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1 **Cryogenically preserved RBCs support gametocytogenesis of *Plasmodium falciparum in***
2 ***vitro* and gametogenesis in mosquitoes.**

3 Ashutosh K. Pathak[1]*, Justine C. Shiau[1], Matthew B. Thomas[2], and Courtney Murdock[1,3,4,5,6,7]

4 [1]Dept. of Infectious Diseases, College of Veterinary Medicine, University of Georgia, Athens, GA
5 30602.

6 [2]Center for Infectious Disease Dynamics and the Department of Entomology, Pennsylvania
7 State University, State College, PA 16803.

8 [3]Odum School of Ecology, University of Georgia, Athens GA 30602.

9 [4]Center for Ecology of Infectious Diseases, University of Georgia, Athens GA 30602.

10 [5]Center for Tropical Emerging Global Diseases, University of Georgia, Athens GA 30602.

11 [6]Center for Vaccines and Immunology, University of Georgia, Athens GA 30602.

12 [7]Riverbasin Center, University of Georgia, Athens GA 30602.

13 * ash1@uga.edu

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21 **Abstract**

22 **Background**

23 The malaria Eradication Research Agenda (malERA) has identified human-to-mosquito
24 transmission of *Plasmodium falciparum* as a major target for eradication. The cornerstone for
25 identifying and evaluating transmission in the laboratory is small membrane feeding assays
26 (SMFAs) where mature gametocytes of *P. falciparum* generated *in vitro* are offered to
27 mosquitoes as part of a blood-meal. However, propagation of "infectious" gametocytes requires
28 10-12 days with considerable physico-chemical demands imposed on host RBCs and
29 thus, "fresh" RBCs that are ≤1-week old post-collection are generally recommended. However,
30 in addition to the costs, physico-chemical characteristics unique to RBC donors may confound
31 reproducibility and interpretation of SMFAs. Cryogenic storage of RBCs (cryo-preserved RBCs
32 herein) is approved by the European and US FDAs as an alternative to refrigeration (4°C) for
33 preserving RBC quality and while cryo-preserved RBCs have been used for *in vitro* cultures of
34 other *Plasmodia* and the asexual stages of *P. falciparum*, none of the studies required RBCs to
35 support parasite development for >4 days.

36 **Results**

37 Using the standard laboratory strain, *P. falciparum* NF54, we first demonstrate that cryo-
38 preserved RBCs preserved in the gaseous phase of liquid nitrogen and thawed after storage for
39 1, 4, 8 and 12 weeks, supported gametocytogenesis *in vitro* and subsequent gametogenesis
40 in *Anopheles stephensi* mosquitoes. Using data from 11 SMFAs and RBCs from 4 separate
41 donors with 3 donors re-tested following various periods of cryo-preservation, we show that
42 overall levels of sporogony in the mosquito, as measured by oocyst prevalence and burdens in
43 the midguts and sporozoites in salivary glands, were similar or better than using ≤1-week old

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44 refrigerated RBCs. Additionally, the potential for cryo-preserved RBCs to serve as a universal
45 substrate for SMFAs is shown for a Cambodian isolate of *P. falciparum*.

46 **Conclusions**

47 Considering the suitability of cryo-preserved RBCs for *P. falciparum* SMFAs, we suggest
48 guidelines for their use and how they can be integrated into an existing laboratory/insectary
49 framework with the potential to significantly reduce running costs and provide greater reliability.
50 Finally, we discuss scenarios where cryo-preserved RBCs may be especially useful in
51 enhancing our understanding and/or providing novel insights into the patterns and process
52 underlying human-to-mosquito transmission.

53 **Keywords**

54 *Anopheles stephensi*, cryogenic preservation, gametogenesis, malaria, *Plasmodium falciparum*,
55 RBCs, SMFAs, transmission.

56 **Background**

57 Incidence of malaria due to *Plasmodium falciparum* has seen a steady decline in the last 15-20
58 years, enabled primarily by the synergy between drug-combination therapies for case/disease
59 management and vector control programs [1, 2]. Since transmission to the mosquito is thought
60 to be a severe bottleneck in the parasite's life history, considerable effort has been channeled
61 towards identifying parasite and vector traits influencing transmission to the mosquito with the
62 aim of designing and testing small molecule inhibitors and vaccines [3-6]. The experimental
63 model for evaluating basic parasite life history or the efficacy of various interventions typically
64 involve small-membrane feeding assays (SMFAs), where mature male and female gametocytes
65 of *P. falciparum* cultured *in vitro* are supplemented with naïve RBCs and offered as part of a
66 blood-meal to female *Anopheles* spp. mosquitoes. These assays are then followed by the
67 collection of various measures of parasite fitness in the mosquito vector [7-11]. However, in the

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68 40 years since Trager and Jensen's first description of the methodology, *in vitro* cultures of
69 transmission-competent parasite stages (the definitive measure of parasite fitness) have proven
70 to be an arduous and labor-intensive venture with the precise mechanism(s) regulating the
71 induction of gametocytogenesis remaining largely elusive [12-15]. Currently available
72 methodology is a synthesis of several tools and techniques that have been elegantly
73 summarized in two recent publications [13, 15].

74 Relative to other etiological agents of human malaria, *P. falciparum* is unique in its life-
75 history in blood wherein the asexual replication period lasts 2 days during which approximately
76 15% of the resulting parasite progeny is pre-destined to invade an RBC and enter an
77 irreversible path of sexual differentiation lasting an additional 10-12 days before maturation into
78 male and female gametocytes capable of infecting a mosquito. Most references outlining
79 methods for growing sexual stages in RBCs *in vitro* emphasize the need for maintaining the
80 sexual stages in fresh RBCs (i.e., storage under refrigeration for no more than 1-week from the
81 day of collection), as the RBC must be able to support gametocytogenesis both physically and
82 energetically during the 10 to 12-day differentiation period [13, 15-18]. However, ensuring a
83 regular supply of commercially sourced RBCs can quickly prove to be prohibitively expensive.
84 Additionally, variation introduced by differences in storage time of RBCs and across blood
85 donors can potentially carry significant consequences for the reproducibility of downstream
86 experiments and across laboratories for validating vaccine and drug efficacy for instance [9, 19-
87 21].

88 Despite >100 years of experience in bio-banking, identifying the true "shelf-life" of
89 refrigerated RBCs is controversial even in case of human blood transfusions [22]. While it may
90 be possible to restore some aspects of RBC metabolism upon rejuvenation of packed RBCs
91 with the addition of fresh nutrient-containing media, most of the storage-associated changes are
92 simply irreversible [23-25]. As an alternative, cryo-preservation of RBCs in glycerol-based cryo-

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93 protectants (storage at -65ºC or below) has been suggested using procedures approved by both
94 the United States Food and Drug Administration and the European Council [26, 27].

95 While cryo-preserved RBCs had been shown to support the growth of other etiological
96 agents of human malaria such as *P. vivax*, a recent study demonstrated that growth rates of the
97 asexual blood stages of *P. falciparum* in cryogenically-preserved RBCs (-196ºC in the gaseous
98 phase of liquid nitrogen) was almost identical to freshly collected blood *in vitro* [28, 29].
99 However, none of these studies required the RBCs to sustain parasite growth for more than four
100 days. According to current FDA guidelines, cryo-preserved RBCs thawed under sterile
101 conditions can retain physicochemical properties for up to (at least) 14 days following the thaw
102 date [26]. Considering how this timeframe encompasses the duration required for
103 gametocytogenesis of *P. falciparum*, we hypothesized that cryo-preserved RBCs should also
104 provide a suitable substrate for the culture of sexual stages of *P. falciparum*. In the current
105 study, we were interested in determining if cryo-preserved RBCs support gametocytogenesis,
106 and if the resulting mature gametocytes undergo gametogenesis using SMFAs with *Anopheles*
107 *stephensi* as the definitive measure of fitness. We also addressed whether infectiousness was
108 retained following extended periods of cryo-preservation. Lastly, although we accomplished
109 these objectives primarily with the standard lab strain (NF54) of *P. falciparum*, we also tested
110 the potential of cryo-preserved RBCs to serve as a substrate for supporting growth and
111 differentiation of infectious stages of a Cambodian isolate of *P. falciparum*, CB132 [30].

112 **Results**

113 <u>Gametocytogenesis of *P. falciparum in vitro*</u>

114 Mature gametocytemia *in vitro* generally increased over time with detectable levels
115 achieved by 12 days post-infection (figure 1). Overall trends were independent of storage
116 method (Odds ratios (OR)= 1.31, standard error (se)=0.41, *p*=0.4) or duration (OR=1.01,

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117 se=0.04, *p*=0.72) with most of the random variation is explained by differences between
118 experimental block and negligible contribution by RBC donor (Table 1).

