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

3

4 Hyeogsun Kwon[1], Rebekah A. Reynolds[1,§], Maria L. Simões[2,§], George Dimopoulos[2],
5 Ryan C. Smith[1]*

6

7 [1]Department of Entomology, Iowa State University, Ames, Iowa, USA

8 [2]Department of Molecular Microbiology and Immunology, Johns Hopkins Bloomberg
9 School of Public Health, Baltimore, Maryland, USA

10 [§]These authors equally contributed to this work.

11

12 *Corresponding author: smithr@iastate.edu

13

Abstract

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

Introduction

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

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

With the ability to complete a gonotrophic cycle approximately every three days, mosquitoes can feed multiple times during their lifespan. Therefore, the consequences of additional feeding behaviors following an initial infection are crucial to our understanding of malaria transmission. In limited studies, *Plasmodium*-infected mosquitoes were more likely to seek an additional blood-meal (Ferguson and Read, 2004), while others have demonstrated that additional feeding increased sporozoite infection of the mosquito 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.

Materials and Methods

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.

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.

Plasmodium berghei infection

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

Plasmodium falciparum infection

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

Additional feeding challenge following *Plasmodium* infection

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

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

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. Midguts 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 oocsts, 20 oocsts 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.

Results

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.

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.

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.

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.

Acknowledgements

References

334

335 Armstrong, P.M., Ehrlich, H., Bransfield, A., Warren, J.L., Pitzer, V.E., and Brackney, D.E.
336 (2018). Successive bloodmeals enhance virus dissemination within mosquitoes and
337 increase transmission potential. BioRxiv 1–26.

338 Arrighi, R.B.G., and Hurd, H. (2002). The role of *Plasmodium berghei* ookinete proteins
339 in binding to basal lamina components and transformation into oocysts. Int. J. Parasitol.
340 *32*, 91–98.

341 Arrighi, R.B.G., Lycett, G., Mahairaki, V., Siden-Kiamos, I., and Louis, C. (2005). Laminin
342 and the malaria parasite's journey through the mosquito midgut. J. Exp. Biol. *208*, 2497–
343 2502.

344 Blandin, S., Shiao, S.H., Moita, L.F., Janse, C.J., Waters, A.P., Kafatos, F.C., and
345 Levashina, E.A. (2004). Complement-like protein TEP1 is a determinant of vectorial
346 capacity in the malaria vector *Anopheles gambiae*. Cell *116*, 661–670.

347 Collins, F.H., Sakai, R.K., Vernick, K.D., Paskewitz, S., Seeley, D.C., Miller, L.H., Collins,
348 W.E., Campbell, C.C., and Gwadz, R.W. (1986). Genetic selection of a *Plasmodium*-
349 refractory strain of the malaria vector *Anopheles gambiae*. Science *234*, 607–610.

350 Costa, G., Gildenhard, M., Eldering, M., Lindquist, R.L., Hauser, A.E., Sauerwein, R.,
351 Goosmann, C., Brinkmann, V., Carrillo-Bustamante, P., and Levashina, E.A. (2018). Non-
352 competitive resource exploitation within mosquito shapes within-host malaria infectivity
353 and virulence. Nat. Commun. *9*, 3474.

354 Dong, S., Balaraman, V., Kantor, A.M., Lin, J., Grant, D.A.G., Held, N.L., and Franz,
355 A.W.E. (2017). Chikungunya virus dissemination from the midgut of *Aedes aegypti* is
356 associated with temporal basal lamina degradation during bloodmeal digestion. PLoS
357 Negl. Trop. Dis. *11*, 1–26.

358 Ferguson, H.M., and Read, A.F. (2004). Mosquito appetite for blood is stimulated by
359 *Plasmodium chaboudi* infections in themselves and their vertebrate hosts. Malar. J. *3*, 12.

360 Fraiture, M., Baxter, R.H.G., Steinert, S., Chelliah, Y., Frolet, C., Quispe-Tintaya, W.,
361 Hoffmann, J. A., Blandin, S. A., and Levashina, E. A. (2009). Two mosquito LRR proteins

362 function as complement control factors in the TEP1-mediated killing of *Plasmodium*. Cell
363 Host Microbe *5*, 273–284.

