

1 **Malaria parasite immune evasion and adaptation to its mosquito host**
2 **is influenced by the acquisition of multiple blood meals**

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14 **Abstract**

15 A minimum of two blood meals are required for a mosquito to acquire and transmit
16 malaria, yet *Anopheles* mosquitoes frequently obtain additional blood meals during their
17 adult lifespan. To determine the impact of subsequent blood-feeding on parasite
18 development in *Anopheles gambiae*, we examined rodent and human *Plasmodium*
19 parasite infection with or without an additional non-infected blood meal. We find that an
20 additional blood meal significantly reduces *P. berghei* immature oocyst numbers, yet does
21 not influence mature oocysts that have already begun sporogony. This is in contrast to
22 experiments performed with the human parasite, *P. falciparum*, where an additional blood
23 meal does not affect oocyst numbers. These observations are reproduced when
24 mosquitoes were similarly challenged with an artificial protein meal, suggesting that
25 parasite losses are due to the physical distension of the mosquito midgut. We provide
26 evidence that feeding compromises the integrity of the midgut basal lamina, enabling the
27 recognition and lysis of immature *P. berghei* oocysts by the mosquito complement
28 system. Moreover, we demonstrate that additional feeding promotes *P. falciparum* oocyst
29 growth, suggesting that human malaria parasites exploit host resources provided with
30 blood-feeding to accelerate their growth. This contrasts experiments with *P. berghei*,
31 where the size of surviving oocysts is independent of an additional blood meal. Together,
32 these data demonstrate differences in the ability of *Plasmodium* species to evade immune
33 detection and adapt to utilize host resources at the oocyst stage, representing an
34 additional, yet unexplored component of vectorial capacity that has important implications
35 for transmission of malaria.

36 Introduction

37 Blood-feeding is an inherent behavior of all hematophagous arthropods, providing
38 nutritional resources for development or reproduction, while enabling the acquisition and
39 transmission of a pathogen from one host to the next. This includes a number of
40 arthropod-borne diseases that influence human health, most notably malaria, which
41 causes more than 200 million infections and 400,000 deaths every year (WHO, 2018).
42 Caused by *Plasmodium* parasites, malaria transmission requires the bite of an *Anopheles*
43 mosquito, such that understanding the factors that influence vectorial capacity are integral
44 to efforts to reduce malaria transmission.

45 Following the ingestion of an infectious blood-meal, malaria parasites undergo substantial
46 development in the mosquito host as they transition from gametes to a fertilized zygote,
47 a motile ookinete, an oocyst, and a sporozoite capable of transmission to a new host
48 (Smith et al., 2014). During this approximate two week period of development (referred
49 to as the extrinsic incubation period, EIP), significant bottlenecks reduce parasite
50 numbers at each of these respective *Plasmodium* stages (Smith et al., 2014). These
51 losses are mediated in part by the mosquito innate immune system that target the
52 *Plasmodium* ookinete or oocyst through distinct immune mechanisms (Gupta et al., 2009;
53 Kwon and Smith, 2019; Kwon et al., 2017; Smith et al., 2015). However, for those
54 parasites that are able to escape immune recognition, the impact of changes to mosquito
55 physiology on the remainder of the parasite life cycle remains unknown. This includes
56 nutritional stress (i.e.-starvation and dehydration) and the potential for multiple blood
57 meals before *Plasmodium* sporozoites reach the salivary glands of the mosquito, which
58 could significantly impact the EIP and the likelihood of transmission (Ohm et al., 2018).

59 With the ability to complete a gonotrophic cycle approximately every three days,
60 mosquitoes can feed multiple times during their lifespan. Therefore, the consequences of
61 additional feeding behaviors following an initial infection are crucial to our understanding
62 of malaria transmission. In limited studies, *Plasmodium*-infected mosquitoes were more
63 likely to seek an additional blood-meal (Ferguson and Read, 2004), while others have
64 demonstrated that additional feeding increased sporozoite infection of the mosquito
65 salivary glands (Ponnudurai et al., 1989; Rosenberg and Rungsiwongse, 1991). However,

66 the impacts of a blood-meal on developing *Plasmodium* parasites have not been fully
67 explored.

68 Herein, we examine the influence of an additional blood-meal on *Plasmodium* oocyst
69 survival and development. Performing experiments with both rodent and human malaria
70 parasites, we see distinct differences in parasite survival and growth in response to
71 additional feeding. Our results suggest that *P. falciparum* oocysts have evolved
72 mechanisms to evade immune detection and to capture host resources to facilitate their
73 growth, arguing that an additional blood-meal increases the likelihood of malaria
74 transmission. Therefore, these findings provide novel insight into the host-parasite
75 interactions that determine vectorial capacity and define important new implications for
76 the role of mosquito feeding behavior in the efficacy of malaria transmission.

77 **Materials and Methods**

78 **Ethics statement**

79 The protocols and procedures used in this study were approved by the Animal Care and
80 Use Committee at Iowa State University (IACUC-18-228) and Johns Hopkins University
81 (M006H300), with additional oversight from the Johns Hopkins School of Public Health
82 Ethics Committee. Commercial anonymous human blood was used for parasite cultures
83 and mosquito feeding experiments, therefore human consent was not required.

