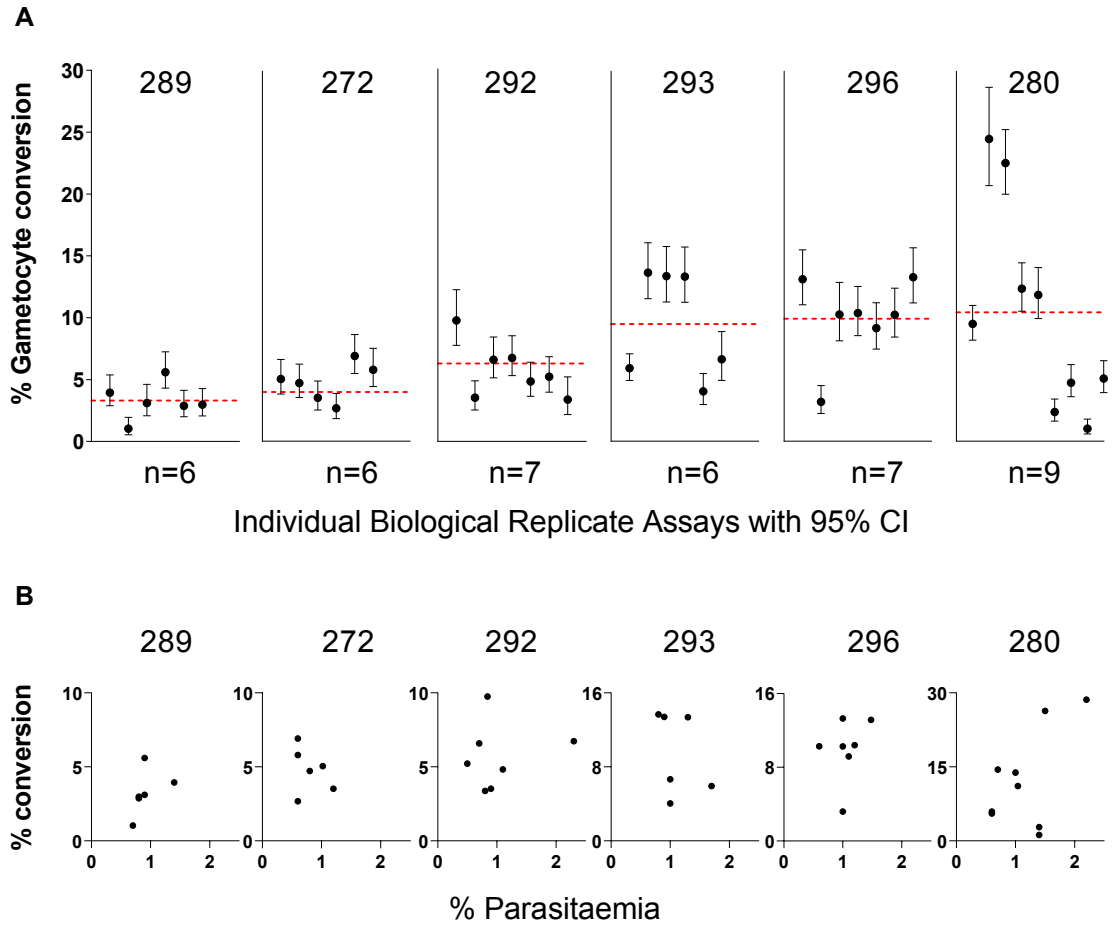
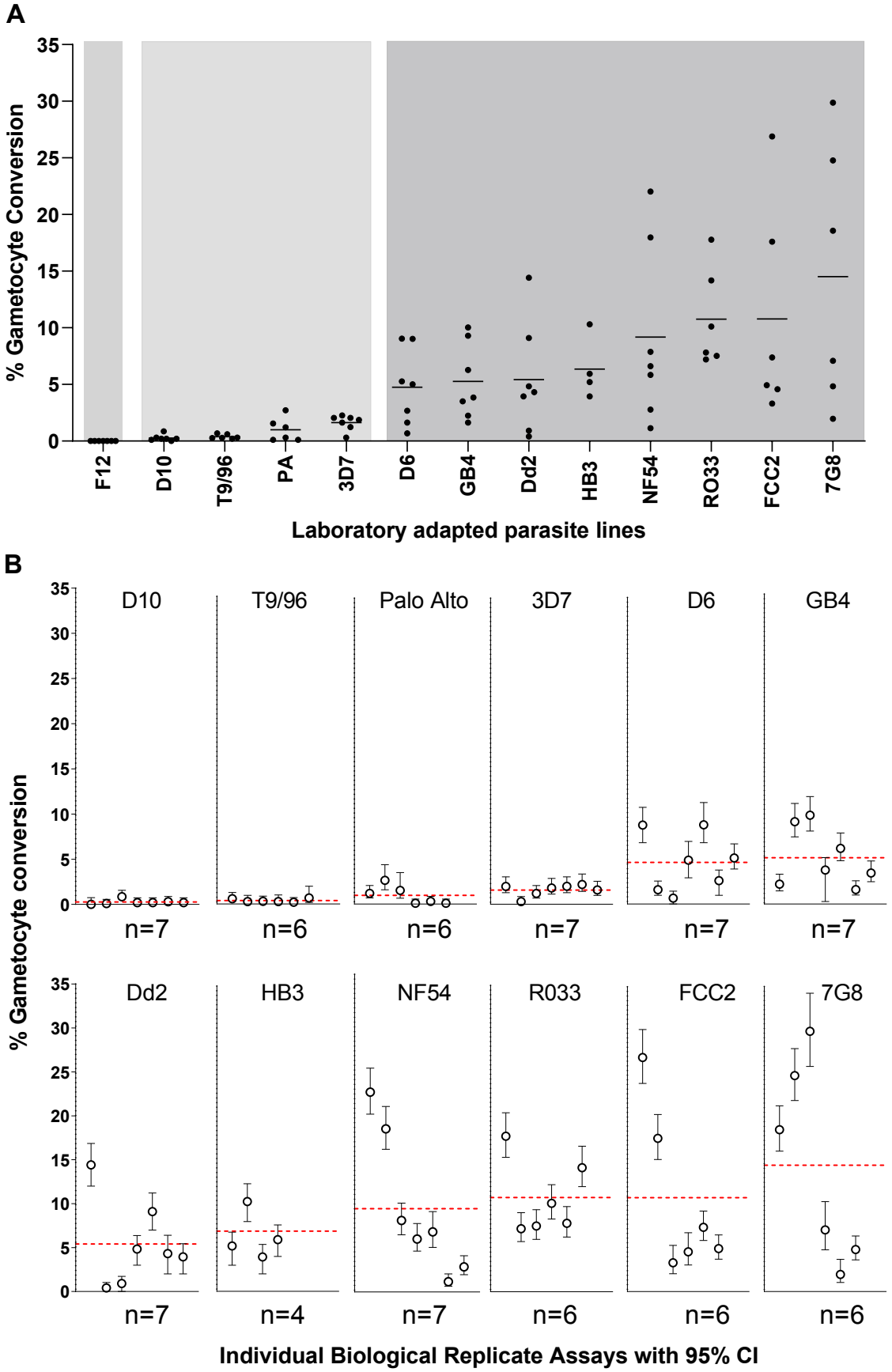