364 Gupta, L., Molina-Cruz, A., Kumar, S., Rodrigues, J., Dixit, R., Zamora, R.E., and Barillas-
365 Mury, C. (2009). The STAT pathway mediates late-phase immunity against *Plasmodium*
366 in the mosquito *Anopheles gambiae*. Cell Host Microbe *5*, 498–507.

367 Hurd, H., Taylor, P.J., Adams, D., Underhill, A., and Eggleston, P. (2005). Evaluating the
368 costs of mosquito resistance to malaria parasites. Evolution *59*, 2560–2572.

369 Hwang, J., Huang, Y., Burwell, T.J., Peterson, N.C., Connor, J., Weiss, S.J., Yu, S.M.,
370 and Li, Y. (2017). *In situ* imaging of tissue remodeling with collagen hybridizing peptides.
371 ACS Nano *11*, 9825–9835.

372 Kwon, H., and Smith, R.C. (2019). Chemical depletion of phagocytic immune cells in
373 *Anopheles gambiae* reveals dual roles of mosquito hemocytes in anti-*Plasmodium*
374 immunity. Proc. Natl. Acad. Sci. *116*, 201900147.

375 Kwon, H., Arends, B.R., and Smith, R.C. (2017). Late-phase immune responses limiting
376 oocyst survival are independent of TEP1 function yet display strain specific differences in
377 *Anopheles gambiae*. Parasit. Vectors *10*, 369.

378 Molina-Cruz, A., DeJong, R.J., Ortega, C., Haile, A., Abban, E., Rodrigues, J., Jaramillo-
379 Gutierrez, G., and Barillas-Mury, C. (2012). Some strains of *Plasmodium falciparum*, a
380 human malaria parasite, evade the complement-like system of *Anopheles gambiae*
381 mosquitoes. Proc. Natl. Acad. Sci. *109*, E1957–E1962.

382 Molina-Cruz, A., Garver, L.S., Alabaster, A., Bangiolo, L., Haile, A., Winikor, J., Ortega,
383 C., van Schaijk, B.C.L., Sauerwein, R.W., Taylor-Salmon, E., et al. (2013). The human
384 malaria parasite Pfs47 gene mediates evasion of the mosquito immune system. Science
385 *340*, 984–987.

386 Molina-Cruz, A., Canepa, G.E., Kamath, N., Pavlovic, N. V, Mu, J., Ramphul, U.N.,
387 Ramirez, J.L., and Barillas-Mury, C. (2015). *Plasmodium* evasion of mosquito immunity
388 and global malaria transmission: The lock-and-key theory. Proc. Natl. Acad. Sci. U. S. A.
389 *112*, 15178–15183.

390 Ohm, J.R., Baldini, F., Barreaux, P., Lefevre, T., Lynch, P.A., Suh, E., Whitehead, S.A.,
391 and Thomas, M.B. (2018). Rethinking the extrinsic incubation period of malaria parasites.
392 Parasit Vectors *11*, 1–9.

393 Paaijmans, K.P., Blanford, S., Bell, A.S., Blanford, J.I., Read, A.F., and Thomas, M.B.
394 (2010). Influence of climate on malaria transmission depends on daily temperature
395 variation. Proc. Natl. Acad. Sci. U. S. A. *107*, 15135–15139.

396 Ponnudurai, T., Lensen, A.H.W., Van Gemert, G.J.A., Bensink, M.P.E., Bolmer, M., and
397 Meuwissen, J.H.E.T. (1989). Sporozoite load of mosquitoes infected with *Plasmodium*
398 *falciparum*. Trans. R. Soc. Trop. Med. Hyg. *83*, 67–70.

399 Povelones, M., Waterhouse, R.M., Kafatos, F.C., and Christophides, G.K. (2009).
400 Leucine-rich repeat protein complex activates mosquito complement in defense against
401 *Plasmodium* parasites. Science *324*, 258–261.

402 Ranford-Cartwright, L.C., McGeechan, S., Inch, D., Smart, G., Richterová, L., and
403 Mwangi, J.M. (2016). Characterisation of species and diversity of *Anopheles gambiae*
404 Keele colony. PLoS One *11*, e0168999.

405 Rosenberg, R., and Rungsiwongse, J. (1991). The number of sporozoites produced by
406 individual malaria oocysts. Am. J. Trop. Med. Hyg. *45*, 574–577.