119 Prevalence of *P. falciparum* in midguts of *An. stephensi* mosquitoes

120 The mean (±se) prevalence of mosquitoes infected with *P. falciparum* oocysts (NF54) was
121 53±6.4% (n=11 SMFAs, mean mosquito midguts sampled/SMFA= 26 (range= 17-44), total
122 number of mosquito midguts sampled=283) (figure. 2a). While prevalence was independent of
123 storage method (OR=1.05, se=0.38, *p*=0.89), the ability of cryo-preserved RBCs to culture
124 infectious gametocytes showed a marginally insignificant decline with duration of storage
125 (OR=0.89, se=0.18, *p*=0.07) with much of the unexplained variation represented by differences
126 across experimental blocks, although some could also be attributed to RBC donor
127 characteristics (Table 1 and additional file 3).

128 Prevalence of *P. falciparum* in the salivary glands of mosquitoes

129 The mean (±se) prevalence of mosquitoes carrying sporozoites of *P. falciparum* NF54 in the
130 salivary glands was 54±5.3% (n=10 SMFAs, mean salivary glands sampled/SMFA= 24 (range=
131 20-30), total number of salivary glands sampled=235) (figure 2b). Overall trends in sporozoite
132 prevalence were independent of storage method (OR=1.35, se=0.84, p=0.63) or duration
133 (OR=0.93, se=0.08, p=0.36) (Table 1). Unlike the midguts however, most of the random
134 variation was caused by differences among infections with negligible contributions from
135 experimental block or RBC donor (Table 1).

136 Oocyst abundance and intensity in the midguts of mosquitoes

137 Mean (±se) oocyst abundance (oocyst counts from all sampled midgut) was 5.4±2.53 (n=11
138 SMFAs, mean mosquito midguts sampled/SMFA= 26 (range= 17-44), total number of mosquito
139 midguts sampled=283) while oocyst intensity (oocyst counts from infected midguts only) was
140 7.75±2.85 (n=11 SMFAs, range of infected midguts/SMFA= 1-28, total number of mosquito

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141 midguts sampled=157) (figure 3). While both abundance and intensity were independent of
142 storage method (Incidence rates ratio for abundance (IRR)= 1.20, se=0.71, p=0. 80, IRR for
143 intensity=1.12, se=0.36, p=0.75) or duration (IRR for abundance= 0.86, se=0.1, p=0.13, IRR for
144 intensity=0.95, se=0.07, p=0.48) (Table 1), most of the random variation was generated by the
145 differences between experimental blocks with some contribution from RBC donor-specific
146 characteristics and the least from the variation among infections.

147 Gametogenesis of Cambodian isolate *P. falciparum* (CB132)

148 To determine if cryo-preservation can support infectious cultures of other strains of *P.*
149 *falciparum,* RBC donor number 4 (additional file 2b) used for the NF54 cultures above was used
150 as a substrate for sexual stage cultures of a clinical isolate of Cambodian origin (*P. falciparum*
151 CB132 [30]) following storage periods of 6 (n=35 midguts and 23 salivary glands) and 8 weeks
152 (n=49 midguts) (additional file 5). Statistical analyses were not performed due to the lack of
153 replication in the study design.

154 **Discussion**

155 Although cryogenically preserved RBCs have been shown to be suitable for culturing other
156 *Plasmodium* species, including the asexual stages of *P. falciparum*, none of the studies ran *P.*
157 *falciparum* cultures for more than four days [28, 29]. In the current study, we demonstrated that
158 in addition to supporting proliferation of the asexual stages for the first 4 days, cryo-preserved
159 RBCs were able to maintain their "qualities" as a substrate for the additional 10-12 days
160 required for progression through the various stages of gametocytogenesis. Crucially, the fitness
161 of the resulting sexually mature gametocytes was confirmed by successful gametogenesis and
162 sporogony in *An. stephensi* mosquitoes.

163 Neither cryo-preservation nor duration of storage up to 12 weeks affected the ability of
164 cryo-preserved RBCs to provide a substrate for parasite growth and differentiation of asexual

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165 stages of *P. falciparum* NF54 into mature gametocytes *in vitro*. Although rates of gametocytemia
166 were apparently higher in cryo-preserved RBCs with a decline in concentrations following a
167 peak at 12 days post-infection (figure 1), this non-linearity was accounted for by the random
168 variation between experimental blocks (Table 1). The most obvious difference between the
169 blocks was the growth rates of asexual stages in the asexual feeder flasks used to initiate the
170 gametocyte cultures in each experimental block. Although statistical models suggested a
171 positive association with the proportions of RBCs infected with parasites at the late trophozoite
172 stage and subsequent rates of gametocytemia, our study design precluded more robust
173 analyses of this relationship. Indeed, future experiments with synchronized asexual feeder
174 cultures seeded at various starting densities should help tease apart the underlying
175 relationships, manipulation of which could help design more streamlined regimes for inducing
176 gametocytogenesis. Nevertheless, the relationship between late trophozoites in asexual feeder
177 flasks and future gametocytemia has been suggested by others [13] and taken together, our
178 observations lend further support to the importance of considering parasite fitness at the
179 asexual stages and subsequent gametocytogenesis *in vitro*.

180 The rates of gametocytemia *in vitro* were not entirely reflected in the patterns of oocyst
181 prevalence of *P. falciparum* NF54 in midguts of *An. stephensi* mosquitoes. Although not
182 significant, the ability of cryo-preserved RBCs to support infectious gametocytes suggested a
183 gradual decline after 12 weeks of storage. Although this trend was likely driven by one of the
184 two biological replicates representing 12 weeks of cryo-preservation, to be conservative, we
185 suggest restricting the use of cryo-preserved RBCs to 8-weeks if the objective is to perform
186 SMFAs. Whether the observed low prevalence is a donor-specific characteristic would have to
187 be confirmed with more RBC donors representing 12 weeks of cryo-preservation, however,
188 when considered along with the other 2 infections performed within the same experimental
189 block (additional file 3), mean oocyst prevalence was lower relative to the other experimental

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190 blocks. Indeed, refitting statistical models after excluding this infection resulted in a significant
191 reduction in random RBC donor-introduced variation (0.17 in Table 1 to 0.04, remaining output
192 not shown) but less so for the experimental block (0.35 to 0.19), suggesting that in general,
193 while RBC donor characteristics may not be a significant source of variability in oocyst
194 prevalence, a blocked experimental design may warrant careful consideration for SMFAs to aid
195 robust interpretation of the results. Future experiments should help tease apart if this variability
196 between experimental blocks is the result of 1) subtle differences between mosquito
197 generations and/or 2) final parasite density and/or infectiousness which can vary significantly
198 between generations [9]. Sporozoite prevalence in the salivary glands is independent of storage
199 method and duration and largely mirrors the profiles of oocyst prevalence in the midguts, albeit
200 with negligible random variation contributed by experimental blocks or RBC donor, with the
201 majority resulting from differences between the 10 infections *per se*. However, this variability
202 was being driven primarily by 1 outlier infection where prevalence was low for one of the RBC
203 donors tested following 8 weeks of cryo-preservation, the exclusion of which was able to
204 account for most if not all the previously unexplained variability between infections after refitting
205 the model (data not shown). Similarly, neither oocyst abundance nor intensity were affected by
206 maturation of gametocytes in 1-week old refrigerated or 1 to 12-week old cryo-preserved RBCs,
207 with significant random variation across experimental blocks, infections and RBC donor. It is
208 important to note that although we follow parasitological convention in differentiating between
209 oocyst abundance and intensity [31], “intensity” has also been used to describe abundance in
210 other studies [7, 9].

211 Lastly, preliminary experiments suggest that cryo-preserved RBCs should, with further
212 refinements, support differentiation of other strains of *P. falciparum*. 6- and 8-week old cryo-
213 preserved RBCs from one of the donors previously verified to support gametocytogenesis of
214 NF54 was used to generate transmission-competent gametocytes of a Cambodian isolate of *P.*

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215 *falciparum*, CB132 (additional file 5) [30]. The lower levels of infection relative to the standard
216 laboratory isolate, NF54, could reflect differences in "infectiousness" between the two strains
217 while the reduction in oocyst prevalence at 8 weeks compared to 6 weeks could be the result of
218 random variation between experimental blocks as seen for NF54.