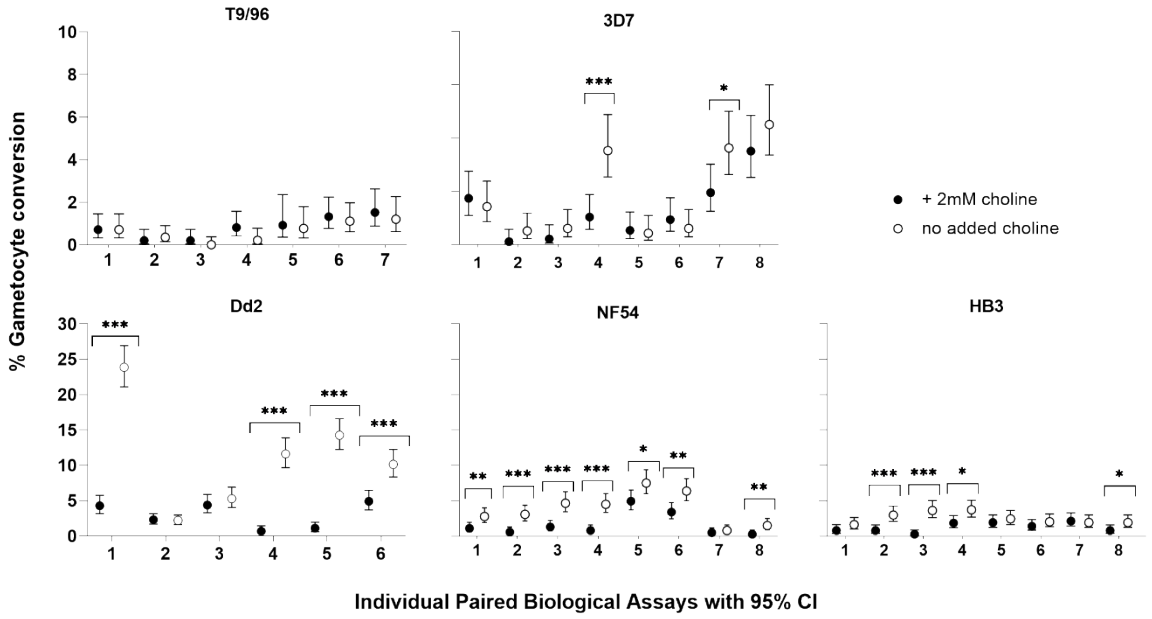

