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ABSTRACT

Plasmodium vivax is now the predominant species causing malarial infection and disease in most non-African areas, but little is known about its transmission efficiency from human to mosquitoes. Because the majority of *Plasmodium* infections in endemic areas are low density and asymptomatic, it is important to evaluate how well these infections transmit. In this study, using direct membrane feeding assays on *Anopheles dirus*, we assessed *P. vivax* infectivity from 94 naturally infected individuals who had parasitemia spanning 4 orders of magnitude. We found that the mosquito infection rate is positively correlated with blood parasitemia and that infection begins to rise when the parasite density is >10 parasites/μl. Below this threshold, mosquito infection is rare and associated with very few oocysts. These findings provide useful information for assessing the human reservoir of transmission and for establishing diagnostic sensitivity required to identify individuals who are most infectious to mosquitoes.

Keywords:

malaria, transmission, *Plasmodium vivax*, *Anopheles dirus*, infectivity, membrane feeding assay

1. Introduction

Over the past decade, malaria incidence has steadily declined in various parts of the worlds. In many places where *P. falciparum* and *P. vivax* coexist, including South America, South East Asia and Western Pacific, the latter has now become the predominant species (Imwong et al., 2015; Oliveira-Ferreira et al., 2010; Rodriguez et al., 2011; Waltmann et al., 2015). The resilience of *P. vivax* relative to *P. falciparum* against malaria controls can be attributed, at least partially, to the parasite's ability to remain dormant as hypnozoites in the host liver (Krotoski et al., 1982; Robinson et al., 2015; White et al., 2014) and its greater transmission efficiency (Boyd, 1937; Pethleart et al., 2004). *P. vivax* thus poses a great challenge for malaria eradication. Because of the lack of an *in vitro* culture system that produces infectious gametocytes, information about *P. vivax* transmission efficiency is limited, and has mostly relied on direct or membrane feeding experiments using blood from human malaria infections (Gamage-Mendis et al., 1993; Rios-Velasquez et al., 2013; Sattabongkot et al., 2003; Sattabongkot et al., 1991; Vallejo et al., 2016; Zollner et al., 2006).

Several studies have reported that the majority of *P. vivax* infections in the endemic areas of Asia, South America, and Oceania are asymptomatic (Baum et al., 2015; Harris et al., 2010; Imwong et al., 2015; Vasquez-Jimenez et al., 2016; Waltmann et al., 2015) and submicroscopic (Cheng et al., 2015), even in areas where malaria transmission intensity has declined. Previous studies have also shown that blood from both *P. vivax* patients and asymptomatic carriers can infect South East Asian vector *Anopheles dirus* (Coleman et al., 2004; Pethleart et al., 2004; Sattabongkot et al., 2003; Sattabongkot et al., 1991), but the relationship between *P. vivax* parasitemia and the mosquito infection rate is often described as weak if not absent (Bharti et al., 2006; Coleman et al., 2004; Gamage-Mendis et al., 1991; Graves et al., 1988). At present, the relative contributions to transmission of asymptomatic and symptomatic *P. vivax* infected populations remain unclear. Such information is important for improving the current disease control and elimination programs. If

asymptomatic carriers are contributing substantially to transmission, then malaria interventions will need to also target these carriers to be effective.

To determine how well *P. vivax* transmits from man to mosquitoes and to assess the contribution of asymptomatic carriers to transmission, here we performed membrane feeding experiments on *An. dirus* using blood samples from both *P. vivax* malaria patients and asymptomatic carriers. These samples covered a broad range of parasitemia, from submicroscopic to 10,000 parasites/µl.

2. Materials and methods

2.1 Study sites

The study was conducted in Tha Song Yang District of Tak Province and Sai Yok District of Kanchanaburi Province in western Thailand between 2014 and 2015. Both areas were mountainous and residents were composed mainly of Thai and Karen ethnicities. The main occupations of the participants were farmers. Malaria in the study areas was seasonal, with the major peak season lasting from May to August, and a secondary peak in November to December. The prevalence of *P. vivax* and *P. falciparum* in Tha Song Yang in 2011-2012 was approximately 10% and 3.7% by PCR (Parker et al., 2015). Four anopheline species were recently found to be infected by *Plasmodium* in this area, including *An. maculatus*, *An. minimus*, *An. annularis* and *An. barbirostris* (Sriwichai et al., 2016). The prevalence of *P. vivax* and *P. falciparum* in our study site in Sai Yok was 3.8% and 1.4 % by PCR in 2012 (Nguitragool et al., submitted). No information is currently available about the vectors in this area.

2.2 Enrollment for membrane feeding experiments

Enrollment was limited to ≥13 years old local residents whose blood was determined positive for *Plasmodium* parasites. Participants were enrolled all year round either at local malaria clinics where *P. vivax* was first identified by light microscopy, or through mass blood surveys of the general

village population by genus-specific LAMP (Han et al., 2007) or qRT-PCR (Wampfler et al., 2013). During the enrollment process, body temperature of each participant was recorded by an infrared thermometer. The history of recent malaria infection was obtained through interview. We classified symptomatic individuals as those who felt sick with malaria-like symptoms (fever, headache, or chill) or had temperature > 37.5°C at the time of the blood survey. Asymptomatic carriers were *Plasmodium* positive individuals whose temperature at the time of blood survey was < 37.5°C and reported absence of fever during the preceding two weeks. Informed consent or assent was obtained from each participant as well as his/her legal guardian if the participant was < 18 years old. This study was approved by the Ethics Committees of Mahidol University and Pennsylvania State University.

In total, 222 individuals were included in the final data analysis. Individuals who were evidently infected with *P. falciparum* were excluded. The 222 participants comprise 93 individuals who were infected with *P. vivax* (70 symptomatic, 23 asymptomatic), 22 individuals who were infected with *Plasmodium* parasites of unknown species, and 107 individuals whose blood was virtually free of *Plasmodium* by genus-specific LAMP and qRT-PCR (details below).