219 **Conclusions**

220 Gametocytogenesis of *P. falciparum* in RBCs cryo-preserved within 1 week of collection
221 retained infectivity to mosquitoes for up to 12 weeks of storage, at levels similar or better than
222 the "gold-standard" of using refrigerated 1-week old RBCs, with the observed patterns likely
223 being governed by similar rate-limiting factors as refrigerated RBCs. However, while our results
224 suggest that cryo-preservation might be suitable for culturing mature gametocytes *in vitro* for up
225 to 12 weeks, their infectiousness to mosquitoes may decline after 8 weeks for some RBC
226 donors. From an empirical perspective, the ability to culture parasites in the same donor of
227 RBCs will increase the reproducibility of SMFAs, especially in scenarios where the contribution
228 of RBC donor may warrant consideration. Further, the use of cryo-preserved RBCs for SMFAs
229 is not restricted to that of the canonical NF54 isolate of *P. falciparum* and should also be
230 applicable to other strains of *P. falciparum*, as we demonstrated for a Cambodian isolate,
231 CB132 (additional file 6). From an economic perspective, 8 weeks offers a significant
232 improvement over the current practice of using RBCs that are ≤1-week old, with the potential to
233 therefore reduce the costs by almost 7-fold. Moreover, considering how this period also aligns
234 with the 2-month duration suggested for storing blood for maintaining breeder colonies of
235 mosquitoes ([32] and unpublished observations), it is conceivable for laboratories to integrate
236 SMFAs using cryo-preserved RBCs into the general mosquito rearing schedule. For instance,
237 we routinely aliquot RBCs for cryo-preservation within 3-4 days of collection for culturing
238 parasites *in vitro* for SMFAs, with the remainder used for maintaining mosquito breeder
239 populations until receipt of RBCs from the next donor.

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240 In a broader context, if growth and differentiation of *P. falciparum* is reduced in RBCs
241 that have been refrigerated for different durations, i.e., >1-week, simultaneous comparisons with
242 cryo-preserved blood may help identify physico-chemical changes in blood that are "naturally"
243 detrimental to parasite fitness. In fact, the adverse effects of prolonged storage of refrigerated
244 RBCs in some cases has led the FDA to recommend identifying the true shelf-life of refrigerated
245 RBCs and towards this end, clinical researchers have started using "omics" technologies to
246 extensively catalog storage-related physico-chemical artefacts, some of which could indeed
247 serve as suitable starting points for comparison [23, 25]. Along the same lines, cryo-
248 preservation has the potential to offer a superior alternative to using older, refrigerated RBCs
249 where "storage-related artefacts", possibly compounded by inherent differences in RBCs
250 between donors, may influence the interpretation of assays quantifying parasite fitness and/or
251 reproducibility across labs, for instance, while comparing mutant strains or testing for drug
252 susceptibility [14]. Finally, if differences between RBC donors are reflected in the mechanisms
253 and consequences of pathogenesis for instance, cryo-preservation would provide an ideal tool
254 for replicating the observations or allow testing other parasite strains [33].

255 In summary, we anticipate our approach to be compatible with a broad range of research
256 questions involving the biology of gametocytogenesis as well as human-to-mosquito
257 transmission, with significant potential for integrating into existing research pipelines in addition
258 to identifying novel avenues for target identification and therapeutic intervention. Blocking this
259 stage of the parasite's life-cycle is predicted to be one of the last remaining hurdles to overcome
260 before we can declare a malaria-free world [1, 2], but for which SMFAs will continue to serve as
261 the penultimate reference.

262 **Materials and Methods**

263 Study design

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264 The overall study design is described below, and in the supplement accompanying this
265 manuscript (additional file 1). In general, each experimental block in the current study
266 progressed in the following manner: 1) gametocytogenesis of *P. falciparum* NF54/CB132 was
267 initiated in refrigerated (4ºC) or cryo-preserved RBCs *in vitro*, 2) cultures were maintained for
268 14-16 days, and 3) fed to mosquitoes in SMFAs to assess the efficiency of gametogenesis and
269 sporogony (additional file 2a).

270 To determine if cryo-preservation alters the ability of RBCs to support *P. falciparum*
271 NF54 gametocytogenesis and mosquito infection, a nested/fully-crossed experimental design
272 was implemented with two unique donors of RBCs serving as the basis of biological replication
273 in two independent experimental blocks (additional file 2b, "nested design") [34-36]. To account
274 for any technical/biological variation arising from unknown factors independent of RBC storage
275 method, each experimental block fulfilled three important criteria. First, mature gametocytes
276 were cultured in refrigerated (4ºC) or cryo-preserved RBCs from the same donor to ensure that
277 any differences observed were not due to inherent differences between donors. Second,
278 gametocyte flasks for comparing the two methods were initiated from the same asexual seed
279 culture since parasite fitness can vary over generations, which in turn can introduce variation in
280 induction of gametocytogenesis. Third, infectious cultures were offered to mosquitoes that were
281 sorted from the same starting population and age post-emergence to minimize any variation in
282 sporogony caused by inter-generational differences across mosquito cohorts. In summary
283 therefore, each experimental block for the comparison between ≤1-week old refrigerated and
284 cryo-preserved RBCs would consist of the following steps: 1) the same asexual seed culture
285 was used to initiate gametocytogenesis of *P. falciparum* NF54 in ≤1-week old refrigerated (4ºC)
286 or cryo-preserved RBCs *in vitro*, 2) cultures maintained and monitored in parallel for 14-16 days,
287 and 3) fed to mosquitoes from the same cohort to assess the efficiency of gametogenesis and
288 sporogony (additional file 2). Lastly, it should be noted that due to practical constraints, that the

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289 comparison between ≤1-week old refrigerated and cryo-preserved RBCs were performed after
290 the latter had been stored for 2-3 days.

291 In the second part of this study, we determined if duration of storage affects the ability of
292 cryo-preserved RBCs to support transmission-competent gametocytes by assessing
293 gametocytogenesis and infectivity to mosquitoes for parasites cultured in RBCs thawed after
294 cryogenic storage for 4, 8 and 12 weeks. Biological replication for each storage period was
295 again provided by RBC donors wherein 2-3 donors were tested independently for their ability to
296 support infectious gametocytes after cryogenic storage for 4, 8 and/or 12 weeks. To fulfil the
297 second and third criteria outlined above, we were unable to determine the effect of storage
298 duration across all RBC donors, which resulted in a partially crossed experimental design
299 (additional file 2b, "partially crossed design). In this part of the study, each experimental block
300 was defined by the following steps: 1) the same asexual seed culture was used to initiate
301 gametocytogenesis of *P. falciparum* NF54 in cryo-preserved RBCs from 2-3 donors at 4, 8
302 and/or 12 weeks of preservation with independent donors representing each storage period, 2)
303 cultures maintained and monitored in parallel for 14-16 days, with 3) mature gametocytes from
304 each flasks offered to mosquitoes from the same cohort and assessed for efficiency of
305 gametogenesis and sporogony (additional file 2).

306 <u>Chemicals and consumables</u>

307 All reagents and consumables described herein were purchased from Fisher Scientific
308 (Hampton, NH) unless noted otherwise.

309 <u>Preparation of media components</u>

310 Parasite culture media was prepared and stored as described previously [15], with minor
311 modifications. Briefly, incomplete media was prepared by dissolving pre-made RPMI-1640
312 powdered media in distilled water before adding 2% sodium bicarbonate (w/v) and 0.005%

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313 hypoxanthine (w/v, Sigma-Aldrich, St. Louis, MO). The incomplete media was then filter-
314 sterilized with 0.2 µm filters under vacuum before moving to storage at -20ºC in aliquot sizes of
315 450 or 900 mls. Complete media was prepared just prior to use by adding 50 or 100mls of 10%
316 A+ non-heat inactivated human serum (Valley Biomedical, Winchester, VA) to 450 or 900mls to
317 thawed, incomplete media respectively. Media was dispensed into 45 ml aliquots in 50 ml
318 conical-bottom tubes and the air-liquid interface sparged for 5-10 seconds with a micro-
319 aerophilic gas mixture of 5% CO_2, 5% O_2 and 90% N_2 (referred to herein as “tri-gas mixture”,
320 Airgas LLC, Kennesaw, GA) prior to storage for 1 week. Since “quality” of serum is critical for
321 culturing transmission competent parasites, serum samples were tested as described previously
322 [15]. Serum from 14 individuals was pooled into pairs and tested for their ability to support
323 gametocytogenesis as well as ex-flagellation of male gametocytes in case of *P. falciparum*
324 isolate NF54 (unpublished observations). The same pool of serum donors was used to culture
325 *P. falciparum* NF54 for the duration of this study.