A
% Gametocyte conversion
289
272
292
293
296
280
n=6
n=6
n=7
n=6
n=7
n=9
Individual Biological Replicate Assays with 95% CI
B
% conversion
% Parasitaemia

Figure 4.


A
% Gametocyte Conversion
35
30
25
20
15
10
5
0
F12
D10
T9/96
PA
3D7
D6
GB4
Dd2
HB3
NF54
RO33
FCC2
7G8
Laboratory adapted parasite lines
B
% Gametocyte conversion
D10
T9/96
Palo Alto
3D7
D6
GB4
n=7
n=6
n=6
n=7
n=7
n=7
Dd2
HB3
NF54
R033
FCC2
7G8
n=7
n=4
n=7
n=6
n=6
n=6
Individual Biological Replicate Assays with 95% CI

792 **Figure 5.**

793



794

795 **Supplementary Table S1.** Gametocyte conversion rate (GCR) estimates (with 95%
796 confidence intervals) and all numerical count data for two different methods with multiple
797 experimental replicates applied to *P. falciparum* line 3D7/iGP under elevated GDV1
798 expression with Shield-1 reagent (+SHLD) and control (-SHLD) conditions.

		Method 1			Method 2		
Replicate	**Condition**	**Pfs16 +ve**	**DAPI +ve**	**Pfs16 GCR (95%CI)**	**D0 % Parasit.**	**D4 % Parasit.**	**D0D4 GCR (95% CI)**
1	-SHLD	84	921	9.12 (7.4 - 11.2)	10.1 (101/999)	0.3 (26/8262)	3.4 (2.2 -5.2)
	+SHLD	474	826	57.4 (54.0 - 60.7)	9.8 (98/999)	1.9 (117/6124)	20.9 (16.0 - 27.3)
2	-SHLD	104	1171	8.9 (7.4 - 10.7)	7.2 (67/932)	0.2 (15/8210)	2.7 (1.6 - 4.7)
	+SHLD	262	978	26.8 (24.1 - 29.7)	3.0 (29/970)	0.4 (49/13415)	12.5 (7.9 - 19.8)
3	-SHLD	41	926	4.4 (3.3 - 6.0)	5.6 (53/946)	0.1 (8/9305)	1.6 (2.8 - 3.4)
	+SHLD	150	595	25.2 (21.9 - 28.9)	4.4 (42/957)	0.4 (36/8625)	9.9 (6.3 - 15.4)
4	-SHLD	47	1043	4.5 (3.4 - 6.0)	5.0 (48/951)	0.4 (30/7278)	8.5 (5.4 - 13.5)
	+SHLD	267	701	38.1 (34.6 - 41.7)	4.4 (42/952)	1.0 (77/8070)	20.1 (14.0 - 28.8)

799

800 **Supplementary Table S2.** Estimates of gametocyte conversion rate (GCR) for 17 assays of
801 the *P. falciparum* inducible 3D7/iGP_D9 line in which two different methods were
802 performed in parallel for comparison (as detailed in Materials and Methods). Varying
803 concentrations of Shield-1 (0.055 – 1.0 µM, and none) were used for the assays. Data are
804 plotted and analysed in Figure 2, showing an overall correlation between the methods, with
805 Method 1 being generally more sensitive (and more precise with narrower confidence
806 intervals as shown here).

Independent assays of *P. falciparum* line	Method 1 Pfs16 GCR (95%CI)	Method 2 D0/D4 GCR (95%CI)
iGP - SHLD	9.1 (7.4 - 11.2)	3.4 (2.2 - 5.3)
iGP +SHLD (1.0)	57.4 (54.0 - 60.7)	20.9 (16.0 - 27.31)
iGP - SHLD	8.9 (7.4 - 10.7)	2.7 (1.6 - 4.8)
iGP + SHLD (0.055)	14.2 (12.3 - 16.4)	4.0 (2.2 - 7.2)
iGP + SHLD (0.15)	16.8 (14.8 - 19.1)	2.4 (1.2 (4.9)
iGP + SHLD (0.5)	17.4 (15.3 - 19.7)	2.6 (1.3 - 5.2)
iGP +SHLD (1.0)	26.8 (24.1 - 30.0)	12.5 (8.0 - 19.8)
iGP - SHLD	4.4 (3.4 - 7.0)	1.6 (0.8 - 3.4)
iGP + SHLD (0.055)	20.5 (17.9 - 23.4)	7.2 (4.6 - 11.3)
iGP + SHLD (0.15)	16.1 (13.8 - 18.7)	4.7 (3.2 - 6.9)
iGP + SHLD (0.5)	26.5 (23.5 - 29.6)	5.1 (3.2 - 8.2)
iGP +SHLD (1.0)	25.2 (21.9 - 28.9)	9.9 (6.4 - 15.4)
iGP - SHLD	4.5 (3.4 - 6.9)	8.5 (5.4 - 13.5)
iGP + SHLD (0.055)	8.9 (7.2 - 11.2)	10.7 (7.3 - 15.7)
iGP + SHLD (0.15)	22.3 (19.2 - 25.8)	8.9 (6.1 - 12.8)
iGP + SHLD (0.5)	37.4 (34.0 - 41.0)	17.5 (12.9 - 23.8)
iGP +SHLD (1.0)	38.1 (34.6 - 41.7)	20.1 (14.0 - 28.8)

807

808 **Supplementary Table S3.** Multiple biological replicate assays of gametocyte conversion
809 rates (GCR) for each of 6 cultured *P. falciparum* clinical isolates from Navrongo, Ghana.