84 **Mosquito rearing**

85 *An. gambiae* mosquitoes of the Keele strain (Hurd et al., 2005; Ranford-Cartwright et al.,
86 2016), as well as the TEP1 mutant and parental control X1 lines (Kwon et al., 2017;
87 Smidler et al., 2013) were reared at 27°C with 80% relative humidity and a 14/10 hour
88 light/dark cycle. At Iowa State University, larvae were fed fish flakes (Tetramin, Tetra),
89 while adult mosquitoes were maintained on 10% sucrose solution and fed on commercial
90 sheep blood for egg production. At the Johns Hopkins Bloomberg School of Public Health,
91 larvae were reared on a diet of fish flakes and cat food, while adult mosquitoes were fed
92 on anesthetized 6- to 8-week-old female Swiss Webster mice for egg production. The
93 Keele colony at Iowa State was derived from the Keele colony at Johns Hopkins and has
94 been independently maintained for ~4 years.

95 ***Plasmodium berghei* infection**

96 Female Swiss Webster mice were infected with *P. berghei*-mCherry strain as described
97 previously (Kwon et al., 2017; Smith et al., 2015) and maintained at 19°C with 80%
98 relative humidity and a 14/10 hour light/dark cycle. Malaria parasite infection was
99 examined by dissecting individual mosquito midguts in PBS to perform counts of
100 *Plasmodium* oocyst numbers by fluorescence microscopy (Nikon Eclipse 50i, Nikon) at
101 either eight- or ten-days post-infection.

102 ***Plasmodium falciparum* infection**

103 Three- to four-day-old female mosquitoes were fed through artificial membrane feeders
104 on an NF54 *P. falciparum* gametocyte culture in human blood as previously (Simões et
105 al., 2017). After removal of the unfed females, *P. falciparum*-infected *An. gambiae*
106 females were maintained at 27°C on a 10% sucrose solution. Midguts were dissected in
107 PBS and stained in 0.2% mercurochrome to determine oocyst numbers at eight- or ten-
108 days post-infection using a light-contrast microscope. Images were captured using an
109 optical microscope.

110 **Additional feeding challenge following *Plasmodium* infection**

111 Naïve female mosquitoes (3- to 6-day old) were initially challenged with a *P. berghei*-
112 infected mouse or a *P. falciparum*-infected blood meal. Infected mosquitoes were
113 provided with an egg cup for oviposition, then separated into two groups, one of which
114 was maintained on a 10% sucrose solution for the duration of the experiment. The second
115 group was challenged with either defibrinated sheep blood, human blood, or a protein
116 meal (consisting of 200 mg/mL of bovine serum albumin, 2 mM ATP, and 20% (v/v) food
117 dye in 1XPBS) using a glass membrane feeder at either four- or eight-days post-infection
118 to examine the effects of an additional feeding on early or mature oocysts.

119 Additional experiments performed with the TEP1 mutant and X1 lines were similarly
120 initially infected with *P. berghei*, then challenged with a naïve mouse at day 4 post-
121 infection or maintained on a sucrose diet without receiving a second blood meal. Parasite
122 numbers were evaluated by counting oocyst numbers at day 8 post-infection.

123 **Measurement of basal lamina integrity using collagen hybridizing peptide (CHP)**

124 Following blood or protein feeding, midguts were dissected from mosquitoes after feeding
125 blood meal or a protein meal at 3, 6, 18, 24, and 48 hrs. Midguts from non-fed mosquitoes
126 served as negative control, while heat-treated midguts (70°C for 10 min) were used as a
127 positive control. After dissection, the blood or protein bolus was removed, washed in
128 1xPBS, then fixed with 2% glutaraldehyde and 2% paraformaldehyde in PBS at pH 7.4
129 (Electron Microscopy Sciences) for 3 h at 4°C. Following fixation, samples were washed
130 three times in 1xPBS, then blocked overnight at 4°C in blocking buffer (5% bovine serum
131 albumin in 1xPBS). To measure the potential disruption of collagen present on the midgut
132 basal lamina, fluorescein conjugated collagen hybridizing peptide (CHP) (Echelon
133 Biosciences) was diluted in 1xPBS (1:20; 5 µM). Before use, the CHP dilution was placed
134 on a heating block at 80°C for 10 min, then chilled on ice. CHP was added to the blocked
135 midgut samples and incubated overnight at 4°C. Midguts were washed five times in PBS,
136 then mounted with ProLong®Diamond Antifade mountant with DAPI (Life Technologies).
137 Staining was visualized by fluorescence on a Nikon Eclipse 50i and captured using NIS
138 Elements (Nikon) imaging software under the same exposure settings. Micrographs were
139 used to quantify fluorescence across samples using Image J software (Schneider et al.,
140 2012).

141 **Immunofluorescence assays**

142 Mosquitoes previously infected with *P. berghei* were either maintained on 10% sucrose
143 or fed on defibrinated sheep blood at day 4 post-infection. Midguts were dissected from
144 ~24 h after additional blood feeding or from non-challenged mosquitoes. Midgut sheets
145 were prepared, removing the blood bolus. Midgut samples were washed in 1xPBS before
146 fixation in 4% PFA for 1 hour at RT. To examine basal lamina integrity based on staining
147 of the oocyst capsule, midgut samples were washed three times in 1x PBS then blocked
148 overnight in 1% bovine serum albumin (BSA)/ 0.1% Triton X-100 in 1xPBS at 4°C. Midgut
149 samples were incubated with mouse circumsporozoite protein (CSP, 1:500) and rabbit-
150 TEP1 (1:500) primary antibodies overnight in blocking buffer (1% BSA/1xPBS) at 4°C.
151 After washing in 1xPBS, midguts were incubated with Alexa Fluor 488 goat anti-mouse
152 IgG (1:500, Thermo Fisher Scientific) and Alexa Fluor 568 goat anti-mouse IgG (1:500,
153 Thermo Fisher Scientific) secondary antibodies in blocking buffer for 2 h at RT. Midguts
154 were washed three times in 1xPBS, then mounted with ProLong®Diamond Antifade

155 mountant with DAPI for visualization. To quantify TEP1 positive oocysts, 20 oocysts were
156 randomly selected from individual mosquito midguts and the percentage displaying
157 TEP1⁺ positive oocysts was recorded. Data were compiled from two independent
158 experiments.