326 Cryo-preservation and thawing of RBCs

327 Cryo-preservation of RBCs was performed 3-4 days after collection as suggested elsewhere
328 [27, 37]. Procedures for freezing and thawing were adapted from Sputtek [37] and Goheen et al
329 [28] with minor modifications. Two-fold concentrated stocks of cryo-protectant were prepared in
330 deionized water by warming a solution of 28% glycerol (v/v), 3% sorbitol (v/v from a 1M stock)
331 and 0.65% (w/v) sodium chloride (NaCl) prior to sterilization with a 0.2 µm filter and storage at
332 room temperature. Whole blood (~500ml units, Valley Biomedical, Winchester, VA or Interstate
333 Blood Bank, Memphis, TN) was dispensed in 45 ml aliquots and either stored refrigerated at 4ºC
334 until use (see next section) or allowed to warm to room temperature prior to cryo-preservation.
335 To ensure reproducible recovery of viable RBCs following cryo-preservation, it is critical for the
336 cryo-protectant and RBCs to be at the same temperature (e.g., room temperature), prior to use
337 [37].

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338 Aliquots of whole blood, no more than 3-4 days post-collection, were centrifuged at
339 1800xg for 10 minutes at room temperature at a low brake setting and plasma and white blood
340 cell layers aspirated under vacuum before an equal volume of cryo-protectant solution (~20-
341 25mls) was added to the packed RBC pellet to achieve a final glycerol concentration of 14%
342 (v/v) and a hematocrit of ~50%. The packed RBCs were then equilibrated with the cryo-
343 protectant for 15-20 minutes with gentle intermittent mixing. Glycerolized RBCs were then
344 dispensed in 2 ml aliquots and snap-frozen by immersing in liquid nitrogen for 2-3 minutes
345 before transferring to the vapor phase for long-term storage. Cryo-preserved RBCs were
346 thawed just before use by incubating cryogenic vials in a dry bath for 5 minutes at 37ºC before
347 transferring the contents into a 15 or 50 ml conical bottom tube prior to de-glycerolization.
348 Unless stated otherwise, all solutions for de-glycerolization were prepared in deionized water
349 and sterilized with 0.2µm filters before storage at room temperature. De-glycerolization was
350 initiated with the addition of successive gradients of sodium chloride concentrations starting with
351 0.4mls (0.2 volumes) of 12% NaCl (w/v in distilled water) added dropwise under gentle agitation
352 (~1 minute). The mixture was then incubated at room temperature for 5 minutes with gentle
353 intermittent mixing before dropwise addition of 10mls (5 volumes) of sterile 1.6% NaCl (w/v in
354 distilled water). The de-glycerolized RBC suspension was centrifuged at 1000xg for 3 minutes at
355 20-23ºC and low brakes and 20mls (10 volumes) of a salt-dextrose solution (0.9% NaCl + 0.2%
356 dextrose, w/v in distilled water) added dropwise to the packed RBC pellet under gentle agitation.
357 If hemolysis was still observed in the supernatant, the packed RBCs were washed once more
358 with the same volume of salt-dextrose solution. Finally, the thawed RBCs were resuspended in
359 complete media before use. Recoveries of ~0.8-0.9mls of packed RBCs should be considered
360 routine.

361 Preparation of refrigerated RBCs

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362 On the day of use, but ≤1-week post-collection, a 45 ml aliquot of whole blood was
363 retrieved from the refrigerator and allowed to warm to room temperature before centrifugation as
364 described in the preceding section. The plasma and white blood cell layers were aspirated
365 before the addition of an equal volume of incomplete media (~20-25mls), also at room
366 temperature. RBCs were gently suspended in incomplete media before centrifugation for 3
367 minutes at 1000xg and low brake setting. Packed RBCs were washed two more times before
368 resuspension in an equal volume of complete media to achieve a final hematocrit of ~50%.

369 Cultures of asexual stage *P. falciparum*

370 *P. falciparum* isolate NF54 was obtained from BEI Resources, NIAID, NIH ("*Plasmodium*
371 *falciparum*, Strain NF54 (Patient Line E), product number MRA-1000, contributed by Megan G.
372 Dowler"). Asexual feeder cultures were routinely sub-cultured in T25 or T75 flasks in a volume
373 of 5 or 15mls of complete media respectively, with final hematocrit of 5% and ring state
374 parasitemia ranging from 0.1-1%. The same volume of media was replaced every 24 hours and
375 then sparged gently at the air-liquid interface with the tri-gas mixture for 15-20 seconds.
376 Parasitemia was monitored every 1-2 days by transferring 50-100 µl of parasite culture to a
377 0.6ml tube, centrifuged at 1800xg for 1 minute and supernatants aspirated until final culture
378 volume was ~15-20 µl. The RBC pellet was re-suspended with repeated pipetting before
379 preparing smears with 2 µl of the concentrate on glass slides. Slides were dried on a slide-
380 warmer for 20-30 seconds and then fixed by submerging in 100% Methanol for 10 seconds prior
381 to Giemsa staining. Concentrated Giemsa stain (Sigma-Aldrich, St. Louis, MO) was filtered in
382 aliquot sizes of 30-35mls with a Whatman no. 1 filter paper and stored at room temperature.
383 Just before use, the filtered stain was diluted to a ratio of 1:20 (v/v) in phosphate-buffered saline
384 (pH 7.2) and fixed slides immersed in the stain in glass Coplin-jars for 10-15 minutes at room
385 temperature. Stained sides were washed under running tap-water for 5-10 seconds with the
386 smeared side facing downwards before being air-dried for microscopy. Smears were mounted

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387 directly in immersion oil (Cargille Labs, Cedar Grove, NJ) and examined at 1000x magnification
388 with a Leica DM2500 upright microscope (Leica Microsystems, Buffalo Grove, IL). Parasitemia
389 was estimated from ~1000 RBCs and staged into early trophozoites (referred to herein as
390 rings), late trophozoites and schizonts following guidelines described previously [13, 38]. Ring-
391 stage parasitemia expressed as a proportion of RBCs counted was used to prepare flasks for
392 routine sub-culture and/or gametocytogenesis [15].

393 Cultures of the sexual stages (gametocytes) of *P. falciparum*

394 Flasks destined for gametocytogenesis were only initiated when ring-stage parasitemia
395 in the asexual seed flasks above showed exponential increase in growth rates (>6-8 fold)
396 relative to the previous sampling point. Flasks for gametocytogenesis were prepared essentially
397 as described above. Briefly, freshly thawed or refrigerated RBCs resuspended in pre-warmed
398 (37ºC) complete media at 5% hematocrit (e.g., 1mls naïve RBCs in 20mls complete media)
399 were seeded with asexual cultures to achieve a final ring-stage density of 0.6-1% in the
400 gametocyte flasks and returned to an incubator maintained at 37ºC. Media in flasks was
401 replaced every 24 hours with 15mls of fresh pre-warmed (37ºC) complete media and sparged
402 with tri-gas mixture for 10-30 seconds. All manipulations outside the incubator were performed
403 by placing the flask on a slide-warming platform maintained at 38ºC (MedSupply Partners,
404 Atlanta, GA). Gametocyte flasks were maintained for 14-16 days and parasitemia monitored
405 with Giemsa staining at 1, 4, 8, 12 and 14 days post-infection as suggested previously [15].
406 Asexual stages were staged as described above and gametocytes were further classified into
407 stages II, III, IV and V male or V female gametocytes using guidelines established previously
408 [13, 38] (additional file 4a and S4b). Stage I gametocytes were excluded from the counts as
409 they are virtually indistinguishable morphologically from late trophozoites, especially in
410 asynchronous cultures [39]. Finally, maturation status of the culture was assessed from 12 days
411 post-infection by quantifying ex-flagellation of male stage V gametocytes in a Neubauer

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412 hemocytometer chamber as suggested previously [15]. Briefly, after adding fresh media, 10 µl of
413 culture was pipetted directly into the chambers of a hemocytometer and incubated at 21-24ºC
414 for 15-20 minutes before quantification (additional file 4c). Imaging was performed at 400x
415 magnification on a Leica DM2500 equipped with differential interference contrast (DIC) optics.
416 Cultures were deemed infectious only when ex-flagellation was observed and offered to
417 mosquitoes within 24-48 hours.

418 *P. falciparum* Cambodian isolate CB132 (kind gift of Prof. Dennis E Kyle, University of
419 Georgia, Athens, GA, USA) [30] was cultured under similar conditions to NF54 with the
420 exception that flasks destined for gametocytogenesis were seeded at a density of 0.5%.