407 Schneider, C.A., Rasband, W.S., and Eliceiri, K.W. (2012). NIH Image to ImageJ: 25
408 years of image analysis. Nat. Methods *9*, 671–675.

409 Serafim, T., Coutinho-Abreu, I. V, Oliviera, F., Meneses, C., Kamhawi, S., and
410 Valenzuela, J.G. (2018). Sequential blood meals promote *Leishmania* replication and
411 reverse metacyclogenesis augmenting vector infectivity. Nat. Microbiol. *3*, 548–555.

412 Shapiro, L.L.M., Murdock, C.C., Jacobs, G.R., Thomas, R.J., and Thomas, M.B. (2016).
413 Larval food quantity affects the capacity of adult mosquitoes to transmit human malaria.
414 Proc. R. Soc. B Biol. Sci. *283*.

415 Shapiro, L.L.M., Whitehead, S.A., and Thomas, M.B. (2017). Quantifying the effects of
416 temperature on mosquito and parasite traits that determine the transmission potential of
417 human malaria. PLoS Biol. *15*, 1–21.

418   Simões, M.L., Mlambo, G., Tripathi, A., Dong, Y., and Dimopoulos, G. (2017). Immune
419   regulation of *Plasmodium* is *Anopheles* species specific and infection intensity
420   dependent. MBio *8*, 1–13.

421   Smidler, A.L., Terenzi, O., Soichot, J., Levashina, E. A., and Marois, E. (2013). Targeted
422   mutagenesis in the malaria mosquito using TALE nucleases. PLoS One *8*, 1–9.

423   Smith, R.C., and Barillas-Mury, C. (2016). *Plasmodium* oocysts: overlooked targets of
424   mosquito immunity. Trends Parasitol. *32*, 979–990.

425   Smith, R.C., Vega-Rodríguez, J., and Jacobs-Lorena, M. (2014). The *Plasmodium*
426   bottleneck: malaria parasite losses in the mosquito vector. Mem. Inst. Oswaldo Cruz *109*,
427   644–661.

428   Smith, R.C., Barillas-Mury, C., and Jacobs-Lorena, M. (2015). Hemocyte differentiation
429   mediates the mosquito late-phase immune response against *Plasmodium* in *Anopheles*
430   *gambiae*. Proc. Natl. Acad. Sci. *112*, E3412-20.

431   Sodja, A., Fujioka, H., Lemos, F.J.A., Donnelly-Doman, M., and Jacobs-Lorena, M.
432   (2007). Induction of actin gene expression in the mosquito midgut by blood ingestion
433   correlates with striking changes of cell shape. J. Insect Physiol. *53*, 833–839.

434   Ukegbu, C.V., Giorgalli, M., Yassine, H., Ramirez, J.L., Taxiarchi, C., Barillas-Mury, C.,
435   Christophides, G.K., and Vlachou, D. (2017). *Plasmodium berghei* P47 is essential for
436   ookinete protection from the *Anopheles gambiae* complement-like response. Sci. Rep. *7*,
437   6026.

438   Werling, K., Shaw, W.R., Itoe, M.A., Westervelt, K.A., Marcenac, P., Paton, D.G., Peng,
439   D., Singh, N., Smidler, A.L., South, A., et al. (2019). Steroid hormone function controls
440   non-competitive *Plasmodium* development in *Anopheles*. Cell *177*, 1–11.