159 **Results**

160 **Additional feeding differentially affects *Plasmodium* survival**

161 To examine the effects of an additional blood meal on rodent malaria parasite infection,
162 we first infected *An. gambiae* (Keele) with *Plasmodium berghei*, then maintained one
163 cohort on sugar, while the second received a second, naive blood meal four days post-
164 infection (Figure 1A). When oocyst numbers were examined at eight days post-infection,
165 mosquitoes receiving a second blood meal displayed significantly reduced oocyst
166 numbers when compared to those maintained on sugar alone after the initial infection
167 (Figure 1B). To determine if this effect could be attributed to blood-feeding or the physical
168 distention of the midgut that results from blood engorgement, we similarly challenged
169 mosquitoes with *P. berghei* and maintained one cohort on sugar, while a second cohort
170 was given a protein meal (2%BSA, 2mM ATP in 1xPBS) four days post-infection (Figure
171 1A). Following the additional protein feeding, oocyst numbers were similarly significantly
172 reduced (Figure 1C). Based on the minimal components of a protein meal, we argue that
173 the shared physical distention of the midgut of both treatments is responsible for this
174 dramatic reduction in *P. berghei* numbers.

175 Using a similar methodology, we also examined the influence of additional blood- or
176 protein-meal on the infection of the human malaria parasite, *Plasmodium falciparum*
177 (Figure 1D). However, an additional blood- or protein-meal did not influence *P. falciparum*
178 oocyst numbers (Figure 1E), suggesting that there are differences in the immune
179 recognition and killing of these two *Plasmodium* species in the mosquito host.

180 We also examined the temporal nature of an additional feeding, where mosquitoes
181 infected with either *P. berghei* or *P. falciparum* received an additional blood-meal eight
182 days post-infection (Figure 1F), a time in which developing oocysts have initiated
183 sporogony (Smith and Barillas-Mury, 2016). At this stage of oocyst development, an
184 additional blood meal does not influence *P. berghei* (Figure 1G) or *P. falciparum* (Figure

185 1H) oocyst numbers, which suggests there is a temporal component that determines *P.*
186 *berghei* losses.

187 **Blood- and protein-feeding degrade the midgut basal lamina**

188 *Plasmodium* oocysts develop in the space between the midgut epithelium and the midgut
189 basal lamina (Smith and Barillas-Mury, 2016), providing protection from the cellular or
190 humoral components of the mosquito immune system. To examine if an additional blood-
191 or protein-meal could influence the integrity of the basal lamina, we utilized a collagen
192 hybridizing peptide (CHP) that specifically binds unfolded collagen chains, to serve as an
193 indicator of tissue damage (Hwang et al., 2017). Collagen IV serves as a primary
194 component of the midgut basal lamina (Arrighi and Hurd, 2002; Arrighi et al., 2005; Dong
195 et al., 2017) that becomes degraded following blood-feeding (Dong et al., 2017). We
196 demonstrate that CHP stains dissected midguts shortly after blood-or protein-feeding
197 (Figure 2A), with the intensity of CHP staining reaching peak levels ~18hr after blood-
198 (Figure 2B) or protein-feeding (Figure 2C), before being repaired shortly thereafter.
199 Together, these results suggest the distention of the midgut and subsequent degradation
200 of the basal lamina enable hemolymph immune components or host resources to interact
201 with developing *Plasmodium* oocysts.

202 **Additional feeding enables TEP1-mediated killing of *P. berghei* oocysts**

203 Since an additional feeding during the immature stages of *P. berghei* oocyst development
204 limits survival (Figure 1) and feeding degrades the midgut basal lamina (Figure 2), we
205 hypothesized that the decrease in *P. berghei* numbers could be attributed to the increased
206 access of circulating immune components in the hemolymph to recognize developing
207 parasites. To address this question, we examined the ability of TEP1, a major determinant
208 of mosquito vector competence (Blandin et al., 2004; Fraiture et al., 2009; Povelones et
209 al., 2009), to recognize the newly exposed *P. berghei* parasites following blood-feeding.
210 Using immunofluorescence assays, we demonstrate that TEP1 recognition of *P. berghei*
211 oocysts requires an additional blood-meal (Figure 3A), arguing that TEP1 may play an
212 integral role in mediating these killing responses. We examined this further using a TEP1
213 knockout line of *An. gambiae* (Smidler et al., 2013), demonstrating that TEP1 is required
214 for the losses in *P. berghei* oocyst numbers following an additional blood-feeding (Figure

215 3B). This argues that the degradation of the midgut basal lamina enables TEP1 to
216 recognize and destroy *P. berghei* oocysts (Figure 3C), a stage of the parasite usually
217 protected from mosquito complement recognition (Kwon et al., 2017; Smith et al., 2015).