421 Mosquito husbandry

422 *An. stephensi* mosquitoes were maintained in a level 2 Arthropod Containment Laboratory at
423 the University of Georgia, which were initiated from eggs kindly provided by the Walter Reed
424 Army Institute of Research ca. 2015 is a wild-type strain referred to as "Strain Indian" [10, 40].
425 Colonies were housed in a dedicated walk-in environmental chamber (chamber (Percival
426 Scientific, Perry, IA) at 27ºC ± 0.5ºC, 80% ±5% relative humidity, and under a 12 hr light: 12 hr
427 dark photo-period schedule. Adult mosquitoes were maintained on 5% dextrose and provided
428 whole human blood offered in glass-jacketed feeders (Chemglass Life Sciences, Vineland, NJ)
429 through parafilm membrane maintained at 37ºC to support egg production. Husbandry
430 procedures were established according to guidelines as suggested elsewhere [32] with minor
431 modifications. Briefly, eggs were rinsed twice with 1% house-hold bleach (v/v, final
432 concentration of 0.06% sodium hypochlorite) before surface-sterilization for 1 minute in the
433 same solution at room temperature. Bleached eggs were washed with 4-5 changes of deionized
434 water and transferred to clear plastic trays (34.6cm L x 21.0cm W x 12.4cm H) containing 500
435 ml of deionized water and 2 medium pellets of Hikari Cichlid Gold fish food (HikariUSA,
436 Hayward, CA) and allowed to hatch for 48 hours. Hatched L1 larvae were dispensed into clear

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437 plastic trays (34.6cm L x 21.0cm W x 12.4cm H) at a density of 300 larvae/1000 ml water and
438 provided the same diet until pupation. The feeding regime consisted of 2 medium pellets
439 provided on the day of dispensing (day 0) followed by the provision of a further 2, 4, 4 and 4
440 medium pellets on days 4, 7, 8 and 9 respectively. This regime allows >85% larval survival and
441 >90% pupation within 11 days with a sex ratio of 1:1 adult males and females (unpublished
442 observations).

443 Mosquito infections

444 All steps outlined below were performed with pre-warmed (38ºC) equipment, including
445 tubes, pipettes, pipette tips and centrifuge rotor buckets. Cultures deemed infectious were
446 concentrated by centrifugation at 1800xg for 1-2 minutes at room temperature in pre-weighed
447 15 or 50 ml conical bottom centrifuge tubes. Supernatants were aspirated under vacuum and
448 the weight/volume of the concentrate estimated by weighing the tube and subtracting the value
449 of the pre-weighed empty tube. 3-6 volumes of a freshly washed and pre-warmed mixture of
450 RBCs resuspended in freshly thawed serum (30% hematocrit) was added to the infected RBC
451 pellet to achieve a final hematocrit of 45-50% and mature gametocytemia of ~0.1%. The
452 volumes of naïve RBCs and serum mixture to be added was informed by the densities recorded
453 in the gametocyte flasks on the day of infection. The mixture of naïve and parasitized RBCs was
454 then fed immediately to 60-80 (3-7 day old) female *An. stephensi* mosquitoes for 15-20 minutes.
455 To facilitate high blood feeding rates, mosquitoes were previously starved for 16-24 hours in
456 dedicated environmental chambers programmed to fluctuate a total of 9ºC around a daily mean
457 of 24ºC with 80% ±5% relative humidity and a 12h light:dark photo-period schedule (Percival
458 Scientific, Perry, IA) as described previously [10]. After the blood-feeds, feeding status was
459 qualitatively ascertained and engorged mosquitoes selected for by extending the starvation
460 period for a further 24-48 hours to eliminate non-blood-fed mosquitoes [41].

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461 To quantify parasite densities in the infectious feed, Giemsa stained smears were
462 prepared from a 2 µl aliquot of the infectious blood meal offered to mosquitoes, as described
463 above. Prevalence and abundance of parasites in the midguts of mosquitoes was assessed at
464 9-13 days post-infection by dissecting midguts and counting oocysts at 400x magnification with
465 a Leica DM2500 under DIC optics (additional file 4d). Sporozoite prevalence was assessed at
466 17-21 days post-infection by dissecting salivary glands into 5 µl of PBS. Glands were ruptured
467 by overlaying a 22mm^2 coverslip and presence/absence of sporozoites was recorded for each
468 mosquito at either 100x or 400x magnification with the same microscope (additional file 4e).

469 <u>Statistical modeling and data analyses</u>

470 All data analyses were performed in RStudio (version 1.1.423) [42] running the statistical
471 software package R (version 3.5) [43]. Due to the experimental design (see above and figures
472 S1 and S2), generalized linear mixed-effects models (GLMMs) were used for all statistical
473 analyses, as suggested previously [34-36]. Choice of GLMM family and corresponding link
474 function were based on five dependent variables modeled- 1) rates of mature gametocytemia *in*
475 *vitro*, 2) oocyst prevalence (proportion of mosquitoes with oocysts on the midgut), 3) oocyst
476 abundance (total number of oocysts per midgut regardless of infection status), 4) oocyst
477 intensity (number of oocysts per midgut from infected midguts only), and 5) sporozoite
478 prevalence (proportion of mosquitoes with sporozoites in the salivary glands). For all the
479 analyses, storage method (refrigerated vs. cryo-preserved RBCs) and duration (4, 8, and 12
480 weeks post cryo-preservation) were specified as independent variables, with storage method
481 and duration classified as a categorical and continuous predictor, respectively. Storage duration
482 was centered and scaled over the grand mean. For analyzing temporal patterns of
483 gametocytogenesis *in vitro*, GLMMs with a binomial distribution ("logit" link) were performed with
484 the total count of RBCs infected with male and female stage V gametocytes (i.e., total mature
485 gametocytes) expressed as a proportion of the sum of RBCs (infected and uninfected) counted

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486 from each sample as the dependent variable. Oocyst and sporozoite prevalence in the midguts
487 and salivary glands of mosquitoes, respectively, were modeled as the probability of mosquitoes
488 being infected and infectious as dependent variables in GLMMs specified with a binomial
489 distribution ("logit" link function). For analyzing oocyst abundance and intensity, GLMMs with
490 negative binomial distributions ("log" link) were utilized. All statistical analyses were performed
491 using the package "lme4" unless stated otherwise [44]. For the *in vitro* analysis, the intercepts of
492 the relationships between the rates of mature gametocytemia and the predictor variables was
493 allowed to vary between RBC donors as well as over sampling period (days post-infection)
494 between experimental blocks and flasks nested within each block. The same random effect
495 structure was specified for analyzing parasite fitness in the mosquitoes except infections/SMFAs
496 were nested within blocks *in lieu* of flasks (additional file 2b). Tabulation of model outputs, along
497 with tests for overdispersion were performed with the "sjstats" package. Where applicable, post-
498 hoc comparisons of storage method and duration were performed using Tukey contrasts of the
499 estimated marginal means derived from the models above, as suggested in the "emmeans"
500 package [45].

501 Finally, cross-validation of the models were performed by 1) testing fit after truncating
502 the number of mosquitoes sampled across each infection and 3) removing experimental
503 block(s) and/or flasks/infections within each block. Where applicable, random effects were
504 checked for normal distribution patterns and the final output tables of statistical modeling
505 prepared with the "sjPlot" package and "sjstats" packages [46, 47]. All figures were prepared
506 using the "ggplot2" package [48].

507 **List of abbreviations**

508 DIC = differential interference contrast, GLMMs = generalized linear mixed-effects models, IRR
509 = incidence rates ratios, OR = odds ratios, SMFAs = small membrane feeding assays.

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654 **Figure 1:** a) Rates of mature gametocytemia of *P. falciparum* NF54 cultured *in vitro* in
655 refrigerated (continuous line) or cryo-preserved (dashed line) RBCs and b), rates of
656 gametocytemia in RBCs cryo-preserved within 3-4 days of collection from each donor and
657 thawed after 1 (closed squares), 4 (closed circles), 8 (closed triangles) and 12 weeks (closed
658 diamonds) of storage. Data represents mean ± standard errors (se).

659 **Figure 2:** Prevalence of a) oocysts and b) sporozoites in midguts and salivary glands
660 respectively of *An. stephensi* mosquitoes infected with ~0.1% mature gametocytemia of *P.*
661 *falciparum* NF54. Each data point represents an SMFA where mosquitoes were provided a
662 blood-meal spiked with mature ex-flagellation competent gametocytes collected from the flasks
663 depicted in Figure 1 and cultured in RBCs refrigerated (4ºC), cryo-preserved ("Cryo") for ≤1
664 week, or following storage for 4, 8 and 12 weeks. See additional file 2 for graphical
665 representation of the study design and additional file 4 demonstrating variation in oocyst
666 prevalence between experimental blocks.

667 **Figure 3:** Oocyst a) abundance (oocyst counts from all midguts regardless of infection status)
668 and b) intensity (oocyst counts from infected midguts only) in mosquitoes depicted in figure 2a.
669 Horizontal bars represent group means with each data point representing oocyst counts from an
670 individual mosquito midgut. For visualization purposes, counts were jittered horizontally to 40%
671 but not vertically to maintain alignment with the gradient on the y-axis.