Isolate	Replicate	RBC Batch	Days in culture	% Parasit.	Pfs16 +ve	DAPI +ve	GCR (95%CI)
289	1	12	92	1.4	38	961	4.0 (2.9 - 5.4)
	2	12	99	0.7	9	870	1.0 (0.6 - 2.0)
	3	12	99	0.9	23	740	3.1 (2.1 - 4.6)
	4	13	106	0.9	53	946	5.6 (4.3 - 7.3)
	5	13	106	0.8	28	971	2.9 (2.0 - 4.1)
	6	15	125	0.8	28	939	3.0 (2.1 - 4.3)
	Mean (SD)						*3.3 (SD = 1.5)*
272	1	12	83	1.0	48	951	5.0 (3.8 - 6.6)
	2	12	83	0.8	45	954	4.7 (3.5 - 6.3)
	3	13	91	1.2	34	965	3.5 (2.5 - 4.9)
	4	13	91	0.6	26	973	2.7 (1.8 - 3.9)
	5	15	110	0.6	69	999	6.9 (5.5 - 8.7)
	6	15	110	0.6	51	880	5.8 (4.4 - 7.5)
	Mean (SD)						*4.8 (SD = 1.5)*
292	1	1	67	0.8	66	677	9.7 (7.7 - 12.2)
	2	12	62	0.9	34	965	3.5 (2.5 - 4.9)
	3	12	62	0.7	58	882	6.6 (5.1 - 8.4)
	4	13	69	2.3	63	936	6.7 (5.3 - 8.5)
	5	13	69	1.1	46	953	4.8 (3.6 - 6.4)
	6	15	88	0.5	49	939	5.2 (4.0 - 6.8)
	7	15	88	0.8	19	564	3.4 (2.2 - 5.2)
	Mean (SD)						*5.7 (SD = 2.2)*
293	1	1	67	1.7	109	1841	5.9 (4.9 - 7.1)
	2	12	55	0.8	120	878	13.7 (11.6 - 16.1)
	3	13	69	0.9	118	881	13.4 (11.3 - 15.8)
	4	13	69	1.3	118	883	13.4 (11.3 - 15.8)
	5	13	76	1.0	39	962	4.1 (3.0 - 5.5)
	6	13	76	1.0	41	617	6.6 (4.9 - 8.9)
	Mean (SD)						*9.5 (SD = 4.4)*
296	1	12	48	1.5	116	883	13.1 (11.1 - 15.5)
	2	12	55	1.0	31	968	3.2 (2.3 - 4.5)
	3	12	55	0.6	65	632	10.3 (8.2 - 12.9)
	4	13	62	1.2	94	905	10.4 (8.6 - 12.5)
	5	13	62	1.1	84	915	9.2 (7.5 - 11.2)
	6	13	69	1.0	93	906	10.3 (8.5 - 12.4)
	7	13	69	1.0	118	887	13.3 (11.2 - 15.7)
	Mean (SD)						*10.0 (SD = 3.4)*
280	1	1	55	1.0	156	1406	11.1 (9.6 - 12.9)
	2	2	64	2.2	103	360	28.6 (24.2 - 33.5)
	3	2	64	1.5	209	794	26.3 (23.5 - 29.6)
	4	3	71	0.7	131	907	14.4 (12.3 - 16.9)
	5	3	71	1.0	109	787	13.9 (11.6 - 16.4)
	6	3	78	1.4	27	973	2.8 (1.9 - 4.0)
	7	3	78	0.6	49	884	5.5 (4.2 - 7.3)
	8	4	85	1.4	12	998	1.2 (0.7 - 2.1)
	9	4	85	0.6	56	943	5.9 (4.6 - 7.6)
	Mean (SD)						*12.2 (SD = 9.8)*

The table shows exact counts and 95% confidence intervals of the GCR estimates using Method 1 (with Pfs16 staining) as plotted and analysed in Figure 3. The number of days in culture is shown as well as the % parasitaemia measured in the cycle before. The assay replicates are presented in the order in which they were performed (the duration of time in culture occasionally does not exactly follow the same sequential temporal order for Isolates 292 and 293 as an intermediate cryopreservation and thawing of the isolates was performed). Erythrocytes from 15 different erythrocyte donors used at various times throughout the series of experiments shown here and in Supplementary Table S5 are numbered (RBC Batch), and inspection of the data indicates that these did not determine the variation between assay replicates.