2.3 Blood collection

Five milliliters of venous blood were collected from each participant in a heparinized tube. From each whole blood sample, 200 µl blood was collected for parasite species identification by species-specific LAMP assays (Han et al., 2007). A second portion of 200 µl was mixed with 1 ml RNAprotect Cell Reagent (Qiagen) and stored at -80°C for RNA extraction and purification. A second portion of thick and thin blood smears were prepared in duplicates using 1 µl whole blood for each spot for microscopic examination. The rest of the sample was kept at 37°C and used in the membrane feeding experiment within 4 h of collection.

2.4 Microscopic examination of blood smears

Thick and thin smears were prepared from whole blood. Thin smear was fixed with methanol, while thick smear was left unfixed, before being stained with 10% Giemsa solution for 10 minutes and examined for *Plasmodium* species and developmental stages. Parasite and gametocyte densities were determined by counting the entire 1 µl blood spots.

2.5 LAMP assays

Genus-specific as well as species-specific (*P. falciparum* or *P. vivax*) LAMP assays (Han et al., 2007) were performed to detect *Plasmodium* parasite infection in blood samples from the cross-sectional surveys. To perform these LAMP assays, 150 µl of distilled water was added to 50 µl blood and boiled for 5 minutes. The sample was then centrifuged for 3 minutes at 13,000 ×g. A total volume of 5.5 µl of the supernatant containing genomic DNA was used as the template in a 25 µl LAMP reaction, using the Loopamp DNA amplification kit (Eiken Chemical Co) and an appropriate primer set as previously described (Han et al., 2007; Sattabongkot et al., 2014). Only samples that were free of *P. falciparum* were included in the final data analysis. The limits of the LAMP assays for *P. falciparum* and *P. vivax* were 100 copies/reaction (Han et al., 2007)."

2.6 qRT-PCR analyses

RNA was extracted from 100 µl of whole blood in 500 µl RNAprotect Cell Reagent and eluted with 50 µl elution buffer, following the instruction of the RNeasy Plus 96 kit (Qiagen). A genus-specific qPCR assay, QMAL (Wampfler et al., 2013), using 4 µl of purified RNA as template, was performed on all RNA samples to ensure no contamination of genomic DNA.

For blood samples from cross-sectional surveys, 4 µl of purified RNA was subjected to genus-specific, as well as *P. vivax*-specific, qRT-PCR assays targeting the 18S rRNA transcripts. The primer and probe sequences for this assay have been described previously (Rosanas-Urgell et al., 2010).

To detect *P. vivax* gametocytes, 4 µl of purified RNA was used as the template in qRT-PCR to amplify the *pvs25* transcripts (Wampfler et al., 2013). Copy numbers were determined from an in-plate standard curve prepared by 10-fold serial dilution of a plasmid harboring the target sequence. Values are reported as copies per µl of equivalent blood volume. To ensure that oocysts in mosquitoes were not due to *P. falciparum* co-infection, qRT-PCR for *pfs25* transcripts was also performed on all samples (Wampfler et al., 2013). Samples positive for *pfs25* transcripts were excluded from the final analysis. The detection limits of qRT-PCR for *pvs25* and *pfs25* were 12 copies/reaction (Nguitragool et al., submitted).

2.7 Mosquito rearing

A colony of *An. dirus* was maintained in the insectary of Mahidol Vivax Research Unit, Faculty of Tropical Medicine, Mahidol University in Bangkok, Thailand. The colony was established in 2011 from the original colony at Armed Force Research Institute of Medical Sciences, Bangkok Thailand. The mosquito has been genetically typed and formally identified as *An. dirus* (originally dirus A, Bangkok strain). The mosquitoes were reared at 27°C (± 1°C), 80% (± 10%) relative humidity, and with a 12 h day/night cycle. For membrane feeding experiments, 200 female mosquitoes/carton were placed inside an insulated plastic cooler and ground transported from Bangkok to the field sites in Tha Song Yang and Sai Yok. Only 5-7 day-old mosquitoes were used for membrane feeding assays. Mosquitoes were starved of sugar for 6 h before blood feeding. Approximately 400 starved mosquitoes were used for each blood sample.

2.8 Membrane feeding assays

Each blood sample was prepared for membrane feeding assays (Sattabongkot et al., 2015) with and without serum replacement. Serum replacement was performed to evaluate the effect of plasma components on mosquito infection. To replace the serum, the original blood was centrifuged at 500 ×g for 3 minutes, and the pellet was washed once with the RPMI medium before mixing with an

186 equal volume of AB-serum from a malaria naïve donor. To feed 200 female mosquitoes, 1 ml of
187 blood was added to a water-jacketed glass membrane feeder covered with a Baudruche membrane
188 and maintained at constant 37°C with a circulating-water system to prevent the transition of
189 gametocytes to gametes. Mosquitoes were allowed to feed for 30 minutes. All unfed mosquitoes
190 were removed and killed by freezing. Engorged mosquitoes were maintained with 10% sucrose
191 solution until transportation back to the insectary of Mahidol Vivax Research Unit in Bangkok.
192 Mosquito infection with *Plasmodium* oocysts was determined 7-9 days after blood feeding. At
193 least 30 mosquito midguts were dissected, stained with 0.05% mercurochrome, and examined for
194 oocysts under microscope. Each dissected midgut was counted once by an experienced
195 microscopist. The distribution of oocyst counts from infective cases of *P. vivax* can be found in
196 Supplementary Fig S1.
197
198 *2.9 Data analysis*
199 Data were analyzed with Graphpad Prism v.6.07, PASW Statistics v.18, and SigmaPlot v.13. For
200 each blood sample, infection rate (% mosquito infected) = 100 × number of mosquitoes with at least
201 one oocyst/total dissected. Oocyst density (oocysts/mosquito) = total number of oocysts found in all
202 mosquitoes / total mosquitoes dissected. Paired Wilcoxon signed rank test was used to compare the
203 median of the mosquito infection rates or of the mean oocyst densities between the feeding assays
204 with and without AB-serum replacement. Spearman's rank correlation coefficient (ρ) was used to
205 determine the correlation between parasite infection rates or oocyst density with parasite densities,
206 gametocyte density, and *pvs25* transcript abundance. Trend lines in Fig 2 represent 3-parameter
207 logistic regression. Trend lines in Fig 3 are thin plate regression splines whose smoothing
208 parameters were estimated with the generalized cross validation criteria. The analysis was
209 performed in R using the mgcv package.
210
211 *2.9 Modeling the relationship between the mosquito infection rate and the oocyst density*