672 **Table 1:** outputs of the statistical models

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677 **Additional file 1**. File format: “pdf”. Title of data: A schematic of the overall study design.
678 Description of data: RBCs 3-4 days old post-collection were either refrigerated at 4ºC or cryo-
679 preserved in the gaseous phase of liquid nitrogen. Aliquots of RBCs were thawed at 1, 4, 8 and
680 12 weeks and assessed for their ability to support 1) gametocytogenesis of *P. falciparum* NF54
681 *in vitro* and 2) gametogenesis *in vivo* relative to refrigerated RBCs which served as the
682 reference (dashed arrows). Black continuous arrows indicate procedures that were common to
683 all treatments.

684 **Additional file 2**: File format: “pdf”. Title of data: A detailed schematic of the experimental
685 design describing biological replication and testing regime. Description of data: a.) The
686 sequence of steps in an experimental block with design objectives in parentheses. See main
687 text for details. b.) Testing regime to demonstrate how RBCs from 4 independent donors were
688 used to compare storage method (refrigeration vs. cryo-preservation) and duration of storage.
689 The colored lines connecting each data point indicate which donors were tested within an
690 experimental block/unit. While the comparison between storage methods was performed for
691 donors 1 and 4 in a nested experimental design, the comparisons for storage duration
692 encompassed a partially crossed design with at least 2-3 donors tested at 4, 8 and 12 weeks
693 respectively with repeated sampling of 3 of the 4 total donors at various periods following cryo-
694 preservation. For instance, RBCs from donor 3 was tested following 4 and 8 weeks of cryo-
695 preservation in experimental blocks 3 and 4 respectively. Additionally, in block 3 (green lines),
696 donors 1 and 2 were also tested after cryo-preservation for 12 and 8 weeks respectively along
697 with donor 3 whose RBCs had been stored for 4 weeks by then.

698 **Additional file 3**: File format: “pdf”. Title of data: Variation between experimental blocks in the
699 prevalence of *P. falciparum* NF54 oocysts in midguts of *An. stephensi*. Description of data: Each
700 data point within an experimental block represents an individual SMFA performed within the

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701 same block as depicted in Additional file 2. Horizontal bars represent mean prevalence within
702 each block.

703 **Additional file 4**: File format: “pdf”. Title of data: Representative images used to classify the
704 various stages of *P. falciparum* NF54 during this study. Description of data: a.) Giemsa-stained
705 images of male gametocytes (1000x, oil immersion, brightfield), b.) Giemsa-stained images of
706 female gametocytes (100x, oil immersion, brightfield), c.) Ex-flagellation of gametocytes *in vitro*
707 with arrowheads depicting flagella (400x DIC, unstained), d.) An oocyst with enclosed
708 sporozoites (arrowheads, 400x, DIC, unstained), e.) ruptured salivary glands with freed
709 sporozoites (arrowheads) at 100x (i.) and 400x (ii) (DIC, unstained). Images were captured with
710 an LG G3 or Samsung Galaxy S7 smartphone using default settings (“Auto”) while attached to
711 the eyepieces on the microscope with a custom-designed apparatus. Except panel e, all images
712 were digitally magnified up to 4x for presentation purposes.

713 **Additional file 5**: File format: “pdf”. Title of data: Cryo-preserved RBCs support SMFAs with a
714 Cambodian isolate of *P. falciparum*. Description of data: a) Oocyst and sporozoite prevalence,
715 b) oocyst abundance and c) intensity of *P. falciparum* CB132 in female *An. stephensi* infected
716 with mature gametocytes of *P. falciparum* CB132 cultured in RBCs from donor 4 (Additional file
717 2) thawed following cryo-preservation for 6 (left panel) or 8 weeks (right panel). Female 3 to 5-
718 day old *An. stephensi* mosquitoes were provided an infectious blood-meal spiked with ~0.6%
719 mature gametocytes and oocyst prevalence and abundance recorded at 12 days post-infection
720 and sporozoite prevalence at 17 days post-infection. Horizontal bars represent group means
721 with each data point representing oocyst counts from an individual mosquito midgut. For
722 visualization purposes, counts were jittered horizontally to 40% but not vertically to maintain
723 alignment with the gradient on the y-axis. NA=not available.

724

Fig 1a

% mature stage V gametocytemia ± se

1.25
1.00
0.75
0.50
0.25
0.00

1 4 8 12 14

Days post-infection

RBC storage method — 4°C - - Cryo

Fig 1b

% mature stage V gametocytemia ± se

1.00
0.75
0.50
0.25
0.00

1 4 8 12 14

Days post-infection

Duration of RBC storage (in weeks) ■ 1 ● 4 ▲ 8 ◆ 12

Fig. 2a

% oocyst prevalence

100
75
50
25
0

4°C (1 week) | Cryo (1 week) | Cryo (4 weeks) | Cryo (8 weeks) | Cryo (12 weeks)

Fig. 2b

% sporozoite prevalence

100
75
50
25
0

4°C (1 week) | Cryo (1 week) | Cryo (4 weeks) | Cryo (8 weeks) | Cryo (12 weeks)

RBC storage method (storage duration in weeks)

Fig 3a

Oocyst abundance (all midguts)

0
10
20
30
40
50
60
70
80

4°C (1 week)
Cryo (1 week)
Cryo (4 weeks)
Cryo (8 weeks)
Cryo (12 weeks)

Fig 3b

Oocyst intensity (infected midguts)

1
10
20
30
40
50
60
70
80

4°C (1 week)
Cryo (1 week)
Cryo (4 weeks)
Cryo (8 weeks)
Cryo (12 weeks)

RBC storage method (storage duration in weeks)

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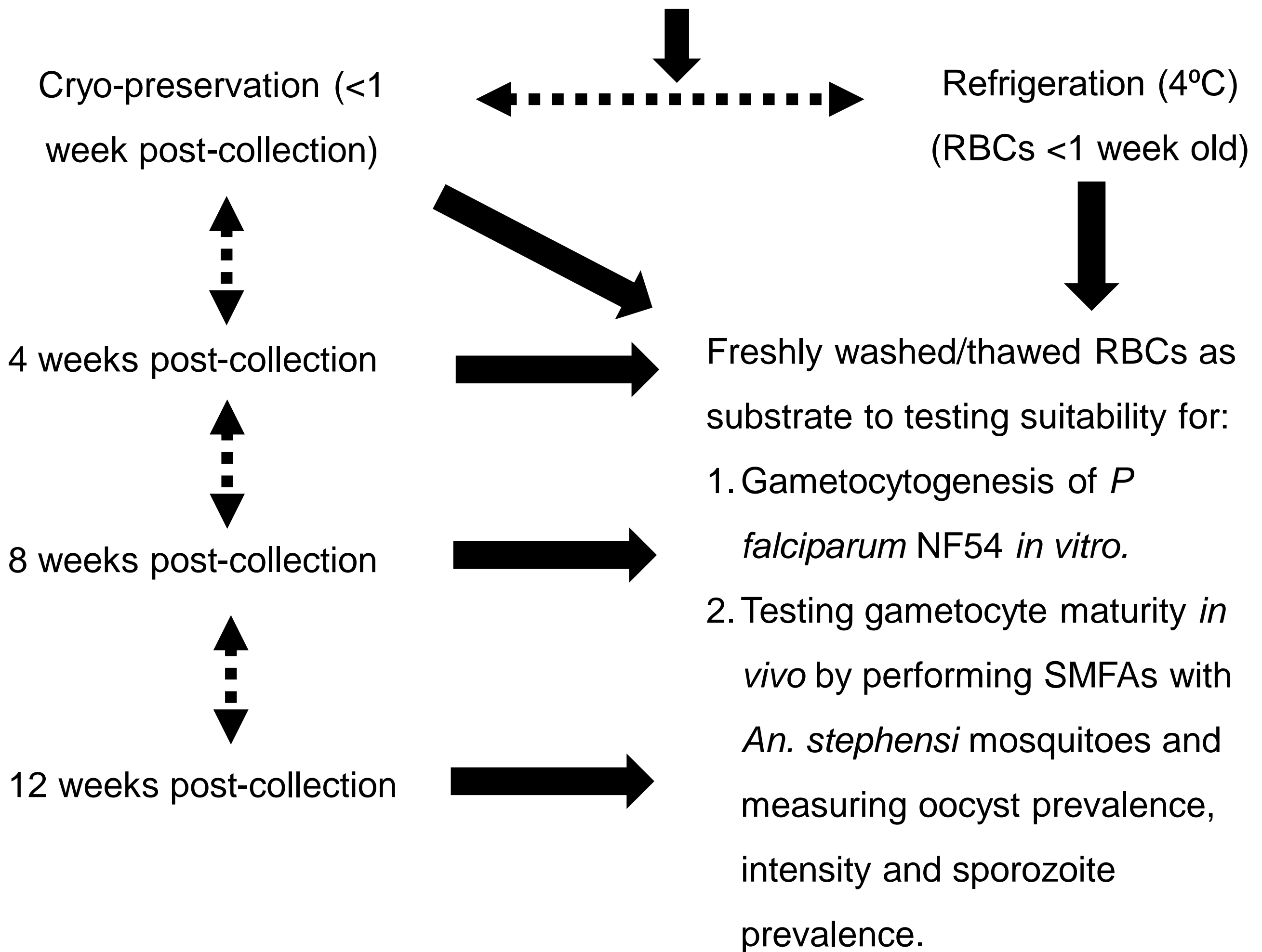
Table 1

