Full length article

Experimental study of the relationship between *Plasmodium* gametocyte density and infection success in mosquitoes; implications for the evaluation of malaria transmission-reducing interventions

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HIGHLIGHTS

- Infection outcome in mosquito increases with *Plasmodium falciparum* gametocyte density.
- At high *Plasmodium berghei* gametocyte densities, infection outcome in mosquito decreases.
- Reduction of infection intensity by mAb 13.1 was constant over oocyst intensities.
- Reduction of infection prevalence by mAb 13.1 decreases at high infection loads.
- Evaluation of TRIs should report reduction of infection intensity and prevalence.

GRAPHICAL ABSTRACT
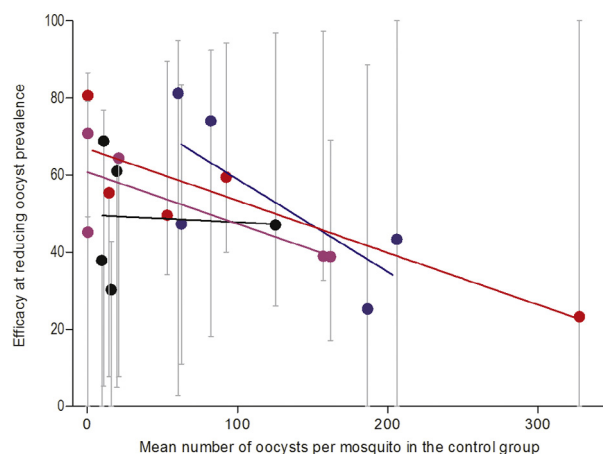
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ABSTRACT

The evaluation of transmission reducing interventions (TRI) to control malaria widely uses membrane feeding assays. In such assays, the intensity of *Plasmodium* infection in the vector might affect the measured efficacy of the candidates to block transmission. Gametocyte density in the host blood is a determinant of the infection success in the mosquito, however, uncertain estimates of parasite densities and intrinsic characteristics of the infected blood can induce variability. To reduce this variation, a feasible method is to dilute infectious blood samples. We describe the effect of diluting samples of *Plasmodium*-containing blood samples to allow accurate relative measures of gametocyte densities and their impact on mosquito infectivity and TRI efficacy. Natural *Plasmodium falciparum* samples were diluted to generate a wide range of parasite densities, and fed to *Anopheles coluzzii* mosquitoes. This was compared with parallel dilutions conducted on *Plasmodium berghei* infections. We examined how blood dilution influences the observed blocking activity of anti-Pbs28 monoclonal antibody using the *P. berghei/Anopheles stephensi* system.

In the natural species combination *P. falciparum/An. coluzzii*, blood dilution using heat-inactivated, infected blood as diluents, revealed positive near linear relationships, between gametocyte densities and oocyst loads in the range tested. A similar relationship was observed in the *P. berghei/An. stephensi* system when using a similar dilution method. In contrast, diluting infected mice blood with fresh uninfected blood dramatically increases the infectiousness. This suggests that highly infected mice blood contains inhibitory factors or reduced blood moieties, which impede infection and may in turn, lead to misinterpretation when comparing individual TRI evaluation assays. In the lab system, the transmission blocking activity of an antibody specific for Pbs28 was confirmed to be density-dependent. This highlights the need to carefully interpret evaluations of TRI candidates, regarding gametocyte densities in the *P. berghei/An. stephensi* system.

1. Introduction

The transmission of malaria requires the survival and development of *Plasmodium* within two individual organisms: the vertebrate host and the mosquito vector. Female anopheline mosquitoes ingest gametocytes when taking a blood meal from infectious vertebrate hosts. Within the mosquito's midgut, the parasite has to overcome a strong population bottleneck; due to the low efficiency of gamete fertilization (Alavi et al., 2003; Gouagna et al., 1998; Vaughan, 2007), and the pressure of both the vertebrate host immunity, still acting in the blood meal (Bousema et al., 2011; Gouagna et al., 2004), and the vector's innate defense mechanisms (Blandin and Levashina, 2004; Yassine and Osta, 2010). For this reason, and the limited antigenic variation exhibited by extracellular proteins expressed in the sexual stages of parasite development (Kaslow et al., 1989; Manske et al., 2012; Shi et al., 1992), the mosquito-stages of the parasite life cycle are logical and attractive targets for transmission reducing interventions (TRIs) (Sinden, 2010). Since the reemergence of the concept of transmission blocking of *Plasmodium* decades ago (Carter and Chen, 1976; Gwadz, 1976; Huff et al., 1958), research efforts have increased in an attempt to find vaccines (Carter et al., 2000; Kaslow, 1997; Saul, 1993, 2007), drugs (Ponsa et al., 2003; Wells et al., 2009) or microorganisms (Boissiere et al., 2012; Cirimotich et al., 2011a, 2011b; Dong et al., 2009; Fang et al., 2011; Hughes et al., 2011) able to disrupt the life cycle of the parasite in the mosquito vector, with the eventual objective of reducing both incidence and prevalence of disease in the vertebrate host.