To describe the relationship between the infection rate and the oocyst density, we assume that the number of oocysts in each midgut (N) arises from two types of ookinetes: i) ones that breach the peritrophic matrix (PM) and mature to oocysts (n_1) and ii) the 'free riders' that cross the PM through an opening generated by the first type of parasites (n_2). We further assume that n_1 is proportional to the ookinete density in the blood meal, and that n_2 scales linearly with the square of the ookinete density, following an empirical second order rate of collision between ookinetes and holes in the PM. Therefore, the number of oocysts in each midgut is: $N = n_1 + n_2 = n_1 + \alpha n_1^2$ where α is an arbitrary constant. If the relationship between the infection rate r (percent) and n_1 follows Poisson statistics, that is $r = 100(1 - e^{-n_1})$, then the relationship between r and N is $r = 100(1 - e^{-\frac{\sqrt{1+4\alpha N}-1}{2\alpha}})$.

3. Results

We performed membrane feeding experiments on 222 blood samples (Table 1). The demographic information of the blood donors as well as the raw membrane feeding data are available in Supplementary Table S1. In total, 94 of the samples were *P. vivax*-positive by light microscopy, Pv-LAMP, or qRT-PCR. 21 samples were *Plasmodium*-positive by genus-specific LAMP or qRT-PCR, but the species were indeterminate due to low parasitemia. The last 107 samples were parasite negative by all diagnostics used (Table 1). None of the 222 samples was positive for *P. falciparum* by Pf-LAMP or *pfs25* qRT-PCR. Of the 94 *P. vivax* positive samples, 70 were from symptomatic individuals and 24 were from asymptomatic individuals. Only 4 of the 24 *P. vivax* asymptomatic infections were positive by light microscopy, all having very low parasitemia in the range 1 – 8 parasites/μl; the other 20 asymptomatic infections were positive for *P. vivax* only by LAMP and/or qRT-PCR.

Because the original blood plasma may contain components that interfere with transmission (Sattabongkot et al., 2003), we performed two feeding experiments on the majority of samples, one with the original plasma and the other after plasma had been replaced with AB-blood group serum

238 from a malaria-naïve person. Due to the limited blood volumes, we could only perform the feeding
239 assay without serum replacement for some samples.
240 Without serum replacement, 84% (59/70) of symptomatic *P. vivax* blood samples were infective
241 (i.e. at least one mosquito became infected with oocysts), with nearly 50% of all 4,389 mosquitoes
242 became infected. In contrast, none of the 23 asymptomatic *P. vivax* samples, and none of the 22
243 indeterminate *Plasmodium* samples, led to infection in the 4,955 mosquitoes dissected.
244 Interestingly, 4% (4/107) of whole blood samples and 5% (5/99) of serum replaced blood samples
245 which were negative for *Plasmodium* by both LAMP and qRT-PCR resulted in mosquito infection.
246 However, the infection rate (0.07%) was vastly lower than that of symptomatic blood (49.33%) and
247 all infected mosquitoes only had 1 – 2 oocysts.
248 AB-serum replacement did not have a big impact on the number of infective cases as well as in
249 the proportion of mosquitoes infected (Table 1). At the level of individual blood samples, AB-
250 serum replacement had variable effects (Fig. 1). It increased the infection rate (% mosquito
251 infected) and/or the mean oocyst density for some samples, but decreased these parameters for
252 others. In line with our findings here, similar blocking and enhancing effects of natural immunity
253 against sexual stages on transmission have been reported (Mendis et al., 1990). When data from all
254 paired samples were compared, the median of the infection rates ($p = 0.003$, $N = 86$) as well as that
255 of the mean oocyst densities ($p = 0.018$, $N = 86$) increased slightly.
256
257 *3.1 An. dirus infection as a function of P. vivax density in blood*
258 Among the blood samples with microscopically patent parasites, the blood parasite density was
259 positively correlated with the infection rate ($\rho = 0.386$, $p = 0.001$, $N = 70$; Fig. 2A). Positive
260 correlation was also found between the infection rate and gametocyte density ($\rho = 0.362$, $p = 0.003$,
261 $N = 65$; Fig. 2C) or *pvs25* transcript abundance ($\rho = 0.499$, $p = 5 \times 10^{-6}$, $N = 75$; Fig. 2E). Despite
262 the large sample-to-sample variation, the relationships between the infection rate and the parasite,
263 gametocyte, and *pvs25* transcript abundance are apparent when the moving median is plotted

against these density measures. The relationship appears to be sigmoidal, with the infection rate beginning to rise sharply at >10 parasites/µl or >1 gametocyte/µl. The trends also became smoother after AB-serum replacement (Fig. 2B, D, F).