Dependent variable →	**Gametocytemia *in vitro* (male + female stage V)**			**Oocyst prevalence in mosquito midguts**			**Sporozoite prevalence in salivary glands**			**Oocyst abundance (infected and un-infected midguts)**			**Oocyst intensity (infected midguts only)**		
Summary statistics →	IRR[a]	std. Error	*p-value*	OR[a]	std. Error	*p-value*	OR[a]	std. Error	*p-value*	IRR[a]	std. Error	*p-value*	IRR[a]	std. Error	*p-value*
Fixed Effects															
(Intercept)	0.00	0.00	<.001	1.09	0.52	0.86	0.82	0.50	0.75	1.96	0.85	0.43	5.19	0.48	<.001
4ºC RBCs vs Cryo-RBCs	1.31	0.41	0.40	1.05	0.38	0.89	1.35	0.84	0.63	1.20	0.71	0.80	1.12	0.36	0.75
Duration of storage	1.01	0.04	0.72	0.89	0.06	0.07	0.93	0.08	0.36	0.86	0.10	0.13	0.95	0.07	0.48
Experimental design															
Total flasks/mosquito infections (SMFAs), nested within experimental block	11			11			10[b]			11			11		
Total number of experimental blocks	5			5			5			5			5		
Total no. of RBC donors tested	4			4			4			4			4		
Total number of observations	51			283			235			283			157		
Random effects															
Group structure and variance for random effects															
Flasks/SMFAs nested within experimental block	0.00			0.00			0.26			0.43			0.10		
Experimental block	0.16			0.35			0.00			0.77			0.40		
RBC donor	0.00			0.17			0.02			0.46			0.21		
Overdispersion tests															
Dispersion ratio	0.98			1.23			1.00			0.75			1.07		
p-value	0.50			0.29			0.40			1.00			0.27		
Notes															
GLMM family (link)	poisson (log)			binomial (logit)			binomial (logit)			negative binomial (log)			negative binomial (log)		
Abbreviations[a]	IRR= Incidence rate ratios, NA=not applicable, OR=Odds ratios														
Comments[b]	[b]no of infections is lower as sporozoite prevalence was not available from 1 infection														