218 ***P. falciparum* oocysts utilize host resources provided with an additional feeding**

219 While an additional feeding does not limit human malaria parasite numbers unlike their
220 rodent malaria counterparts (Figures 1 and 3), when evaluating oocyst numbers we
221 noticed stark differences in parasite growth between *Plasmodium* species in the surviving
222 oocysts (Figure 4). *P. falciparum* oocysts are significantly larger when mosquitoes receive
223 an additional blood- or protein-meal when compared to mosquitoes maintained on
224 sucrose alone after the infectious bloodmeal (Figure 4A), arguing that human malaria
225 parasites are able to utilize host resources to accelerate their growth as previously
226 suggested (Costa et al., 2018; Werling et al., 2019). Differences in oocyst size between
227 blood- or protein-meal were not significant (Figure 4A). In contrast to these results with
228 *P. falciparum*, similar experiments with *P. berghei* did not influence oocyst size (Figure
229 4B), suggesting that rodent malaria parasites are unable to utilize the extra mosquito host
230 resources provided with an additional blood meal (summarized in Figure 4C). Taken
231 together, our results support a model in which the human malaria parasite has evolved
232 with its natural vector to evade immune recognition and to utilize host resources to
233 increase the likelihood of its transmission.

234 **Discussion**

235 Studies of vectorial capacity in *Anopheles* have traditionally focused on measurements
236 of *Plasmodium* oocyst or sporozoite numbers to evaluate the potential to transmit malaria.
237 While insightful, these predominantly lab-based studies have not adequately addressed
238 how mosquito physiology influences parasite survival and growth during the approximate
239 2-3 week extrinsic incubation period (EIP). Evidence suggests that larval nutrition
240 (Shapiro et al., 2016) and temperature (Paaijmans et al., 2010; Shapiro et al., 2017)
241 contribute to the EIP. Moreover, specific host-parasite interactions between different
242 parasite and mosquito species have been suggested to influence vectorial capacity (Ohm
243 et al., 2018; Simões et al., 2017), yet our current understanding of what defines these
244 relationships remains limited. Since mosquitoes may feed multiple times during their

245 lifespan, we hypothesized that an additional blood meal could influence parasite infection.
246 Here we demonstrate that an additional blood meal significantly impacts *Plasmodium*
247 development in terms of parasite survival and growth, with stark differences between
248 human and rodent malaria parasites to evade immune recognition and to utilize nutrients
249 provided by their mosquito host. These traits have likely evolved in *P. falciparum* through
250 interactions with *An. gambiae* as its natural vector, while the laboratory model, *P. berghei*,
251 has not been under similar selective pressures.

252 When challenged with an additional blood meal four days post-infection (the approximate
253 time to potentially find a new host after completing a gonotrophic cycle), we see a
254 dramatic reduction in the number of *P. berghei* oocysts, a phenotype recapitulated by
255 similarly feeding on a protein meal. These results suggest that the effects of *P. berghei*
256 killing are independent of the host blood meal, and more likely caused by the distention
257 of the mosquito midgut following feeding. Ingestion of a blood- or protein-meal results in
258 dramatic changes to midgut epithelium cell morphology, causing a flattening of the
259 columnar cells, the loss of microvilli, and substantial degradation of the basal lamina
260 (Dong et al., 2017; Sodja et al., 2007). This agrees with our CHP assays demonstrating
261 the presence of degraded collagen shortly after taking a blood- or protein-meal,
262 suggesting that the integrity of the basal lamina is compromised during distention,
263 enabling the exposure of developing oocysts to components of the mosquito hemolymph.

264 Experiments with a TEP1 mutant line demonstrate the involvement of TEP1 and mosquito
265 complement function in the killing of *P. berghei* oocysts following an additional blood-
266 meal, contrasting previous results arguing that TEP1-mediated killing responses only
267 target *Plasmodium* ookinetes (Blandin et al., 2004; Kwon et al., 2017; Smith et al., 2015).
268 However, our data suggest that TEP1 can only recognize *P. berghei* early oocysts once
269 the integrity of the basal lamina has been compromised by the distention of the midgut
270 following an additional feeding. This supports a model in which the mosquito basal lamina
271 serves as an integral physical barrier to protect the development of *P. berghei* from the
272 mosquito innate immune system. In addition, these data argue that TEP1 recognition is
273 temporally sensitive, where TEP1 binding and lysis is limited to the narrow time window

274 before damage to the basal lamina is repaired at either the ookinete or oocyst stages of
275 the parasite.

276 Therefore, it is of interest that an additional blood- or protein-meal does not similarly
277 influence *P. falciparum* oocysts four days post-infection, suggesting that parasites
278 surviving the transition into early oocysts can evade immune detection by mosquito
279 complement. This is supported by previous work demonstrating the role of a parasite
280 surface protein, P47, in complement evasion by *Plasmodium* ookinetes (Molina-Cruz et
281 al., 2013, 2015; Ukegbu et al., 2017). As a result, we speculate that *P. falciparum*
282 ookinetes able to evade complement recognition are also likely protected during the
283 oocyst stage.