The mean oocyst density calculated from all dissected mosquitoes (infected and uninfected) generally increased with blood parasite density ($\rho = 0.432$, $p = 2 \times 10^{-4}$, $N = 70$; Fig. 3A), gametocyte density ($\rho = 0.424$, $p = 4 \times 10^{-4}$, $N = 65$; Fig. 3C), and *pvs25* transcript abundance ($\rho = 0.602$, $p = 1 \times 10^{-8}$, $N = 75$; Fig. 3E). However, in the highest decade of parasitemia or gametocytemia, a reduction in oocyst density was observed (Fig. 3A, C, E), perhaps reflecting inhibition by plasma components. Consistently, AB-serum replacement abrogated this inhibition, leading to monotonically increasing relationships (Fig. 3B, D, F).

3.2 Relationship between the mosquito infection rate and the mean oocyst density

The infection rate was strongly correlated with the mean oocyst density ($\rho = 0.825$, $p = 9 \times 10^{-16}$, $N = 59$; Fig. 4). At low parasite densities, the relationship closely followed the prediction of independent formations of individual oocysts. However, at higher parasite loads, the data deviated from this *Poissonian* prediction – the mean oocyst density was higher than expected. These results thus suggest some form of parasite-parasite interaction that becomes more prominent at higher parasite density.

4. Discussion

We used membrane feeding assays to examine mosquito infectivity of blood samples from acute *P. vivax* patients, asymptomatic carriers, and individuals living in the endemic areas who appeared to be free of blood-stage parasites. Although the relationship between *P. vivax* parasitemias and infection rates has often been described as weak or absent (Bharti et al., 2006; Coleman et al., 2004; Gamage-Mendis et al., 1991; Graves et al., 1988), we could detect significant positive correlation between *P. vivax* parasitemia and infection in *An. dirus*. We also found that parasitemia is as good a

290 predictor of infectivity as gametocytemia is, consistent with a previous report that for *P. vivax*
291 parasitemia and gametocytemia are tightly linked (Koepfli et al., 2015). That gametocyte counts did
292 not yield a stronger correlation with mosquito infectivity may also be due to our inability to
293 differentiate fully mature gametocytes from early non-infectious stages of gametocytes. The
294 probability that a single *An. dirus* would become infected with *P. vivax* from a symptomatic blood
295 meal was approximately 50%, similar to the previous report of 43% when the mosquito was
296 allowed to feed directly from the skin of symptomatic men in Thailand (Sattabongkot et al, 1991),
297 alleviating the concern that the membrane feeding assay may not represent natural infection.
298 Similar infection rates (45-60%) were also measured in *An. aquasalis* and *An. albitarsis* in
299 Brazillian Amazon and *An. albimanus* in Colombia by membrane feeding using patient blood
300 (Rios-Velasquez et al., 2013; Solarte et al., 2011; Vallejo et al., 2016). However, direct comparison
301 between membrane feeding and skin feeding remains an important step in the future to fully assess
302 how well the membrane feeding assay reflects natural infection, especially by asymptomatic
303 carriers whose parasitemia are generally very low and whose parasite distribution in the skin may
304 have great impact on mosquito infection.
305
306 We did not detect any infection among the 4,835 mosquitoes that had fed on the 23
307 asymptomatic *P. vivax* blood samples. This is likely due to the very low parasitemia of these
308 samples; 19 of them had submicroscopic parasite density (i.e. <1 parasite/µl for this study) and four
309 had only 1-8 parasites/µl. However, 5% of samples from qRT-PCR/LAMP negative participants
310 resulted in mosquito infections. These observations are consistent with the 10-100 parasites/µl
311 threshold for robust mosquito infection (Fig. 1A). Because of the limited sample size as well as the
312 skewed age distribution towards adults (13-59 years), these 23 samples may not represent the true
313 distribution of asymptomatic *P. vivax* parasitemias in the population. Interestingly, a recent
314 membrane feeding study from Colombia using *An. albimanus* found that 57% (8/14) of
315 submicroscopic asymptomatic blood samples led to mosquito infection at 2.5-5% infection rates

316 (Vallejo et al., 2016). These values are much higher than ours. We do not know the reason for this
317 discrepancy, but it is possible that different mosquito species are differently susceptible to the
318 parasite. Several anopheline species have been found our study area, including, but not limited to,
319 *An. maculatus*, *An. minimus*, *An. annularis*, *An. barbirostris, and An. dirus* (Sriwichai et al., 2016).
320 However, it still remains unclear which species are the main drivers of malaria transmission. We
321 used *An. dirus* in this study because it has high vectorial competence and wide geographical
322 distribution in South East Asia (Manguin et al., 2008). Our infectivity data here should therefore be
323 used judiciously in the context of malaria control programs where a different or multiple mosquito
324 species contribute to transmission.
325
326 Through a series of malaria cross-sectional surveys conducted in several endemic villages in
327 northwestern Thailand and western Cambodia, Imwong and coworkers recently estimated the
328 overall prevalence of *P. vivax* to be ~17% (Imwong et al., 2016). They also determined the *P. vivax*
329 parasitemia distribution in these populations to be unimodal and log normal, peaking at 5
330 parasites/µl. Assuming this distribution, 14% of all asymptomatically infected individuals are
331 predicted to have >100 parasites/µl, the density at which efficient *An. dirus* infection was observed
332 (Fig. 2). This translates to 2.4% of the population (i.e. 14% of the 17% asymptomatic *P. vivax*
333 prevalence) being able to transmit. This number of infectious asymptomatic individuals exceeds
334 that of the symptomatic malaria patients at any given time. It thus follows that, if *An. dirus* is
335 representative of Southeast Asian vectors, asymptomatic *P. vivax* carriers should contribute
336 substantially to transmission. The outdoor biting behaviors of *An. dirus* (Tananchai et al., 2012),
337 which favor transmission from physically active individuals, can only enhance the contribution of
338 the asymptomatic carriers relative to that of the symptomatic patients.
339
340 Although the threshold for *P. v*ivax transmission in *An. dirus* appears to be 10-100 parasites/µl,
341 we believe that it is important for targeted elimination approaches to employ a diagnostic tool that