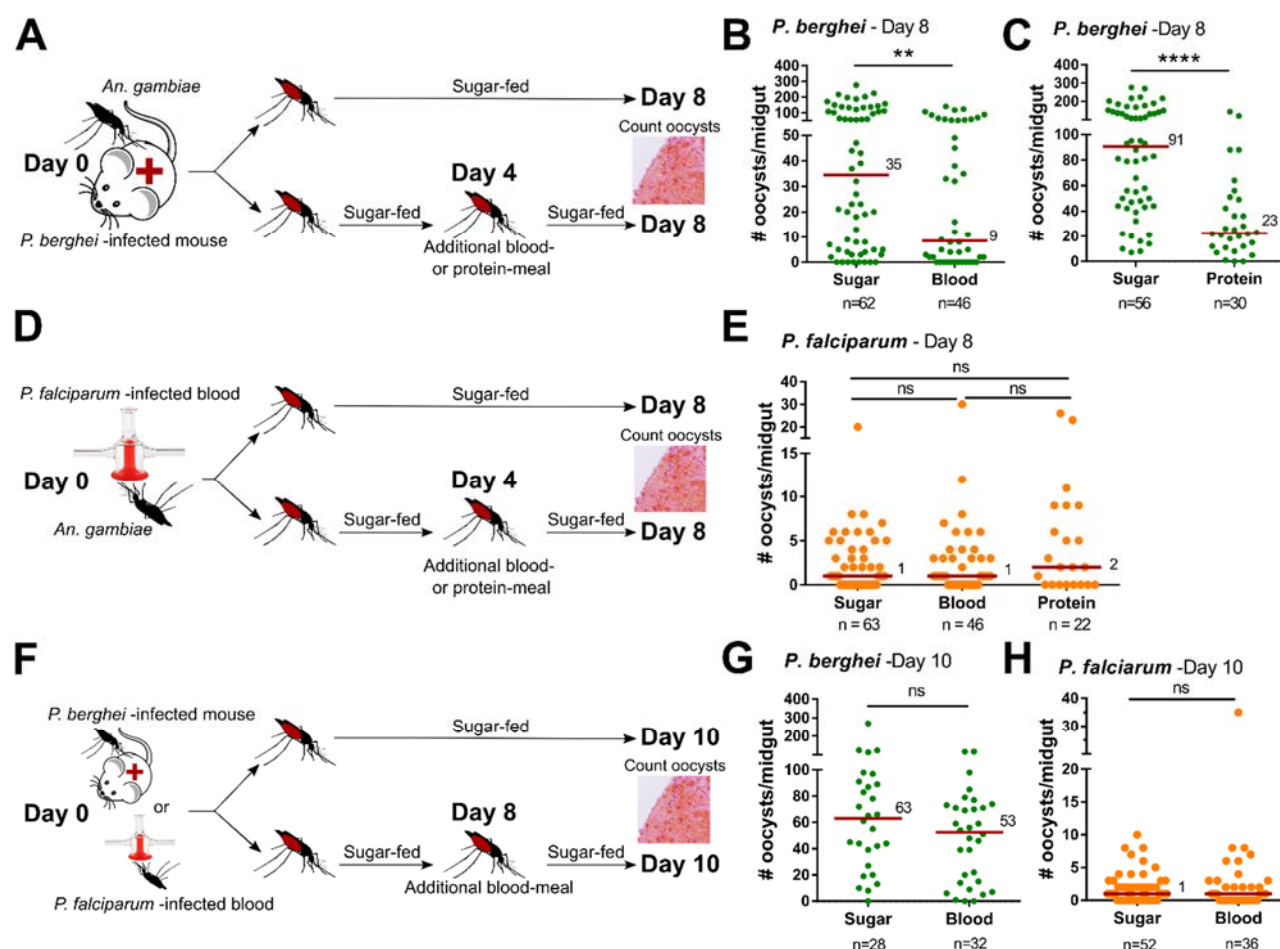441   WHO (2018). World Malaria Report. 2018. ISBN 978 92 4 156469 4.