To assess the potency of potential TRIs, experiments comprising the feeding of *Anopheles* mosquitoes with *Plasmodium* containing blood are widely used. The "gold-standard" assay of this class is the Membrane Feeding Assay (MFA), in which infectious blood mixed with transmission-reducing agents is fed to mosquitoes through an artificial membrane (Sinden, 1996). In the MFA, the number of infectious parasites ingested by the mosquito from the vertebrate host has been identified as one of the main variables that influence the success of *Plasmodium* infection in the vector. Indeed, previous studies established a positive relationship between gametocyte densities and infection outcome in mosquitoes in artificial *Plasmodium*–*Anopheles* species combinations (Dawes et al., 2009; Huff et al., 1958; Poudel et al., 2008; Sinden et al., 2007) and in natural *Plasmodium*–*Anopheles* systems (Boudin et al., 2004; Carter and Graves, 1988; Drakeley et al., 1999; Gouagna et al., 1998, 1999; Huff et al., 1958; Mulder et al., 1994; Ponnudurai et al., 1989; Robert et al., 1998). However the precise shape of the relationship between gametocyte density and subsequent infection remains largely unclear (Churcher et al., 2013). This is unfortunate as the process of density-dependent sporogonic development is thought to be instrumental to the perceived success of a TRI in the MFA, and will have crucial implications in the identification, evaluation and comparison of new TRI candidates (Churcher et al., 2010). A major limitation to our understanding of the effect of parasite density on infection success is the inaccuracy in gametocyte density estimates using the standard methods of microscopy. Indeed, there is considerable sampling variability in the numbers of gametocytes in blood, there is uncertainty in the density of white-blood cells which the gametocytes are measured against (McKenzie and Bossert, 2005; McKenzie et al., 2005) and as high as 80% of all gametocytes might be missed during the staining and reading procedure (Dowling and Shute, 1966). Additionally, gametocytes vary in their maturity (Lensen et al., 1999), sex ratio (Mitri et al., 2009; Paul et al., 2002; Reece et al., 2008), genetics (Ferguson and Read, 2002; Harris et al., 2010; Lambrechts et al., 2005) or multiplicity of infection (Nsango et al., 2012; Reece et al., 2008) which can all influence mosquito infectivity. An additional factor that can cause variation in assay output is the method of sample dilution when performing the MFA. The addition of potentially transmission-blocking agents to infectious blood prior to mosquito blood meal can vary e.g. the volume and type of diluent used to dilute can independently affect both gametocyte concentration in the sample, and oocyst intensity/prevalence. The impact of these variables needs to be fully understood to understand the implications of assay output, particularly with regard to the comparison between potential TRI agents within individual MFA experiments. To control this variation when evaluating TRI candidates, a feasible method of directly comparing the ability of different samples of blood containing gametocytes to transmit is to dilute samples, ensuring that gametocyte numbers are directly comparable between replicates.

Potential TRIs are commonly tested in the MFA with both *Plasmodium falciparum* and the rodent malaria parasite, *Plasmodium berghei*. The *Anopheles stephensi*–*P. berghei* species combination has

been widely used due to its tractability and exceptional robustness (Blagborough et al., 2013; Mlambo and Kumar, 2008; Ramjanee et al., 2007; Tirawanchai et al., 1991). 'Laboratory' parasite–mosquito combinations such as this allow the design of experiments that would be technically or ethically impossible in natural species combinations. Conversely, *Anopheles–Plasmodium* interactions in natural species combinations (e.g. *P. falciparum* and *Anopheles coluzzii*) are shaped by long term co-evolution and may differ from model systems (Boete, 2005; Cohuet et al., 2006; Dong et al., 2006). In particular, gametocyte densities and the resulting infectivity of murine and human *Plasmodium* species can differ markedly (Taylor and Read, 1997). Therefore it is imperative that results obtained in model systems are interpreted with due regard to studies using natural species combinations.

Understanding how vertebrate-to-mosquito transmission changes with dilution of parasites and how this influences the perceived effectiveness of TRI in commonly used natural and model species combinations is essential, to understand the relationship between gametocyte density and infection outcome, to standardize current procedures and to enhance accurate and consistent evaluation of TRI candidates. To address this issue, natural isolates of *P. falciparum* were diluted to generate a wide range of parasite densities and used to perform experimental infections of *An. coluzzii* mosquitoes in conditions representative of those used to measure TRI efficacy in a semi-field context. These are compared with dilutions conducted on *P. berghei* fed to *An. stephensi* mosquitoes in conditions representative of those used to measure TRI efficacy in the laboratory. Additionally, using the *P. berghei/An. stephensi* system, the impact of differing methods of parasite dilution on assay output was investigated. We then examined how blood dilution influenced the observed transmission-reducing capabilities of anti-Pbs28 monoclonal antibody 13.1 (13.1 mAb) at a constant concentration, and discussed the implications for the evaluation of TRIs in lab and field conditions.

2. Materials and methods

2.1. Experiments using the natural system:
P. falciparum–An. coluzzii

P. falciparum gametocytes were collected from naturally infected volunteers recruited in malaria endemic localities. Gametocyte containing blood was used to infect females from a local mosquito colony (Harris et al., 2012) of the species recently named *An. coluzzii* (Coetzee et al., 2013). The protocol was approved by the Centre MURAZ ethical review committee (003-2009/CE-CM). *P. falciparum* gametocyte carriers were selected from 5 to 11 year old children screened in villages (Soumousso and Dandé) surrounding Bobo-Dioulasso in Burkina Faso. Gametocyte density was estimated by microscopy read against 1000 leucocytes on thick blood smears, assuming a standard leukocyte count of 8000/μL. High density carriers of *P. falciparum* gametocytes were selected to generate a wide range of densities by dilution. Eligible children were driven to the lab after health examination and venous blood was collected. The gametocyte density was re-estimated in the venous blood used for mosquito feeding. Each smear was evaluated independently by two expert microscopists and any discordance was resolved by a third microscopist by using the mean of the two closest values.