284 In addition to evaluating the effects of additional blood-feeding on parasite survival at four
285 days post-infection, we also examined mature oocyst numbers when challenged at eight
286 days post-infection once parasites have begun sporogony. However, at this later stage of
287 parasite development, an additional blood-feeding did not affect either rodent or human
288 malaria parasites. This suggests that there are differences in recognition of early and
289 mature *P. berghei* oocysts, where only early oocysts are recognized by mosquito
290 complement following an additional blood-meal. At present, why mature *Plasmodium*
291 oocysts are no longer susceptible to killing remains unclear. This may be due to
292 differences in immune detection, or alternatively, mature oocysts may have reached a
293 stage of development in which they become immune privileged. There is support for the
294 former due to the turnover of oocyst capsule proteins at the onset of sporogony (Smith
295 and Barillas-Mury, 2016), which could potentially remove protein(s) involved in mosquito
296 complement recognition of developing oocysts.

297 Based on differences in oocyst size observed during the initial evaluations of our infection
298 experiments, we measured *Plasmodium* oocysts in response to an additional blood- or
299 protein-meal. While no differences in *P. berghei* oocyst size were detected, *P. falciparum*
300 oocysts significantly increased following additional feeding, suggesting that human
301 malaria parasites utilize the added resources provided in a blood- or protein-meal to
302 increase their growth. Previous work argues that the increase in lipid resources that
303 accompany feeding are utilized by the developing oocyst for growth and sporozoite

304 production (Costa et al., 2018; Werling et al., 2019). Moreover, an additional feeding
305 increases the number of *P. falciparum* salivary gland sporozoites (Ponnudurai et al.,
306 1989; Rosenberg and Rungsiwongse, 1991), arguing that this increased growth may
307 enhance the potential for malaria transmission.

308 The impacts of an additional blood-meal were recently described in other vector-pathogen
309 systems. In *Aedes aegypti* and *Ae. albopictus*, an additional blood-meal enhances
310 arbovirus dissemination, increasing the transmission potential of ZIKV, DENV, and
311 CHIKV (Armstrong et al., 2018). Moreover, sequential blood-feeding in sand flies leads
312 to increased *Leishmania* parasite numbers and an improved frequency of transmission
313 (Serafim et al., 2018). These examples suggest that blood-feeding is a conserved, yet
314 relatively unexplored, mechanism for pathogens to decrease the EIP in their respective
315 vector hosts.

316 Together, our experiments argue that human malaria parasites have developed the ability
317 to evade immune detection and to utilize host resources in their natural mosquito vector.
318 This in contrast to our experiments with rodent malaria parasites, which represent a non-
319 natural system widely used in laboratory studies. This argues that *Plasmodium* species
320 have evolved within their mosquito host, not only to evade immune detection as
321 previously described (Collins et al., 1986; Molina-Cruz et al., 2012, 2015), but to also
322 exploit resources provided with an additional blood-meal to accelerate their development
323 and increase the chances of transmission. As a result, we believe our findings are an
324 important advancement in our understanding of host-parasite interactions and the
325 mechanisms that define vectorial capacity for the transmission of malaria.