442

443

Figure 1. **Additional feeding differentially impacts rodent and human malaria parasite survival.** Experimental overview of *An. gambiae* feeding experiments where mosquitoes were initially challenged with *P. berghei*, then either maintained on sugar or received an additional uninfected blood- or protein-meal four days post infection (A). Oocyst numbers were examined at eight days post-infection for mosquitoes receiving an additional blood- (B) or protein-meal (C). Similar experiments were performed with *P. falciparum* (D), where oocyst numbers were evaluated at eight days post-infection following an additional blood- or protein-meal (E). To examine potential temporal effects on survival, experiments were outlined where mosquitoes were initially infected with *P. berghei* or *P. falciparum* and were maintained on sugar or received an additional uninfected blood-meal eight days post infection (F). Oocyst numbers were evaluated at 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
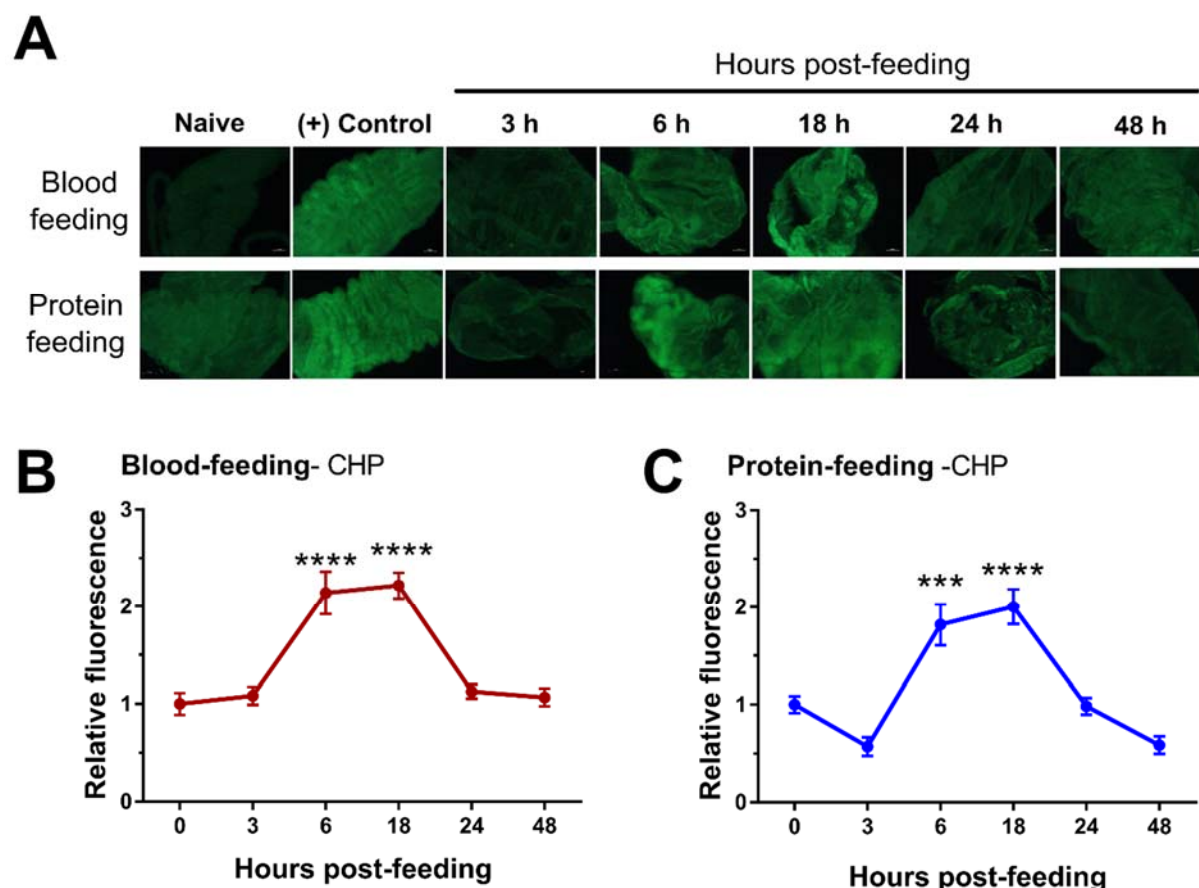
460   group, ns = not significant.