820 **Supplementary Table S4.** Pairwise tests for significant differences in gametocyte conversion
821 rates between different Ghanaian *P. falciparum* clinical isolates.

Clinical Isolates	272	280	289	292	293	296
272						
280	0.1447					
289	0.1797	0.0663				
292	0.5594	0.2523	0.0350*			
293	0.0649	0.7756	0.0043**	0.1375		
296	0.0221*	0.7577	0.0047**	0.0379*	0.7308	

822

823 P values are from Mann-Whitney comparisons of all of the individual biological replicate
824 measures for each of the six isolates (as shown in Supplementary Table S3). Statistically
825 significant comparisons are highlighted in green.

826 **Supplementary Table S5.** Multiple biological replicate assays of gametocyte conversion
827 rates (GCR) for each of 13 long-term culture adapted *P. falciparum* lines of diverse origins.

Line	Replicate	RBC batch	Pfs16 +ve	DAPI +ve	GCR (95% CI)
F12	1	3	0	1000	0 (0)
	2	3	0	1000	0 (0)
	3	4	0	1023	0 (0)
	4	10	0	394	0 (0)
	5	11	0	600	0 (0)
	6	11	0	711	0 (0)
	7	11	0	380	0 (0)
	Mean (SD)				0 (SD = 0)
D10	1	2	0	515	0 (0)
	2	2	1	999	0.1 (0 - 5.6)
	3	2	10	1177	0.8 (0.5 - 1.6)
	4	3	2	997	0.2 (0.1 - 0.7)
	5	3	2	1000	0.2 (0.1 -0.7)
	6	9	3	997	0.3 (0.1 - 0.9)
	7	9	2	997	0.2 (01 - 0.7)
	Mean (SD)				0.26 (SD = 0.27)
T996	1	12	6	993	0.6 (0.3 - 1.3)
	2	13	2	733	0.3 (0.1 - 1.0)
	3	13	3	992	0.3 (0.1 - 0.9)
	4	13	2	703	0.3 (0.1 - 1.0)
	5	14	2	997	0.2 (0 - 0.7)
	6	14	3	433	0.7 (0.2 - 2.0)
	Mean (SD)				0.39 (SD = 0.20)
Palo Alto	1	6	12	987	1.2 (0.7 - 1.2)
	2	7	14	514	2.7 (1.6 - 4.5)
	3	7	5	321	1.6 (0.7 - 3.6)
	4	9	1	998	0.1 (0 - 0.6)
	5	9	3	996	0.3 (0.1 - 0.9)
	6	9	1	998	0.1 (0 - 0.6)
	Mean (SD)				1.00 (SD = 1.04)
3D7	1	2	20	980	2.0 (1.3 - 3.1)
	2	3	3	994	0.3 (0.1 - 0.9)
	3	3	12	979	1.2 (0.7 -2.1)
	4	4	20	977	2.0 (1.3 -3.1)
	5	4	17	916	1.9 (1.2 - 3.0)
	6	12	20	885	2.3 (1.5 - 3.5)
	7	12	16	983	1.6 (1.0 - 2.6)
	Mean (SD)				1.62 (SD = 0.67)

Line	Replicate	RBC batch	Pfs16 +ve	DAPI +ve	GCR (95% CI)
D6	1	5	83	920	9.0 (7.3 -11.1)
	2	5	16	983	1.6 (1.0 - 2.6)
	3	5	6	885	0.7 (0.3 - 1.5)
	4	9	28	559	5.0 (3.5 - 7.1)
	5	9	86	905	9.5 (7.8 - 11.6)
	6	9	26	973	2.7 (1.8 - 3.9)
	7	9	50	950	5.3 (4.0 - 6.9)
	Mean (SD)				4.70 (SD = 3.36)
GB4	1	6	22	978	2.2 (1.5 - 3.4)
	2	6	85	914	9.3 (7.6 -11.4)
	3	6	92	917	10.0 (8.3 - 12.2)
	4	6	37	964	3.8 (2.8 - 5.3)
	5	7	59	940	6.3 (4.9 - 8.0)
	6	7	16	983	1.6 (1.0 - 2.6)
	7	9	34	969	3.5 (2.5 - 4.9)
	Mean (SD)				5.26 (3.35)
Dd2	1	2	130	902	14.4 (12.3 - 16.9)
	2	3	4	995	0.4 (0.2 - 1.0)
	3	3	9	986	0.9 (0.5 - 1.7)
	4	4	46	953	4.8 (3.6 - 6.4)
	5	4	84	916	9.2 (7.5 - 11.2)
	6	12	23	532	4.3 (2.9 -6.4)
	7	12	35	885	4.0 (2.9 - 5.5)
	Mean (SD)				5.42 (SD = 4.89)
HB3	1	2	50	691	7.2 (5.5 - 9.4)
	2	2	103	1000	10.3 (8.6 - 12.3)
	3	9	57	942	6.1 (4.7 -7.8)
	4	9	56	944	5.9 (4.6 -7.6)
	Mean (SD)				6.87 (SD = 2.32)
NF54	1	2	225	1022	22.0 (19.6 - 24.7)
	2	2	182	1013	18.0 (15.7 - 20.5)
	3	3	73	926	7.9 (6.3 - 9.8)
	4	3	54	925	5.8 (4.5 - 7.5)
	5	13	41	619	6.6 (4.9 - 8.9)
	6	13	11	975	1.1 (0.6 - 2.0)
	7	13	27	975	2.8 (1.9 - 4.0)
	Mean (SD)				9.18 (SD = 7.83)