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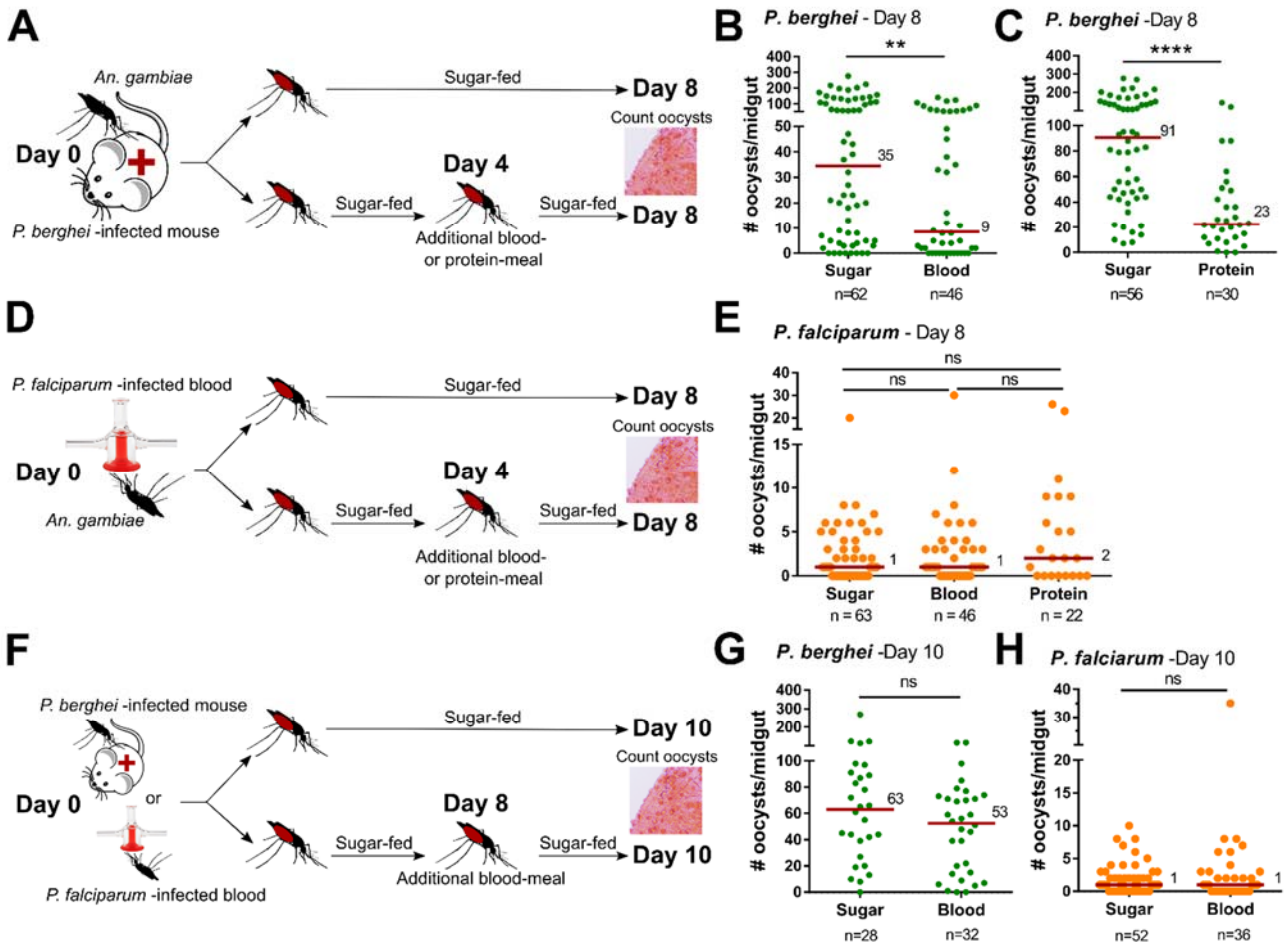
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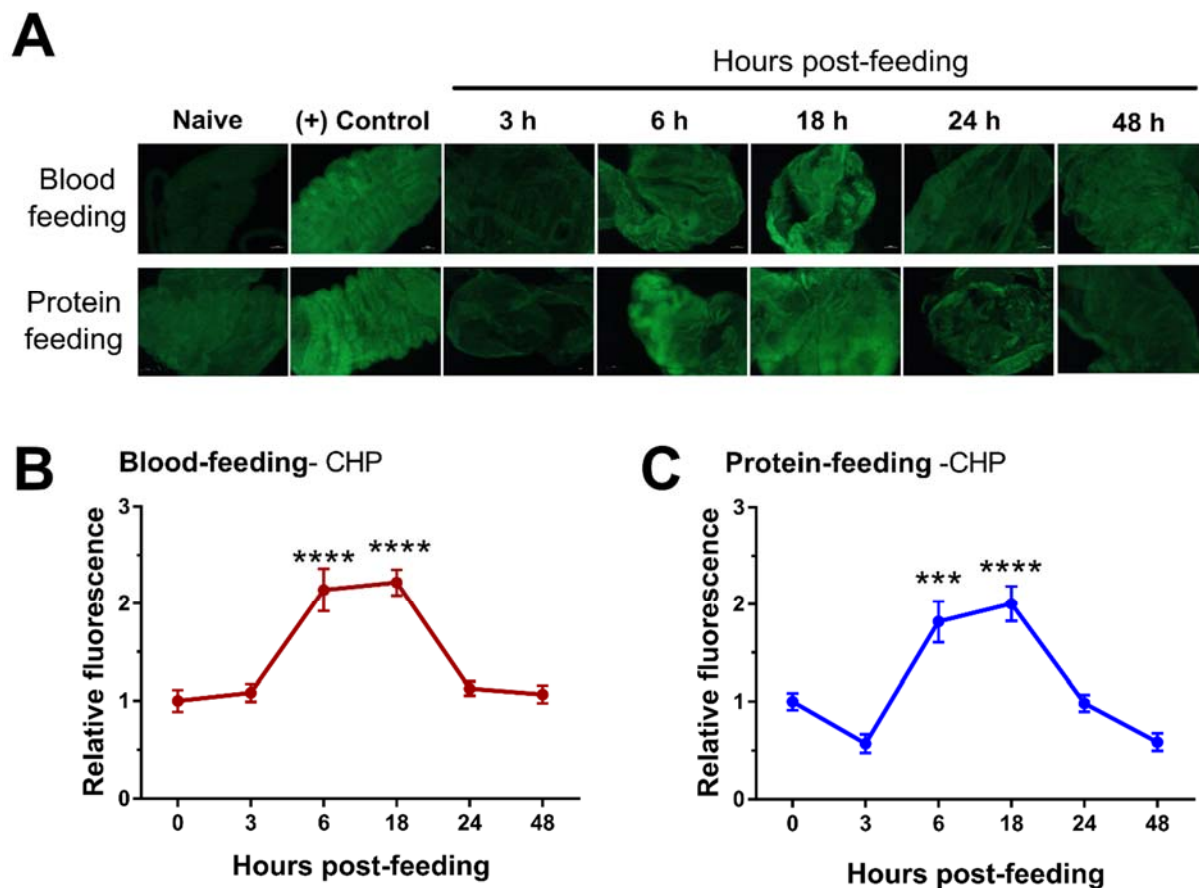


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444 **Figure 1. Additional feeding differentially impacts rodent and human malaria**
 445 **parasite survival.** Experimental overview of *An. gambiae* feeding experiments where
 446 mosquitoes were initially challenged with *P. berghei*, then either maintained on sugar or
 447 received an additional uninfected blood- or protein-meal four days post infection (A).
 448 Oocyst numbers were examined at eight days post-infection for mosquitoes receiving an
 449 additional blood- (B) or protein-meal (C). Similar experiments were performed with *P.*
 450 *falciparum* (D), where oocyst numbers were evaluated at eight days post-infection
 451 following an additional blood- or protein-meal (E). To examine potential temporal effects
 452 on survival, experiments were outlined where mosquitoes were initially infected with *P.*
 453 *berghei* or *P. falciparum* and were maintained on sugar or received an additional
 454 uninfected blood-meal eight days post infection (F). Oocyst numbers were evaluated at
 455 ten days post-infection for *P. berghei* (G) and *P. falciparum* (H). For all experiments, each