Experimental infections of *An. coluzzii* female mosquitoes were performed by membrane feeding assays with serum replacement as previously described (Bousema et al., 2012). Venous blood samples were collected into heparinized tubes and were immediately centrifuged at 1300 *g* for 3 minutes. The gametocyte carrier's plasma was removed and the RBC pellet mixed with serum from a European AB blood donor. This procedure removes natural human transmission blocking immune mechanisms, which vary between individual patients (Carter and Graves, 1988). The gametocyte density in this substituted-serum preparation was diluted sequentially: one part of substituted-serum blood was kept at 37 °C (infectious blood) while the second part was heated on a thermo-mixer at 43 °C for 15 minutes to inactivate gametocytes (non-infectious blood). Heat treatment was previously demonstrated to fully inhibit gametocyte infectivity (Mendes et al., 2008, 2011) without measurably affecting other blood characteristics of mosquito fitness (Sangare et al., 2013). Inactivated-gametocyte blood was then used to dilute the infectious blood to obtain a range of samples that contained different dilutions of infectious gametocytes, yet retaining the same overall cell composition. Blood mixtures were used to feed 24 h starved *An. coluzzii* females, through pre-warmed (37 °C) membrane feeders for 30 min. Fully fed females were sorted and maintained in cages at 28 °C ± 2, 80% ± 05 RH, with 10% glucose solution available. Mosquitoes were dissected day 7 post-feeding in a drop 0.5% mercurochrome and their midguts examined for oocysts by light microscopy. When high oocyst intensities were observed (~200 oocysts), numbers were estimated by two microscopists; any discordance was resolved by a third microscopist by using the mean of the two closest values.

2.2. Experiments using the laboratory system:
P. berghei–An. stephensi

All procedures were performed in accordance with the terms of the UK Animals (Scientific Procedures) Act Project Licence and were approved by the Imperial College Ethical Review Committee (PPL 70/7185). The Office of Laboratory Animal Welfare (OLAW) Assurance for Imperial College covers all Public Health Service (PHS) supported activities involving live vertebrates in the US (#A5634-01).

General parasite maintenance was carried out as previously discussed (Sinden et al., 2002). *P. berghei* ANKA 2.34 parasites or *P. berghei* GFP 507 cl 1 parasites, constitutively expressing GFP (Janse et al., 2006), were maintained in 4–10 week old female Tuck Ordinary (TO) mice by serial mechanical passages (up to a maximum of 8 passages). Hyper-reticulocytosis was induced 2–3 days before infection by treating mice with 200 μL *i.p.* phenylhydrazinium chloride (PH; 6 mg/mL in PBS; ProLabo UK). Mice were infected by intraperitoneal (*i.p.*) injection and infections were monitored on Giemsa-stained tail-blood smears.

To assess total gametocyte numbers in individual blood samples for MFA, following terminal anesthesia and cardiac puncture, harvested blood was pooled from mice and gametocytemia was estimated by counting the percentage of erythrocytes containing gametocytes on a Giemsa smear. Absolute gametocyte numbers were calculated using a mean of 8,360,000 RBC/μL, which was determined by using a Neubauer hemocytometer from the used blood samples. Pooled blood samples were stored briefly at 37 °C, diluted and used as described below.

To produce a wide range of parasite densities, mature infectious *P. berghei* gametocytes freshly harvested from infected mice were serially diluted to prepare samples, which were then fed to previously starved *An. stephensi* mosquitoes. Within the scope of our experiments, to examine the impact of the diluent used for sample dilution when performing the MFA, *P. berghei* gametocytes were diluted either 1) by dilution in naïve blood, from non-infected, non phenylhydrazinium chloride-treated mice, freshly harvested by cardiac puncture immediately prior to MFA; or 2) by dilution with (gametocyte) heat-inactivated infected mouse blood (as described in the parallel *P. falciparum* experiments above). Mixtures were prepared using the following ratios (inf/non-inf); 1:0, 3:4, 2:2, 1:4, 1:10 and 1:50.