828

Line	Replicate	RBC Batch	Pfs16 +ve	DAPI +ve	GCR (95% CI)
RO33	1	5	153	861	17.8 (15.4 - 20.5)
	2	5	68	943	7.2 (5.7 - 9.0)
	3	7	70	932	7.5 (6.0 - 9.4)
	4	7	92	910	10.1 (8.3 - 12.2)
	5	10	72	921	7.8 (6.3 - 9.7)
	6	10	124	875	14.2 (12.0 - 16.6)
	Mean (SD)				10.77 (SD = 4.31)
FCC2	1	5	211	785	26.9 (23.9 - 30.1)
	4	5	147	835	17.6 (15.2 - 20.3)
	5	5	16	484	3.3 (2.0 - 5.3)
	6	5	23	503	4.6 (3.1 - 6.8)
	7	10	69	935	7.4 (5.9 - 9.2)
	8	10	47	952	4.9 (3.7 - 6.5)
	Mean (SD)				10.78 (SD = 9.44)
7G8	1	6	159	856	18.6 (16.1 - 21.3)
	2	6	199	803	24.8 (21.9 - 27.9)
	3	10	135	452	29.9 (25.8 - 34.2)
	4	10	24	339	7.1 (4.8 - 10.3)
	5	10	9	456	2.0 (1.0 - 3.7)
	6	11	46	953	4.8 (3.6 - 6.4)
	Mean (SD)				14.52 (SD = 11.52)

829 Exact counts and 95% confidence intervals of the GCR estimates were performed using
830 Method 1 (with Pfs16 staining) as plotted and analysed in Figure 4. Erythrocytes from fifteen
831 different erythrocyte donors used at various times throughout the series of experiments
832 shown here and in Supplementary Table S3 are numbered (RBC Batch), and inspection of the
833 data shows that these did not determine the variation between assay replicates.

Supplementary Table S6. Pairwise tests for significant differences in gametocyte conversion rates between different *P. falciparum* laboratory lines. P values are from Mann-Whitney comparisons of all of the individual biological replicate measures for each of the 13 lines (as shown in Supplementary Table S5). Statistically significant comparisons are highlighted in green.

Lab Isolates	F12	D10	T9/96	Palo Alto	3D7	D6	GB4	Dd2	HB3	NF54	RO33	7G8	FCC2
F12													
D10	0.0047**												
T9/96	0.0022**	0.1696											
Palo Alto	0.0022**	0.2459	0.6082										
3D7	0.0012**	0.0017**	0.0052**	0.1521									
D6	0.0012**	0.0012**	0.0023**	0.0216*	0.0781								
GB4	0.0012**	0.0006***	0.0012**	0.0047**	0.0117*	0.6457							
Dd2	0.0012**	0.0012**	0.0047**	0.0338*	0.1282	>0.9999	>0.9999						
HB3	0.0048**	0.0030**	0.0095**	0.0061**	0.0012**	0.2303	0.4121	0.3124					
NF54	0.0012**	0.0006***	0.0012**	0.0082**	0.0175*	0.3176	0.535	0.3176	0.9273				
RO33	0.0022**	0.0012**	0.0022**	0.0022**	0.0012**	0.035*	0.035*	0.0734	0.026*	0.4452			
7G8	0.0022**	0.0012**	0.0022**	0.0043**	0.0082**	0.1807	0.1807	0.1101	0.3939	0.4452	>0.9999		
FCC2	0.0022**	0.0012**	0.0022**	0.0022**	0.0012**	0.336	0.2949	0.2343	0.9143	0.9452	0.3939	0.6991	

839 **Supplementary Table S7.** Numerical counts and calculated values of assays measuring the
840 effect of choline-free medium on gametocyte conversion rates (GCR) of five different *P.*
841 *falciparum* lines. The effect of each biological replicate assay is shown as a Rate Ratio with
842 95% confidence intervals, based on the GCR with and without choline. Mantel-Haenszel
843 adjusted mean Rate Ratios show the overall effects across all replicates with 95% confidence
844 intervals.