456 dot represents the number of parasites on an individual midgut, with the median value
457 denoted by a horizontal red line. Data were pooled from 3 or more independent
458 experiments with statistical analysis determined by Mann–Whitney analysis. Asterisks
459 denote significance (** $P < 0.01$, **** $P < 0.0001$). n = number of mosquitoes examined per
460 group, ns = not significant.

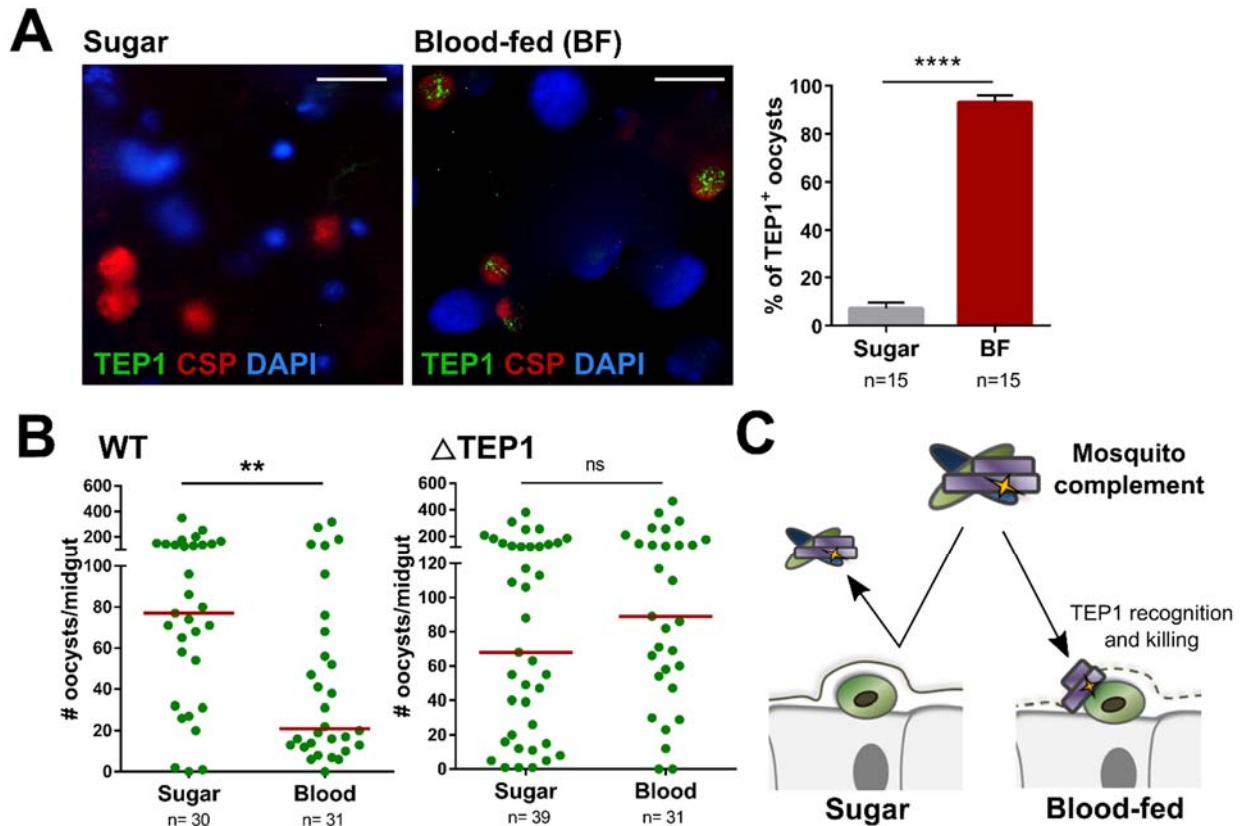
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463 **Figure 2.** Mosquito feeding promotes the degradation of the midgut basal lamina. Using
464 a fluorescein-labeled collagen hybridizing peptide (CHP) to detect degraded collagen,
465 midgut basal lamina integrity was examined temporally at 3, 6, 18, 24 and 48 hours
466 following blood- or protein-feeding (A). Heat-treated midguts (70° for 10 min. in 1x PBS)
467 were used as a (+) control sample. The CHP fluorescence signal was quantified with
468 Image J for each sample, and used to determine the relative fluorescence at each time
469 point following blood-feeding (B) or protein-feeding (C). Significance was determined
470 using a one-way ANOVA with a Holm-Sidak's multiple comparisons test for comparison
471 to the naïve (0 hr) timepoint. Asterisks denote significance (** $P < 0.001$, **** $P < 0.0001$).