To examine the impact of parasite dilution on TBI, pairs of serial dilutions from the originating *P. berghei* infected blood were prepared, one containing the anti-Pbs28 mAb 13.1, and the other containing the non-specific IgG UPC10 (Sigma Aldrich): blood
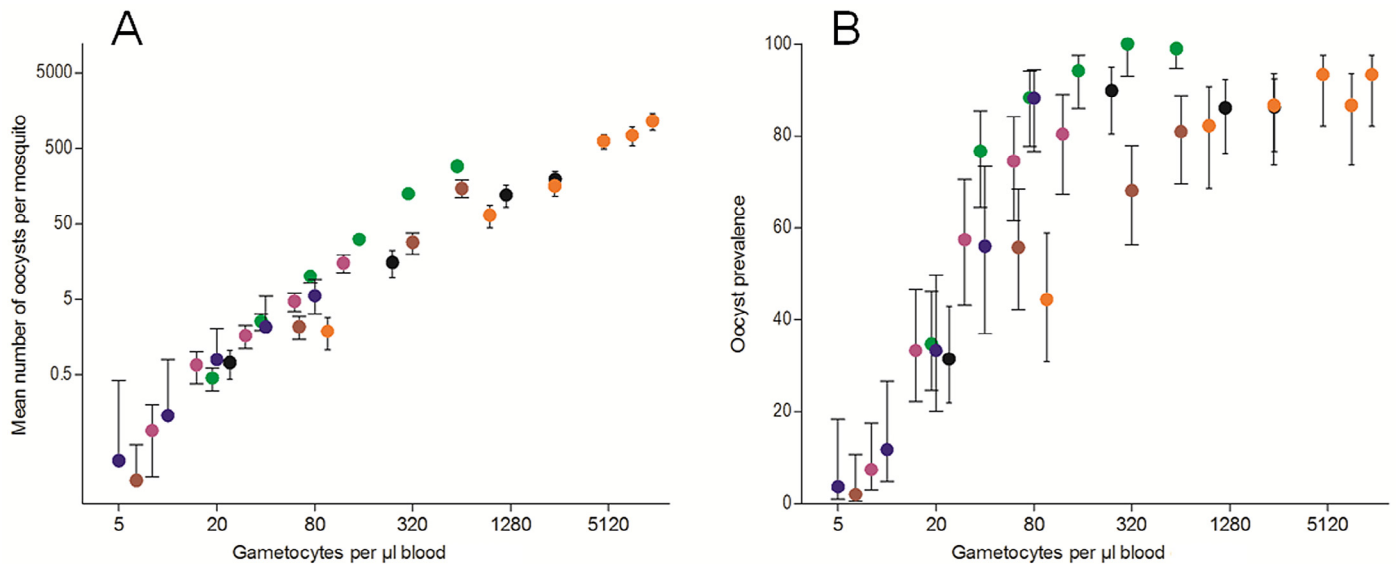
Fig. 1. Relationship between *P. falciparum* gametocyte density and infection outcome in *An. coluzzii*. Individual colors represent sets of feeds performed from a single donor blood sample, with gametocyte dilutions made with the same sample of heat inactivated blood. Gametocyte densities were estimated from undiluted blood samples and subsequent densities were predicted from the dilution factor. (A) Intensity of infection; i.e. mean number of oocyst observed within the midguts post-dissection. (B) Prevalence of infection; i.e. percentage of infected mosquitoes. Error bars indicate 95% confidence intervals.

samples, diluted or not, were mixed with 100 μL of PBS containing mAb 13.1 or UPC10 (negative control) to give a final antibody concentration of 25 μg/mL. The potent transmission-blocking ability of 13.1 has been demonstrated in numerous studies (Tirawanchai et al., 1991; Yoshida et al., 1999), and the use of UPC10 as a isotypic control that exhibits a total lack of non-transmission-blocking effect has been demonstrated previously (Ramjanee et al., 2007).

Membrane feeding assays were carried out as follows; batches of *An. stephensi* (SDA 500 strain) were starved for 24 hours and then fed on 400 μL previously prepared *P. berghei* infected blood. In all assays >50 fed mosquitoes were assayed per sample. 24 hours after feeding, mosquitoes were briefly anesthetized with CO_2, and unfeds were removed. Mosquitoes were then maintained on fructose [8% (w/v) fructose, 0.05% (w/v) *p*-aminobenzoic acid] at 19–22 °C and 50–80% relative humidity. Day 12 post-feeding, midguts were dissected from the remaining mosquitoes, and the number of oocysts was recorded (Blagborough et al., 2013). For *in vivo* observation of GFP-positive ookinetes, midguts were dissected 24 hours post-feed. Midguts were then incubated in 4% para-formaldehyde (PFA) for 45 minutes, washed three times with PBS and mounted in a drop of VectaShield mounting medium for fluorescence with DAPI (4′,6-diamidino-2-phenylindole), covered with a coverslip that was sealed with nail varnish. Ookinetes were counted using a 63× objective magnification on a Leica DMR fluorescence microscope.

2.3. Statistical analysis

To determine whether the impact of the dilution procedure on oocyst prevalence and intensity was consistent across blood samples a generalized linear mixed model was fitted to the observed data (Churcher et al., 2012). A binomial error structure was used for the parasite presence/absence data while a zero-inflated negative binomial distribution was used to describe mosquito oocyst intensity. A suite of linear models was fitted to oocyst prevalence and intensity estimates and the most parsimonious identified using the likelihood ratio test. The most simple described the number of oocyst against gametocyte density. More complex models allowed the intercept and gradient of the best fit lines to vary between dilution series. To determine whether overall there was a change in the ef-

ficacy (point estimates) with gametocyte density all data were included within a linear mixed effects model. The gradient and intercept of the relationship between efficacy and gametocyte density were allowed to vary independently between dilution series. These models were compared to a model without information on gametocyte density to determine whether it influenced efficacy. The same analyses were repeated with mean oocyst intensity in the control group as the variable instead of gametocyte density.

To assess transmission-blocking activity, prevalence and intensity in samples containing mAb 13.1 or UPC10 at corresponding dilutions were compared. Treatment with mAb 13.1 was included as the fixed effect and models with or without treatment information were compared using the likelihood ratio test to determine whether the intervention significantly reduced oocyst development. To test whether there were any consistent changes in TRI efficacy within the different dilution series; the blood source was included in these models as a random effect. Confidence interval estimates were generated by the best fit model or using bootstrapping methodology.