342 can detect lower parasite densities. This is because 1) parasitemia in asymptomatic carriers can
343 fluctuate and reach the transmissible densities if left untreated and 2) other local mosquito species
344 may be more susceptible to infection that *An. dirus*. Our data suggested that microscopy and
345 commercial malaria rapid diagnostic tests, which typically have the limit of detection of roughly 50
346 and 100-200 parasites/µl respectively (Ochola et al., 2006; WHO, 2012), will likely miss a
347 significant portion of the infectious reservoir. Molecular diagnostics such as low-volume LAMP
348 and PCR, which can detect a few parasites/µl (Cordray and Richards-Kortum, 2012), should fare
349 much better in the programmatic settings. They should be able to identify the large majority of
350 currently transmitting individuals. However, even people with no detectable *P. vivax* blood stage
351 infection can harbor hypnozoites and may thus experience relapsing *P. vivax* infection and
352 contribute to transmission (Robinson et al., 2015). Programs aiming at vivax malaria elimination
353 will thus not only need to identify people that can transmit now, but also address the silent
354 hypnozoite reservoir and reintroduction of the parasite through migration.
355

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Legends to Figures

Fig 1. Distribution of fold changes (log_2 transformed) in the infection rate (A) and the mean oocyst density (B) between experiments with AB-serum replacement (AB) and the orignal whole blood (WB). A positive value indicates enhancement by serum replacement. A negative value indicates inhibition by serum replacement. Only experiments with non-zero oocysts in both experimental arms were included.

Fig 2. Relationship between the mosquito infection rate and *P. vivax* parasite, gametocyte, and *pvs25* transcript densities in blood. Circles represent the infection rates of individual feeding experiments. Trend lines are 3 parameter logistic regression; the shaded areas represent the 95% confidence intervals. A, C, E: experiments without AB-serum replacement. B, D, F: experiments with AB-serum replacement. nd, not detected.

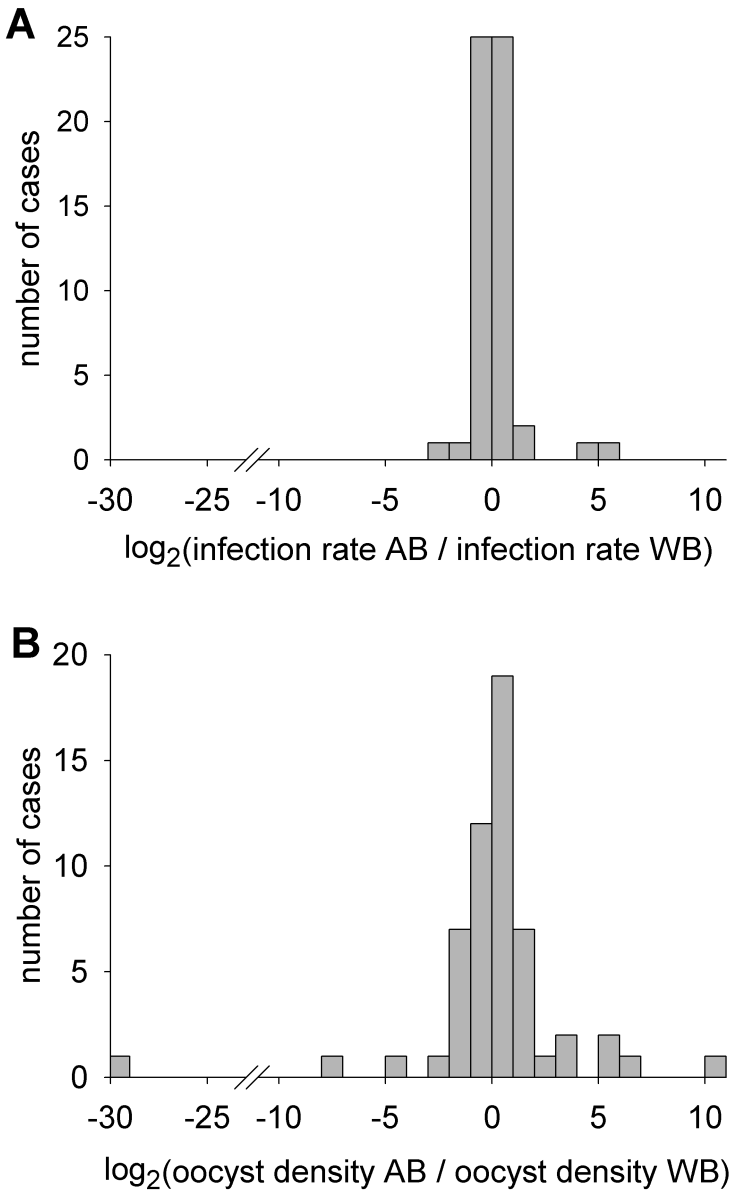
Fig 3. Relationship between the mean oocyst density of each membrane feeding experiment and *P. vivax* parasite, gametocyte, and *pvs25* transcript densities in blood. Circles represent the mean oocyst densities of individual experiments. Trend lines are regression splines; the shaded areas represent the 95% confidence intervals. A, C, E: experiments without AB-serum replacement. B, D, F: experiments with AB-serum replacement. nd, not detected.