Biological Replicate Assays	Condition	Pfs16 +ve	Pfs16 -ve	Rate Ratio (± 95%CI)	GCR (± 95%CI)	Adjusted mean Rate Ratio (± 95%CI)
			***P. falciparum* line HB3**			
R1	-choline	16	967	2.03 (0.93 - 5.46)	1.6 (1.0 - 2.6)	**1.98 (1.55 - 2.52)**
	+ choline	7	864		0.8 (0.4 - 1.6)	
R2	-choline	29	943	3.69 (1.69 - 8.04)	3.0 (2.1 - 4.3)	
	+ choline	8	983		0.8 (0.4 - 1.6)	
R3	-choline	34	902	12.05 (3.71 - 39.13)	3.6 (2.6 -5.0)	
	+ choline	3	993		0.3 (0.1 - 0.9)	
R4	-choline	36	929	2.01 (1.15 - 3.51)	3.7 (2.7 - 5.1)	
	+ choline	18	950		1.9 (1.2 - 2.9)	
R5	-choline	24	954	1.26 (0.70 - 2.30)	2.5 (1.7 - 3.6)	
	+ choline	19	961		1.9 (1.2 - 3.0)	
R6	-choline	14	970	1.00 (0.48 - 2.09)	1.4 (0.8 - 2.4)	
	+ choline	14	971		1.4 (0.8 - 2.4)	
R7	-choline	20	959	0.95 (0.52 - 1.74)	2.0 (1.3 - 3.1)	
	+ choline	21	957		2.1 (1.4 - 3.3)	
R8	-choline	19	962	2.40 1.06 - 5.45)	1.9 (1.2-3.0)	
	+ choline	8	983		0.8 (0.4 - 1.6)	
			***P. falciparum* line Dd2**			
R1	-choline	194	619	5.58 (4.03 - 7.71)	23.9 (21.1 - 26.9)	**3.51 (3.00 - 4.11)**
	+ choline	41	917		4.3 (3.2 - 5.8)	
R2	-choline	42	1867	0.95 (0.61 - 1.47)	2.2 (1.6 - 3.0)	
	+ choline	37	1562		2.31 (1.7 - 3.2)	
R3	-choline	51	912	1.21 (0.81 - 1.80)	5.3 (4.1 - 6.9)	
	+ choline	42	915		4.4 (3.3- 5.9)	
R4	-choline	105	799	16.56 (7.75 - 35.40)	11.6 (9.7 - 13.9)	
	+ choline	7	991		0.7 (0.3 - 1.4)	
R5	-choline	141	847	12.82 (6.98 - 23.53)	14.3 (12.2 - 16.6)	
	+ choline	11	977		1.1 (0.6 - 2.0)	
R6	-choline	93	824	2.07 (1.47 - 2.90)	10.1 (8.4 - 12.3)	
	+ choline	47	910		4.9 (3.7 - 6.5)	