3. Results

3.1. Relationship between blood dilution and mosquito infectivity

P. falciparum gametocyte-infected blood from six naturally infected volunteers was used for dilution of infectious gametocytes and feeding of *An. coluzzii* females. Initial gametocyte densities ranged from 80 to 9520 gametocytes/μL and were each used to perform 3–5 serial dilutions. Infection outcomes were estimated by dissecting a total of 1636 mosquitoes (mean number of dissected midguts per experimental condition = 54.53, SD = 15.86, range: 25–101). We observed some exceptionally high oocyst loads with, for instance, a mean of 1152 oocysts among the females fed on the blood that contained 9520 gametocytes/μL.

Undiluted *P. falciparum* samples were more infectious than diluted blood. The mean number of oocysts observed decreases with dilution in (non-infectious) heat-inactivated blood samples, as shown by a positive correlation between estimated gametocyte density and oocyst intensity (Fig. 1A). The relationship between gametocyte
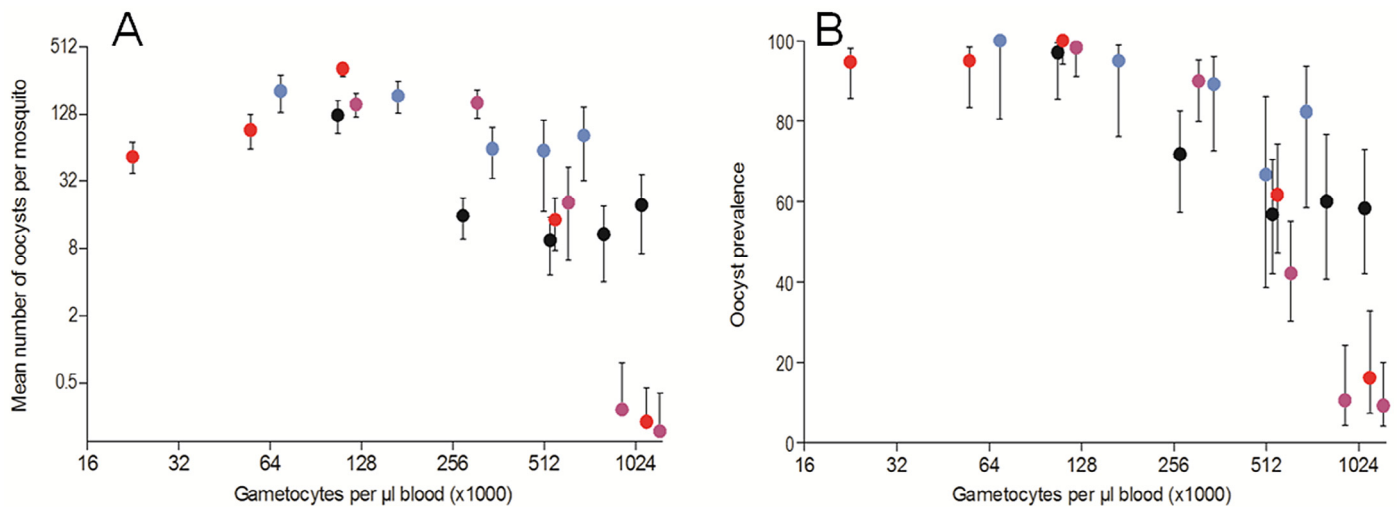
Fig. 2. Relationship between *P. berghei* gametocyte density and infection outcome in *An. stephensi*. Each color represents a set of feeds performed from a single originating blood source, with subsequent gametocyte dilutions made with naïve, uninfected blood. Gametocyte densities were estimated from the undiluted blood samples and subsequent densities were predicted from the dilution factor. (A) Intensity of infection; i.e. mean oocyst numbers. (B) Prevalence of infection; i.e. percentage of infected mosquitoes. Error bars indicate 95% confidence intervals. Note the log log scale in panel A which reduces the appearance of variability.

density and oocyst intensity appears to be adequately described by a simple linear relationship (LL, = −6755.9). Allowing the intercept of the line to vary between dilution series significantly improves the fit of the model (LL = −6575.0, *p* value <10^{-8}) suggesting that factors other than just gametocyte density (as determined by microscopy) are influencing oocyst intensity. Enabling the gradient of the line to also vary (in addition to the intercept) gives the best fit model to these data (LL = −6202.4, *p* value <10^{-8}) suggesting that the exact impact of the dilution procedure also varies between blood samples. Correspondingly, oocyst prevalence also increases with the concentration of infectious blood until a plateau (Fig. 1B).