461

Figure 2. Mosquito feeding promotes the degradation of the midgut basal lamina. Using a fluorescein-labeled collagen hybridizing peptide (CHP) to detect degraded collagen, midgut basal lamina integrity was examined temporally at 3, 6, 18, 24 and 48 hours following blood- or protein-feeding (A). Heat-treated midguts (70° for 10 min. in 1x PBS) were used as a (+) control sample. The CHP fluorescence signal was quantified with Image J for each sample, and used to determine the relative fluorescence at each time point following blood-feeding (B) or protein-feeding (C). Significance was determined using a one-way ANOVA with a Holm-Sidak's multiple comparisons test for comparison to the naïve (0 hr) timepoint. Asterisks denote significance (***$P < 0.001$, ****$P < 0.0001$).
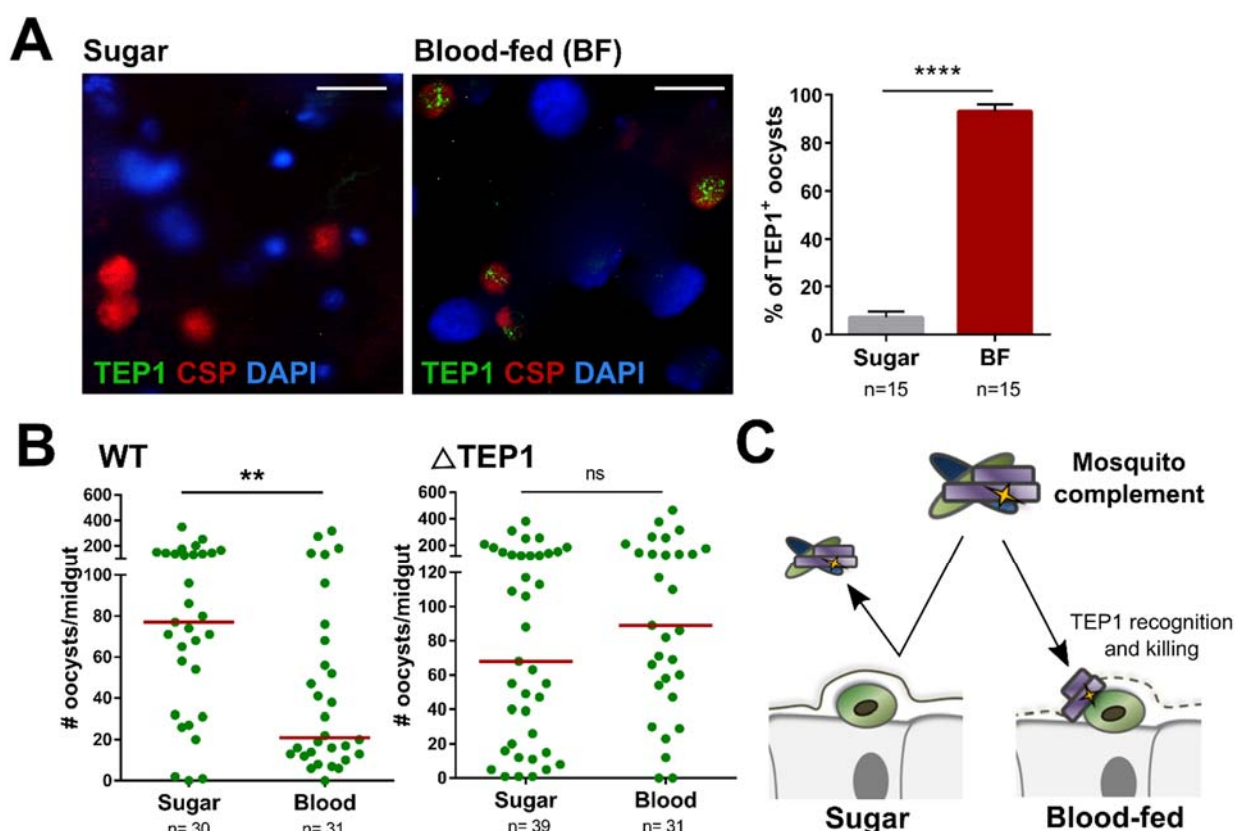
Figure 3. **Additional feeding enables the recognition and killing of *P. berghei* oocysts by mosquito complement.** Immunofluorescence assays were performed to examine TEP1 localization on developing oocysts when maintained on sugar or following an additional blood-meal. (A). Oocysts were identified by circumsporozoite protein (CSP) staining, enabling the ability to determine the percentage of TEP1+ oocysts from both experimental conditions. Additional feeding experiments were performed on either wild-type (WT) or mutant TEP1 (ΔTEP1) lines to confirm the involvement of mosquito complement in oocyst recognition and killing (B). Oocyst numbers were evaluated eight days post-infection. (C) Model for the role of mosquito complement via TEP1 recognition and killing of *P. berghei* oocysts. All results were examined by Mann-Whitney analysis where asterisks denote significance (**$P < 0.01$, ****$P < 0.0001$). Scale bar: 10 μm. n = number of mosquitoes examined, ns = not significant.