Additional file 1

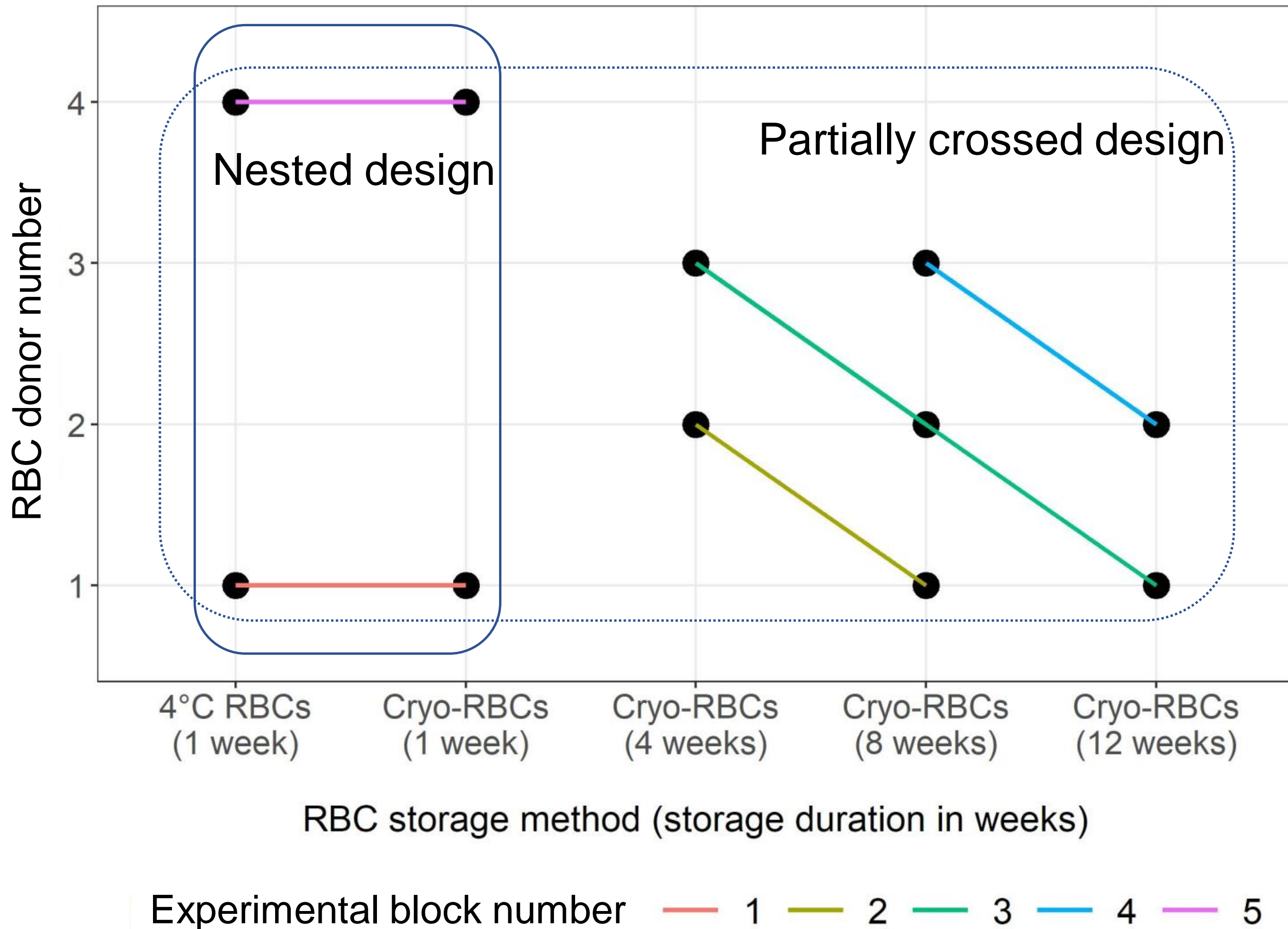


Additional file 2a

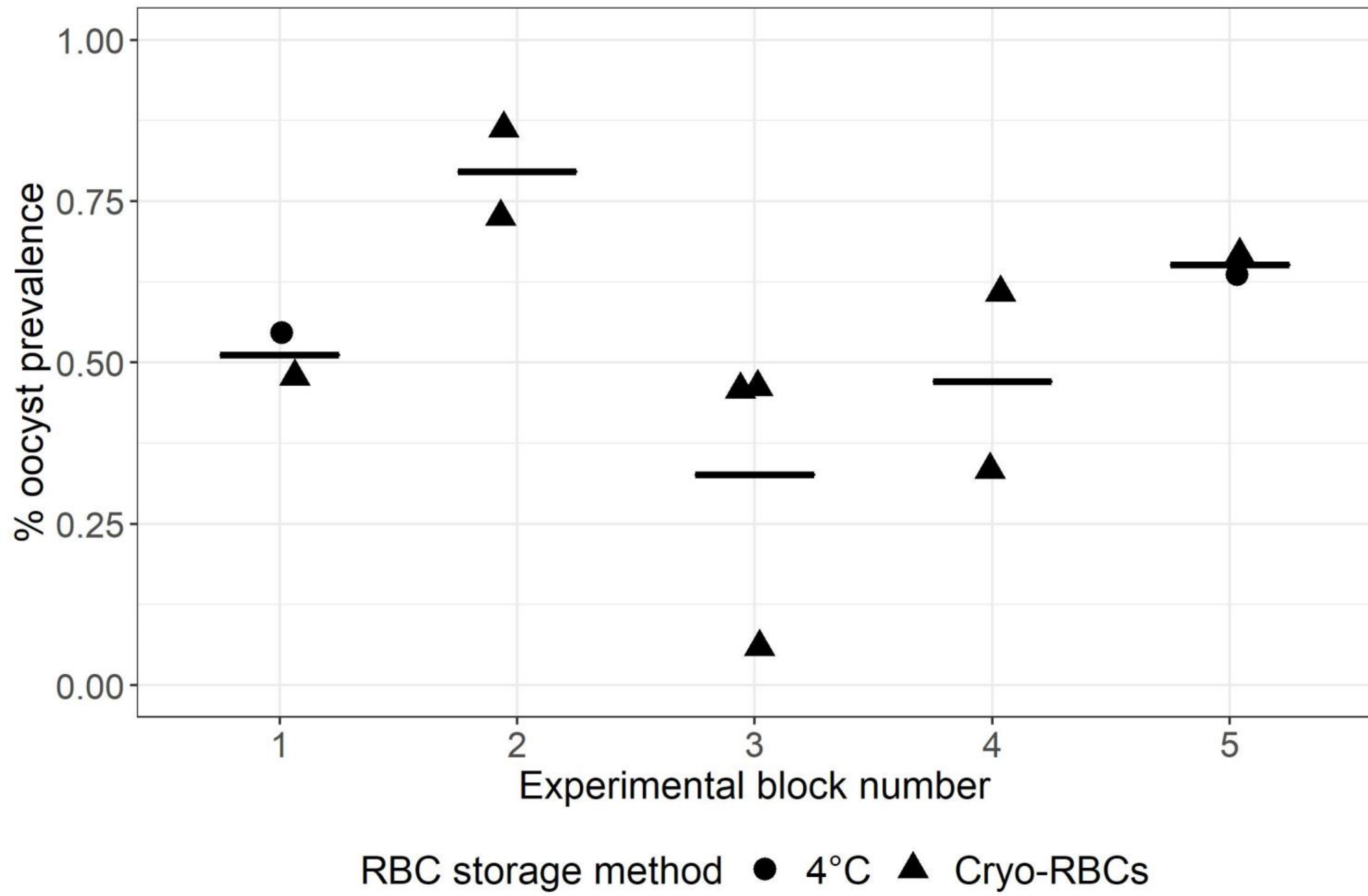
Components of an experimental block

1. Asexual cultures for 2-4 days (Same asexual seed culture/cohort)
→
2. Gametocyte flasks for 14-16 days (2-3 flasks comparing storage methods with same donor or various donors following 4, 8, or 12 weeks of cryo-preservation.
→
3. SMFAs (2-3) representing each treatment (with same mosquito cohort)

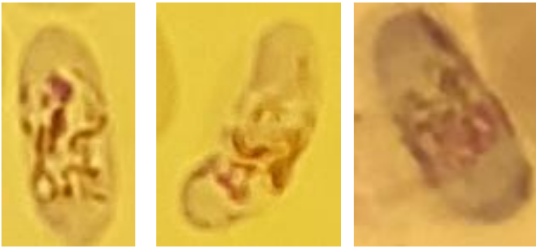
Additional file 2b



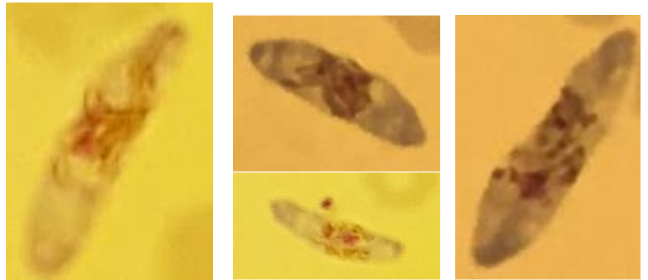
Additional file 3



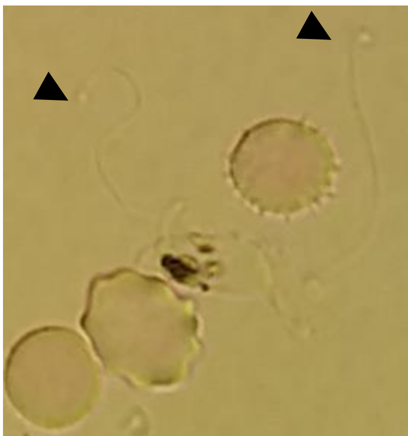
Additional file 4a



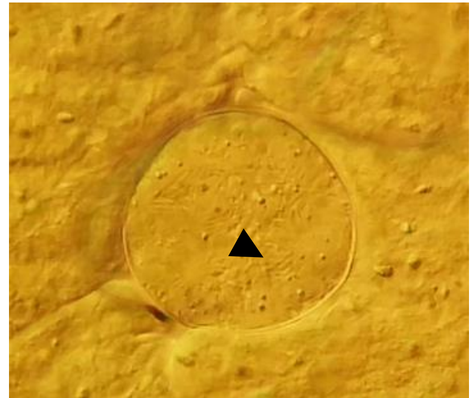
Additional file 4b



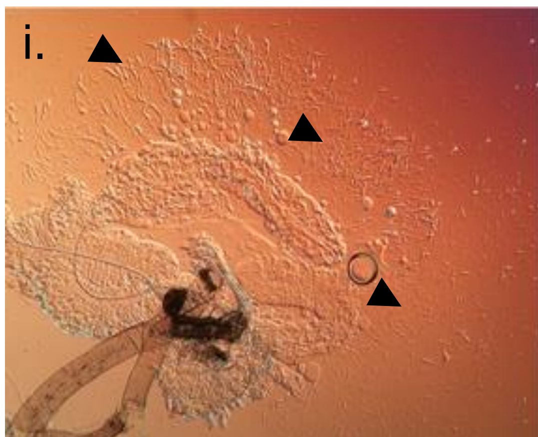
Additional file 4c

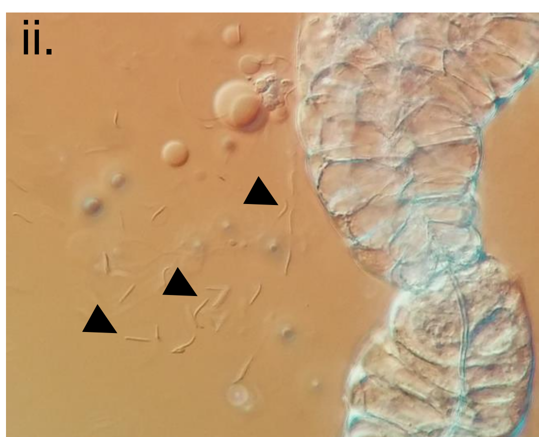


Additional file 4d



Additional file 4e

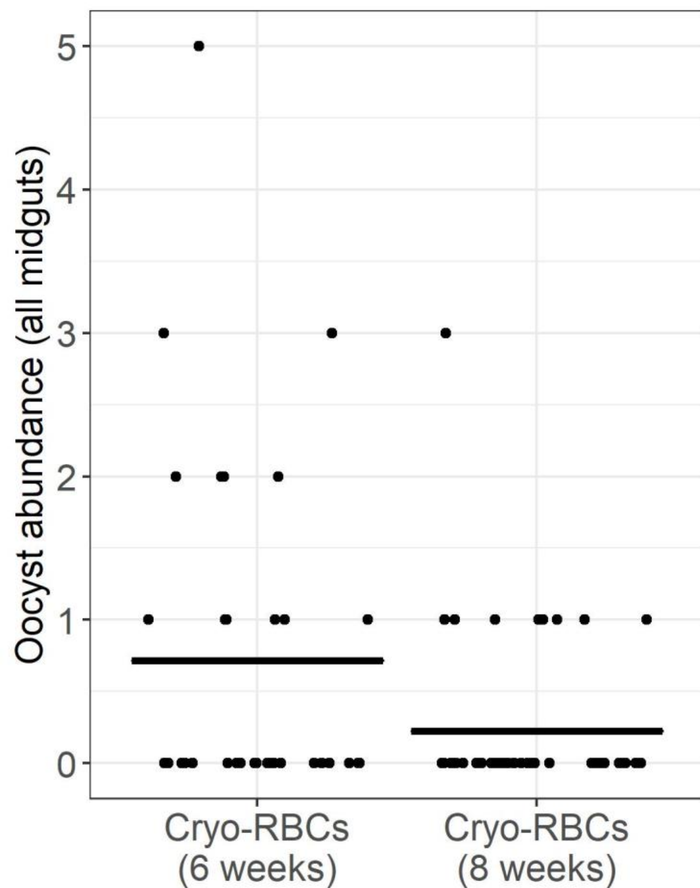




Additional file 5a

Cryo-RBCs (6 weeks)
Cryo-RBCs (8 weeks)
% prevalence
100
75
50
25
0
NA
Midguts (oocysts)
Salivary glands (sporozoites)
Midguts (oocysts)
Salivary glands (sporozoites)

Additional file 5b



Additional file 5c