In corresponding laboratory system experiments, the initial (i.e. non diluted) gametocyte densities observed (post-bleed) ranged from 690,000 to 1,230,000 gametocytes/µL. In total, over multiple assays, 783 mosquitoes (mean number of dissected midguts per experimental condition = 39.15, SD = 15.97, range: 12–60) fed on blood samples containing potentially infectious gametocytes (either undiluted or diluted) and control antibodies without transmission reducing activity (UPC10). Infection outcomes at the oocyst stage are presented in Fig. 2. When performing the MFA with *P. berghei*, diluting samples with naïve blood increases vertebrate-to-mosquito transmission, in terms of both infection intensity and prevalence. The mean oocyst numbers/midgut indicate a "humped" relationship, with peak oocyst intensity observed at ~100,000 gametocytes/µL, although we obtained only few data points at low gametocyte densities (Fig. 2A). The positive relationship between gametocyte densities and infection intensity at lower gametocyte densities is consistent with previous studies (Sinden et al., 2007). At higher gametocyte densities, a largely negative relationship between gametocyte density and infection intensity was observed. This "dilution effect" can be highly significant: for example, an average of 0.22 (95% CI [0.020; 0.431]) oocysts per midgut was observed at an estimated gametocyte density of 1,110,000/µL, whereas an average of 327 (95% CI [278.20; 377.00]) oocysts was observed per mosquito midgut when the same gametocyte containing sample was diluted 1:10 in fresh, uninfected mouse blood. Models which allowed the gradient and the intercept of the best fit lines to vary between dilution series (log likelihood estimate, LL, = −3410.9) fit significantly better than those which assumed that oocyst intensity was described by gametocyte density alone (LL = −3335.4, *p* value < 10^{-8}). However, no linear model was a good fit to these data indicating

that more complex functional forms will be required to capture this relationship. In terms of prevalence, highly diluted blood samples (with an estimated gametocyte densities between 22,500 and 100,000 gametocytes/µL) appear to infect more than 90% of the exposed mosquitoes while feeds performed on non-diluted blood achieve infection prevalence as low as 15%. This pattern is broadly similar between all feeds performed (Fig. 2B). The oocyst intensities and prevalence revealed a positive relationship, in agreement with previous observations (Churcher et al., 2012; Medley et al., 1993).

To clarify at which life-cycle stage this "dilution effect" impacts on transmission to the mosquito, the observed complex relationship between gametocyte density and mosquito infection was examined further. One hundred *An. stephensi* females were individually fed on 5 serial dilutions of mouse blood containing *P. berghei* 507 cl1 GFP gametocytes, with estimated densities of 77,000–770,000 gametocytes/µL. From feeds at each dilution, batches of 50 mosquitoes were dissected at 24 h to examine ookinete infection, the remaining mosquitoes were examined at 13 days post infection to observe oocyst prevalence and intensity. In the dynamic range tested a negative relationship between gametocyte density and both ookinete density and oocyst density was observed (Fig. 3A), so that a positive but non-linear relationship between ookinete and oocyst numbers was also exhibited (Fig. 3B) in agreement with previous studies (Sinden et al., 2007). This result readily suggests that the dilution of *P. berghei* infected blood can improve the success of infection, and this enhancement in transmission occurs at, or before the ookinete stage of parasite development.

To further investigate the mechanisms involved in the observed positive relation between dilution factor and infection success, experiments were carried out by diluting *P. berghei* gametocyte infected blood in parallel; either with 1) naïve, non-infected rodent blood, or 2) by dilution with infected *P. berghei* mouse blood, with gametocytes heat-inactivated (as performed in the *P. falciparum* experiments described in previous experiments). These experiments revealed that the nature of blood used for dilution induced contrasting patterns: when diluting infectious blood with naïve uninfected blood, gametocyte density was inversely correlated to infection success as observed in previous experiments (Fig. 2), whereas dilution with heat inactivated *P. berghei* infected blood demonstrates a positive correlation between gametocyte densities and
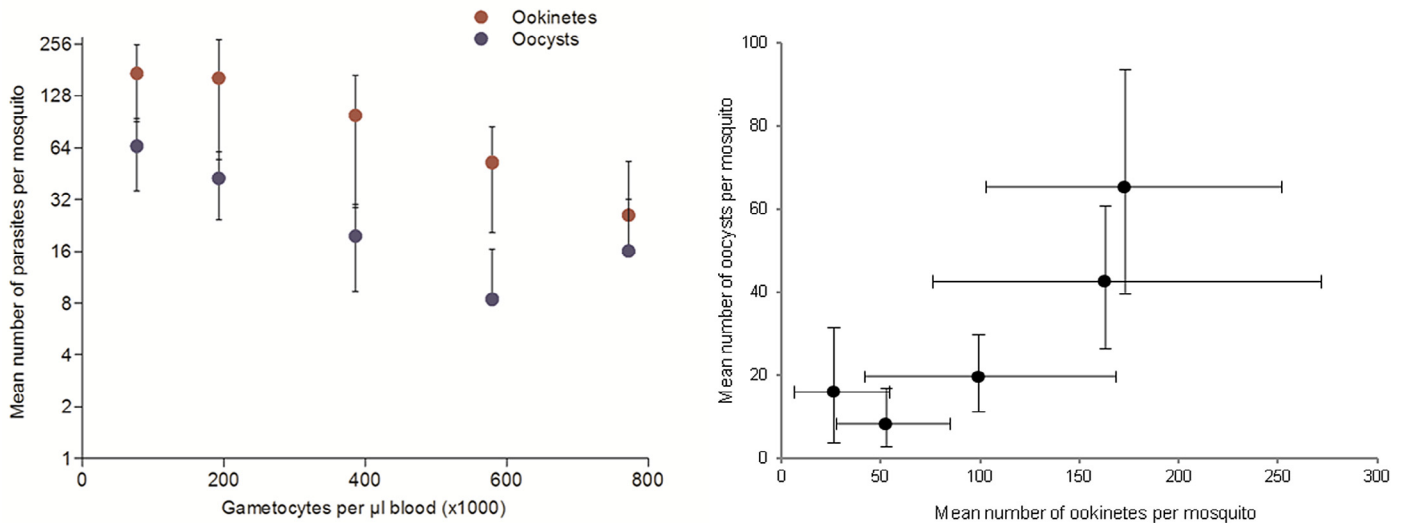
Fig. 3. Mean number of ookinetes and oocysts (A) and relationship between mean number of ookinetes and mean number of oocysts (B) in *An. stephensi* following feeds with diluted GFP *P. berghei* cl 507 gametocyte samples. Error bars indicate 95% confidence intervals.