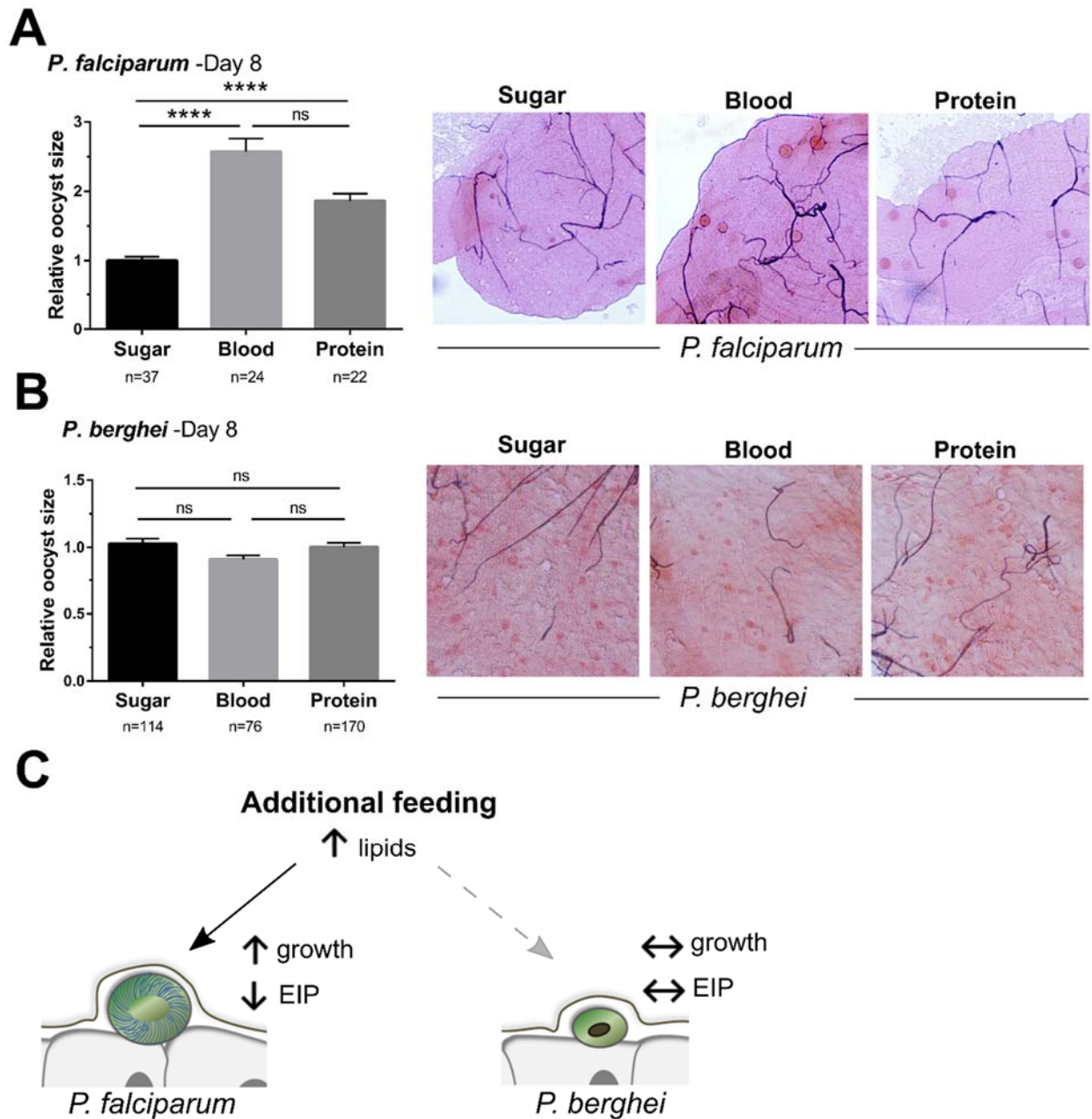
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474 **Figure 3. Additional feeding enables the recognition and killing of *P. berghei***
475 **oocysts by mosquito complement.** Immunofluorescence assays were performed to
476 examine TEP1 localization on developing oocysts when maintained on sugar or following
477 an additional blood-meal. (A). Oocysts were identified by circumsporozoite protein (CSP)
478 staining, enabling the ability to determine the percentage of TEP1⁺ oocysts from both
479 experimental conditions. Additional feeding experiments were performed on either wild-
480 type (WT) or mutant TEP1 (Δ TEP1) lines to confirm the involvement of mosquito
481 complement in oocyst recognition and killing (B). Oocyst numbers were evaluated eight
482 days post-infection. (C) Model for the role of mosquito complement via TEP1 recognition
483 and killing of *P. berghei* oocysts. All results were examined by Mann-Whitney analysis
484 where asterisks denote significance (** $P < 0.01$, **** $P < 0.0001$). Scale bar: 10 μ m. n =
485 number of mosquitoes examined, ns = not significant.

486



487

488 **Figure 4. Human malaria parasites utilize host resources provided by an additional**

489 **feeding to enhance their growth. *P. falciparum* (A) or *P. berghei* (B) oocysts were**

490 examined at eight days post-infection. The size of individual oocysts from mosquitoes

491 maintained on sugar or that received an additional blood- or protein-meal were measured

492 using Image J and compared by relative size across conditions. Representative images

493 are shown on the right. Based on growth differences and supported literature, we propose

494 a model in which human malaria parasites are able to utilize host resources to increase
495 growth and increase the chances of transmission (C). Comparisons of oocyst size were
496 analyzed using Kruskal-Wallis with a Dunn's multiple comparison test. Asterisks denote
497 significance (****P < 0.0001). n= number of oocysts examined, ns = not significant.