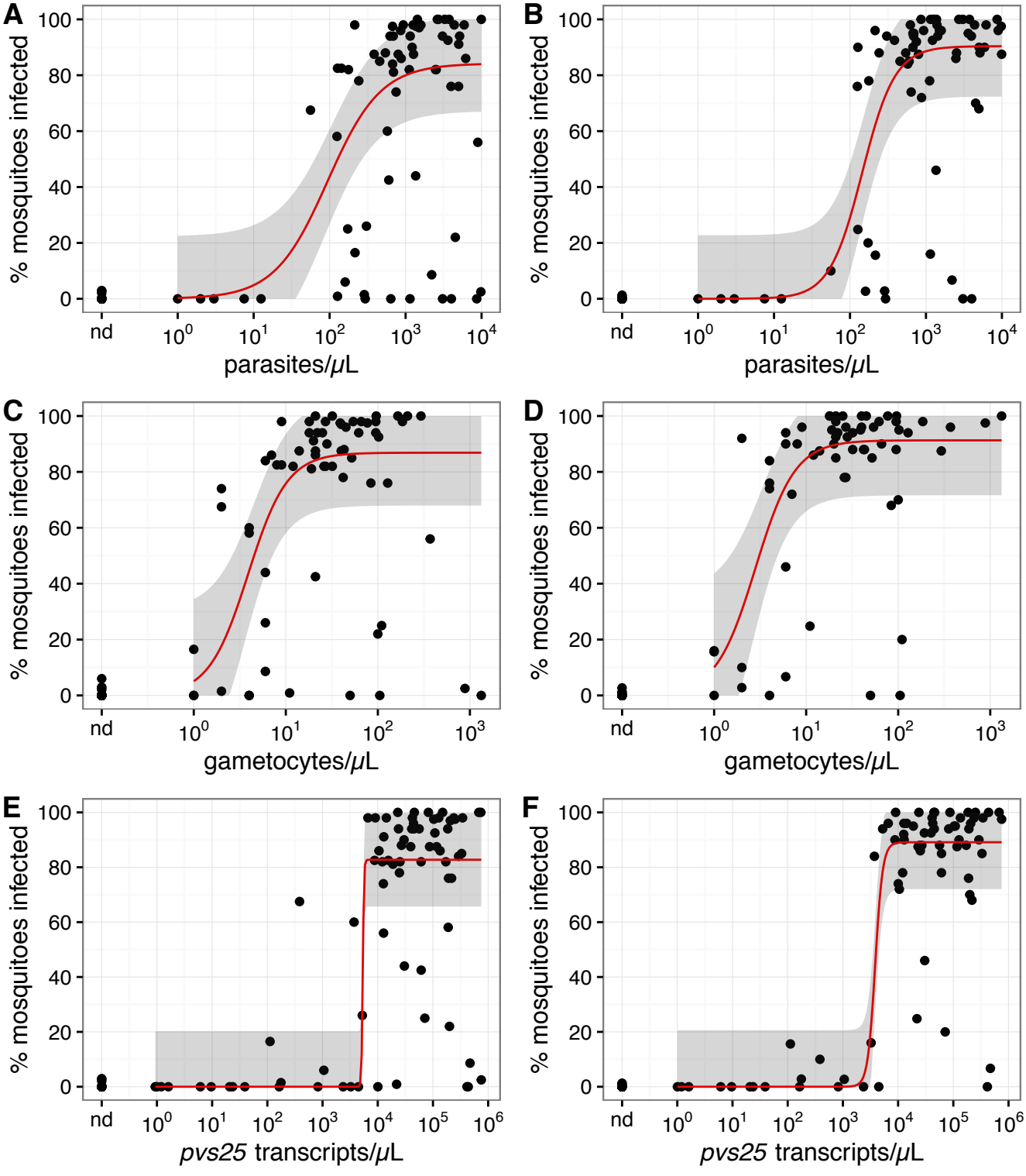
Fig 4. Relationship between the mean oocyst density and the infection rate of *P. vivax*. Circles represent values from individual feeding experiments with AB serum replacement. Dash line, $y = 100(1 - e^{-x})$, depicts the prediction of the Poisson infection process. Solid red line is the best fit ($\alpha = 2.29$) of the same infection process but allowing *free rider* parasites.

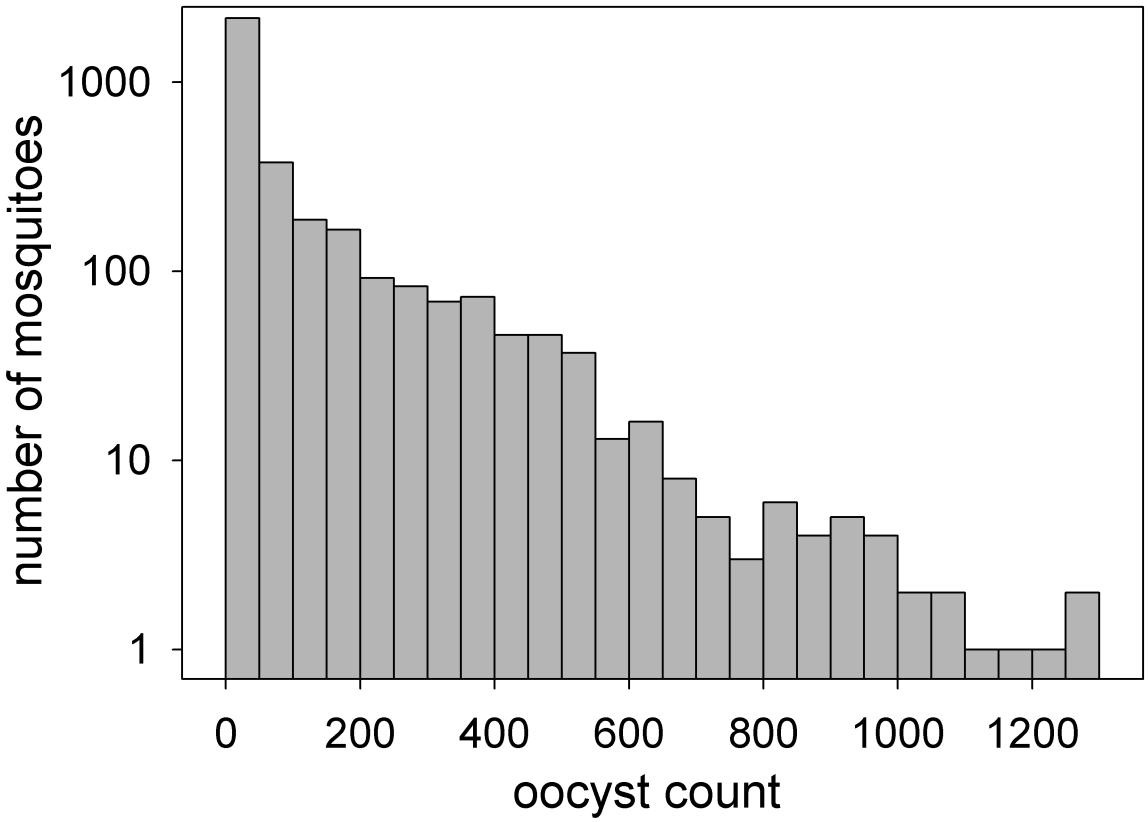
Table 1. Summary of the mosquito membrane feeding assays