oocyst intensity or prevalence (Fig. 4), as observed in corresponding experiments with *P. falciparum* (Fig. 1). This clearly demonstrates that dilution of *P. berghei* gametocytes with naïve uninfected blood enhances mosquito infection, potentially as transmission-inhibiting factors are diluted and depleted moieties are replaced. Conversely, dilution of *P. berghei* gametocytes by blood/parasite preparations where both cells and molecules are heat-inactivated gives rise to a relationship where infection success is only related to the infective dose, as observed in feeds performed with *P. falciparum* (Fig. 1).

3.2. Infection intensity dependence of mAb 13.1 efficacy

Our experiments confirmed the efficacy of mAb 13.1 at 25 µg/mL. The efficacy exhibited pronounced variability, depending on expression as either reduction of oocyst intensity or prevalence, and depending on the parasite density in ingested blood and infection load in control groups. Measured as a reduction in mean number of oocyst per midgut, the antibody had a mean efficacy at 86.67%

(CI 83%–89%). The linear mixed effect models showed that there was no evidence that efficacy changed with gametocyte density ($p = 0.8734$). (Fig. 5A). Consistently, no correlation was observed between intensity of infection in control mosquitoes and transmission reducing activity measured as a reduction of oocyst intensity (Fig. 5B, $p = 0.2485$). In contrast, considering the efficacy as the reduction of oocyst prevalence, the antibody showed increased efficacy in less diluted *P. berghei* containing blood samples (Fig. 5C, $p = 0.0064$), so that efficacy decreases as *P. berghei* mean oocyst number in control mosquitoes increases (Fig. 5D, $p = 0.0161$). The negative relation between the mean number of oocysts in control mosquito groups and the transmission blocking efficacy of the antibody at reducing prevalence suggests that TRI is less efficient at higher infection intensities, observed in feeds where blood samples are diluted. The density dependant efficacy of TRI is consistent with the previously observed relationship between oocyst prevalence and intensity based on a negative binomial distribution of oocyst (Churcher et al., 2012): at high infection intensities, a partial trans-
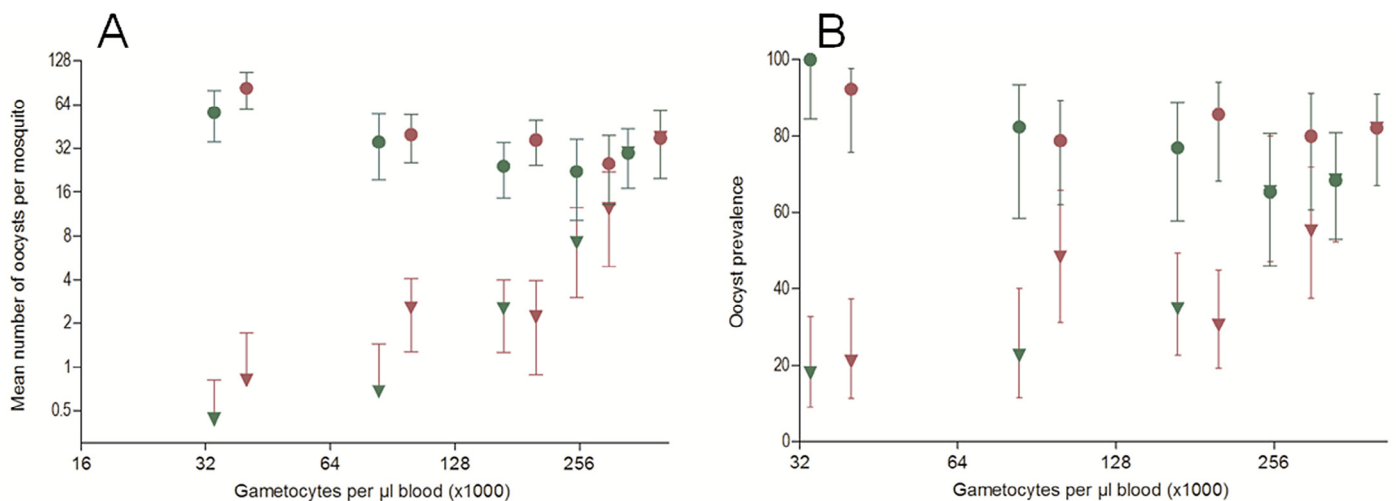