845

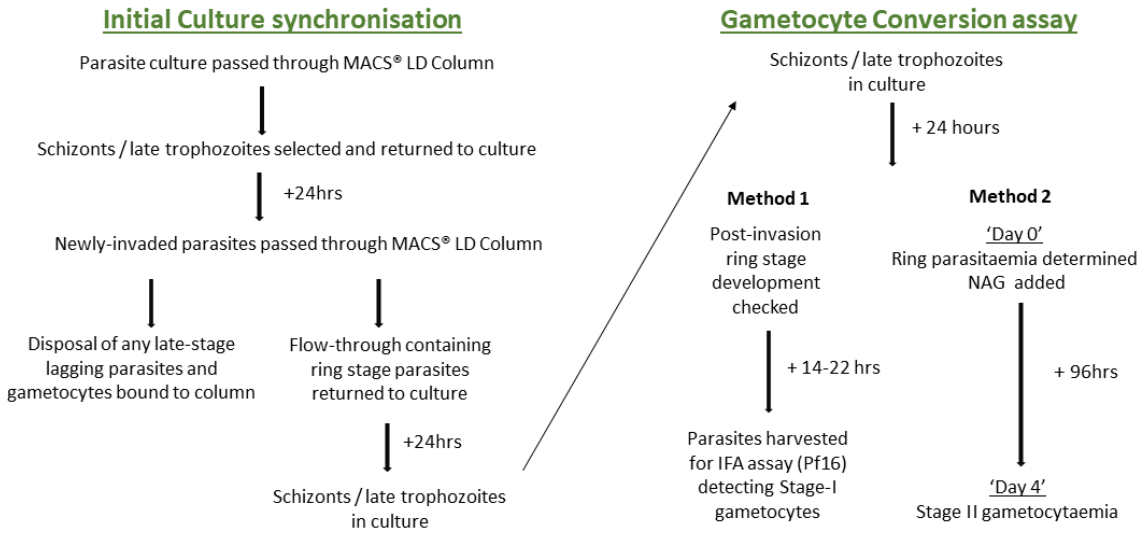
Biological Replicate Assays	Condition	Pfs16 +ve	Pfs16 -ve	Rate Ratio (± 95%CI)	GCR (± 95%CI)	Adjusted mean Rate Ratio (± 95%CI)
			***P. falciparum* line 3D7**			
R1	-choline	17	965	1.22 (0.60 - 2.45)	1.7(1.1 - 2.8)	**1.50 (1.16 - 1.94)**
	+ choline	14	969		1.4 (0.9 - 2.4)	
R2	-choline	1	997	0.20 (0.02- 1.70)	0.1 (0.0 - 0.6)	
	+ choline	5	989		0.5 (0.2 - 1.2)	
R3	-choline	6	987	3.01 (0.61 - 14.88)	0.6 (0.3 - 1.3)	
	+ choline	2	995		0.2 (0.0 - 0.7)	
R4	-choline	34	931	3.45 (1.72 - 6.95)	3.5 (2.5 - 4.9)	
	+ choline	10	970		1.0 (0.6 - 1.9)	
R5	-choline	4	940	0.82 (0.22 - 3.04)	0.4 (0.2 - 1.1)	
	+ choline	5	961		0.5 (0.2 - 1.2)	
R6	-choline	6	987	0.65 (0.23 - 1.82)	0.6 (0.3 - 1.3)	
	+ choline	9	961		0.9 (0.5 - 1.8)	
R7	-choline	35	931	1.86 (1.08 - 3.24)	3.6 (2.6 - 5.0)	
	+ choline	19	961		1.9 (1.2 - 3.0)	
R8	-choline	43	913	1.28 (0.83 - 1.99)	4.5 (3.4 - 6.0)	
	+ choline	34	938		3.5 (2.5 - 4.8)	
			***P. falciparum* line T9/96**			
R1	-choline	7	985	1.00 (0.35 - 2.84)	0.7 (0.3 - 1.4)	**0.8 (0.51 - 1.19)**
	+ choline	7	985		0.7 (0.3 - 1.4)	
R2	-choline	2	995	0.57 (0.10 - 3.10)	0.2 (0.0 - 0.7)	
	+ choline	4	1131		0.4 (0.1 - 0.9)	
R3	-choline	2	995	no estimate	0.2 (0.0 - 0.7)	
	+ choline	0	999		0 (0)	
R4	-choline	2	934	0.26 (0.06 - 1.24)	0.2 (0.0 - 0.8)	
	+ choline	8	983		0.8 (0.4 - 1.6)	
R5	-choline	5	643	0.83 (0.23 - 3.09)	0.8 (0.3 - 1.8)	
	+ choline	4	429		0.9 (0.4 - 2.4)	
R6	-choline	11	978	0.84 (0.38 - 1.88)	1.1 (0.6 - 2.0)	
	+ choline	13	973		1.3 (0.8 - 2.2)	
R7	-choline	9	739	0.79 (0.34 - 1.87)	1.2 (0.6 - 2.3)	
	+ choline	12	780		1.5 (0.9 -2.6)	

846

Biological Replicate Assays	Condition	Pfs16 +ve	Pfs16 -ve	Rate Ratio (± 95%CI)	GCR (± 95%CI)	Adjusted Risk Ratio (± 95%CI)
			***P.falciparum* line NF54**			
R1	-choline	27	945	2.50 (1.25 - 5.01)	2.8 (1.9 - 4.0)	**2.41 (1.97 - 2.96)**
	+ choline	11	978		1.1 (0.6 - 2.0)	
R2	-choline	30	943	5.11 (2.13 - 12.24)	3.1 (2.2- 4.4)	
	+ choline	6	990		0.6 (0.3 - 1.3)	
R3	-choline	40	819	3.53 (1.90 - 6.56)	4.7 (3.4- 6.3)	
	+ choline	13	973		1.3 (0.8 - 2.2)	
R4	-choline	43	913	5.57 (2.63 - 11.79)	4.5 (3.4 - 6.0)	
	+ choline	8	983		0.8 (0.4 - 1.6)	
R5	-choline	70	861	1.52 (1.06 - 2.18)	7.5 (6.0 - 9.4)	
	+ choline	47	905		4.9 (3.7 - 6.5)	
R6	-choline	60	880	1.86 (1.23 - 2.83)	6.4 (5.0 - 8.1)	
	+ choline	33	933		3.4 (2.4 - 4.8)	
R7	-choline	8	983	1.60 (0.53 - 4.87)	0.8 (0.4 - 1.6)	
	+ choline	5	986		0.5 (0.2 - 1.2)	
R8	-choline	15	970	5.06 (1.47 - 17.41)	1.5 (0.9 - 2.5)	
	+ choline	3	993		0.3 (0.1 - 0.9)	

847

848 **Supplementary Figure S1.** Scheme outlining the timing of processes undertaken for two different
849 methods to assay parasite gametocyte conversion rates of different *P. falciparum* lines in culture
850 (details given in Materials and Methods section).



851