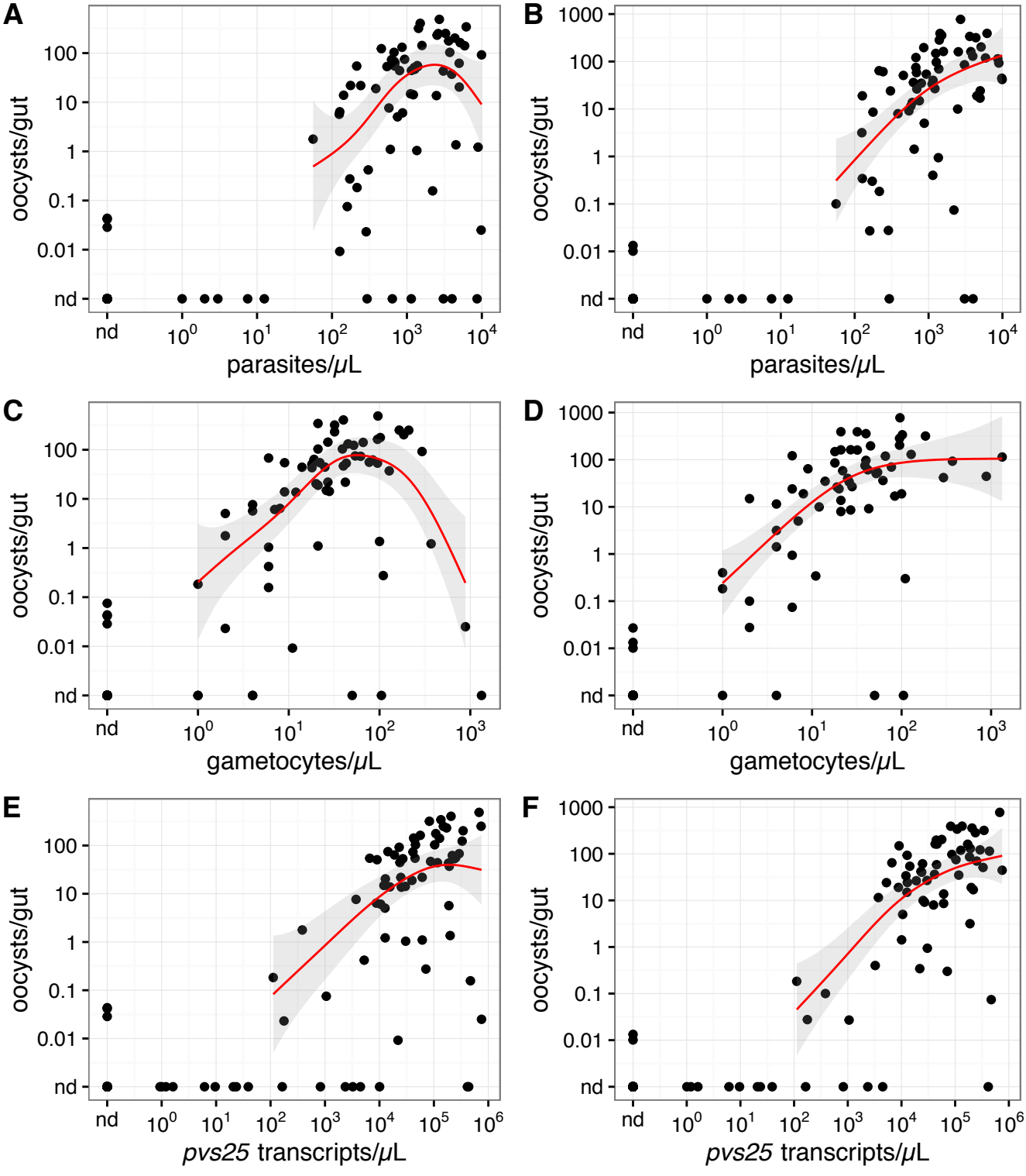
blood infection	**% infective cases** (infective cases/total fed)		**% mosquitoes infected** (infected mosquitoes/total dissected)	
	WB[5]	AB[6]	WB	AB
P. vivax, symptomatic[1]	84 (59/70)	88 (59/67)	49.33 (2,165/4,389)	55.51 (2,352/4,237)
P. vivax, asymptomatic[2]	0 (0/24)	0 (0/19)	0 (0/2,678)	0 (0/2,157)
indeterminate Plasmodium[3]	0 (0/21)	0 (0/20)	0 (0/2,277)	0 (0/2,182)
parasite not detected[4]	4 (4/107)	5 (5/99)	0.07 (8/11,681)	0.05 (6/11,334)