486

487
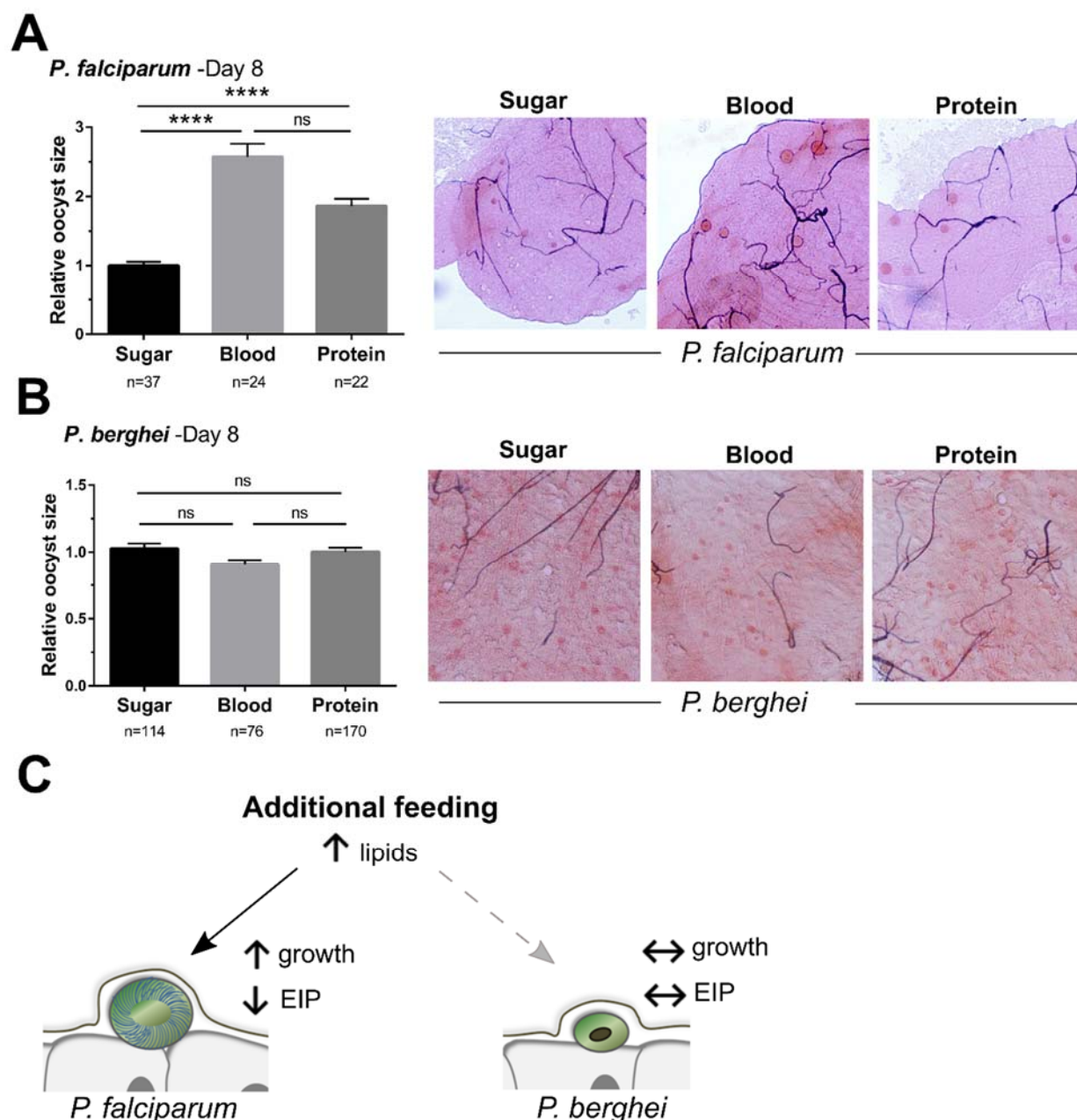
Figure 4. **Human malaria parasites utilize host resources provided by an additional feeding to enhance their growth.** *P. falciparum* (A) or *P. berghei* (B) oocysts were examined at eight days post-infection. The size of individual oocysts from mosquitoes maintained on sugar or that received an additional blood- or protein-meal were measured using Image J and compared by relative size across conditions. Representative images 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.