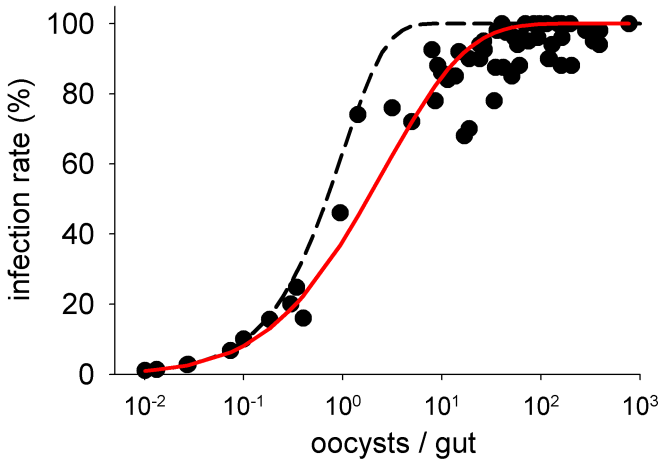
[1] *P. vivax* was detected by light microscopy or qRT-PCR.
[2] *P. vivax* was detected by LAMP or qRT-PCR.
[3] *Plasmodium* parasite was detected by genus-specific LAMP or qRT-PCR, but the species of the parasite could not be identified due to low parasitemia. All samples were negative by light microscopy.
[4] Parasite was not detected by LAMP and qRT-PCR.
[5] Original whole blood
[6] AB-serum replaced blood

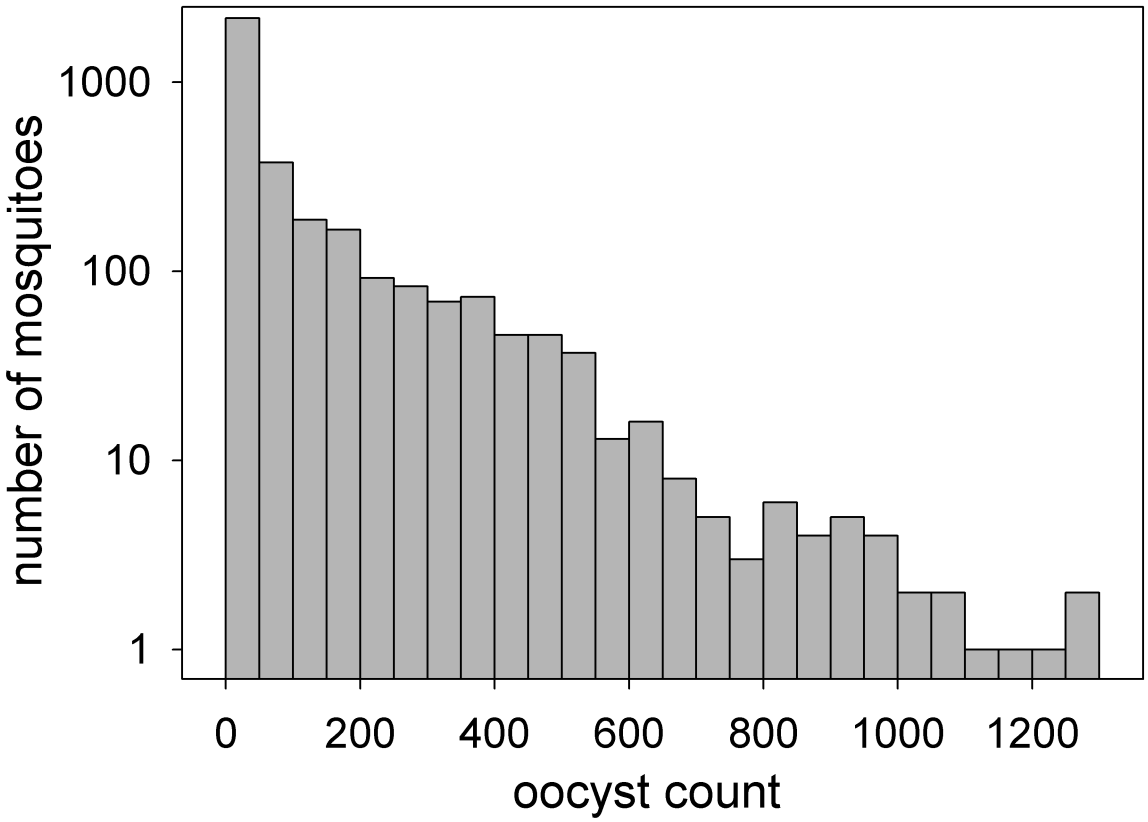

A
25
20
15
10
5
0
number of cases
-30
-25
-10
-5
0
5
10
log2(infection rate AB / infection rate WB)
B
20
15
10
5
0
number of cases
-30
-25
-10
-5
0
5
10
log2(oocyst density AB / oocyst density WB)


A
B
C
D
E
F
% mosquitoes infected
0
20
40
60
80
100
nd
10^0
10^1
10^2
10^3
10^4
10^5
10^6
parasites/μL
gametocytes/μL
pvs25 transcripts/μL


number of mosquitoes
1000
100
10
1
0
200
400
600
800
1000
1200
oocyst count


A
B
C
D
E
F
oocysts/gut
nd
0.01
0.1
1
10
100
1000
parasites/μL
gametocytes/μL
pvs25 transcripts/μL
10^0
10^1
10^2
10^3
10^4
10^5
10^6


100
80
60
40
20
0
infection rate (%)
10-2
10-1
100
101
102
103
oocysts / gut


number of mosquitoes
1000
100
10
1
0
200
400
600
800
1000
1200
oocyst count