Fig. 4. Relationship between *P. berghei* gametocyte density and infection outcome in *An. stephensi*. Each color represents a set of feeds performed from a single originating blood source, with subsequent gametocyte dilutions made with naïve non infected blood (circles), uninfected blood or heat inactivated blood (triangles). Gametocyte densities were estimated from the undiluted blood samples and subsequent densities were predicted from the dilution factor. (A) Intensity of infection; i.e. mean oocyst numbers. (B) Prevalence of infection; i.e. percentage of infected mosquitoes. Error bars indicate 95% confidence intervals.
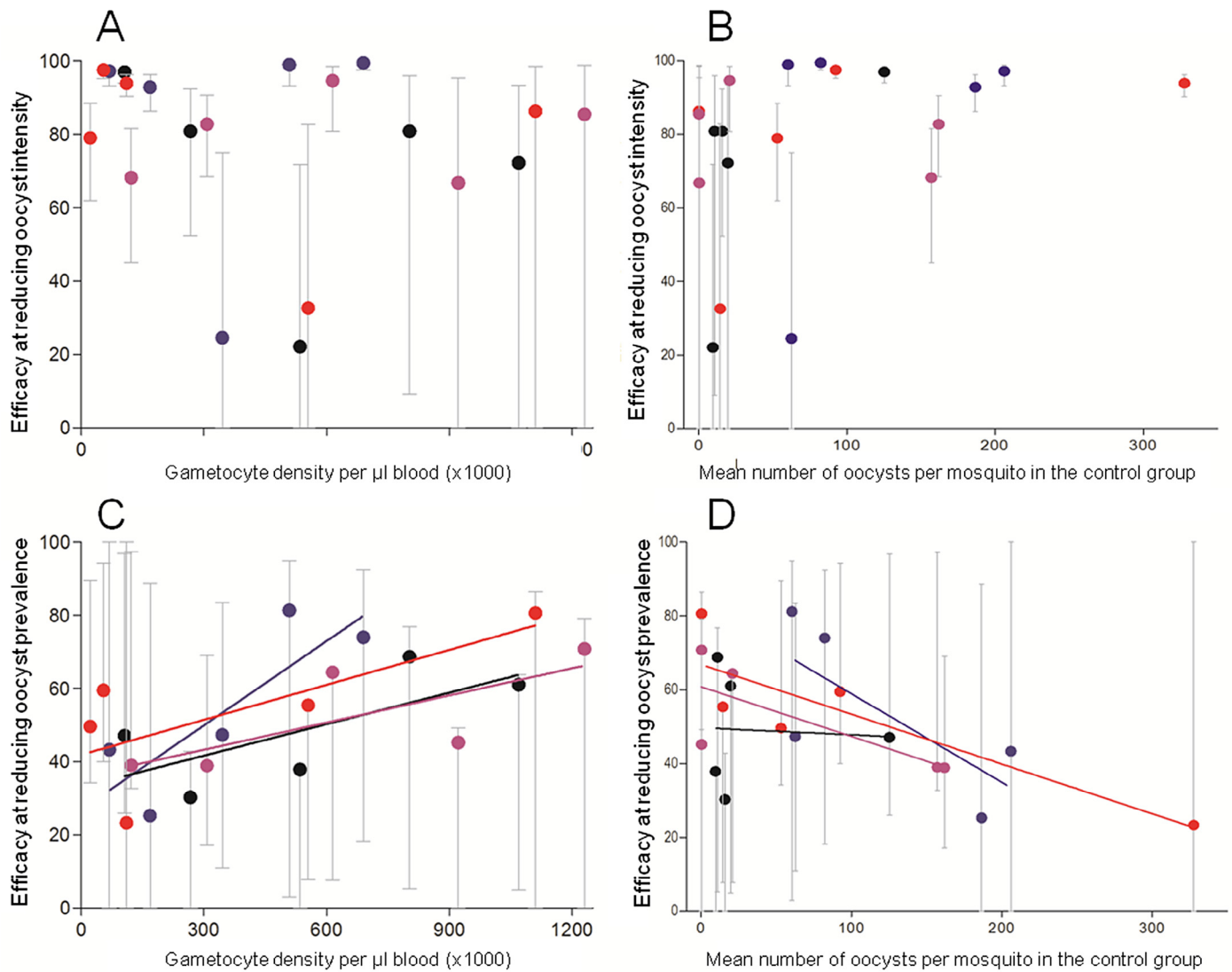
Fig. 5. TRI efficacy for 13.1 MAb at reducing oocyst intensity according to gametocyte density (A) or mean number of oocysts in control groups of mosquitoes (B). TRI efficacy for 13.1 McAb at reducing oocyst prevalence according to gametocyte density (C) or mean number of oocysts in control groups of mosquitoes (D). Each color represents a set of gametocyte dilutions. Vertical lines indicate 95% confidence interval estimates in TBA.

mission reducing activity of TRIs may not affect the prevalence of infection while the same efficacy at reducing infection intensity affects the prevalence of infection when initial infection load is lower.

4. Discussion

In the natural system *P. falciparum* and *An. coluzzii*, dilutions of gametocyte containing blood, using heat inactivated blood as diluent, demonstrated a positive linear relationship between gametocyte density and intensity of the infection in the mosquito for the range of gametocyte densities observed here (estimated from 5 to 9520 gametocytes/μL). The fit of the model was significantly improved when analyzing the dilutions series individually, with linear regressions of different gradients and intercepts for each diluted blood sample. This is indicative of a different effect of dilution for the gametocyte samples obtained from distinct patients. Gametocyte density and oocyst prevalence also revealed a positive relation until a plateau. The differences between series of dilutions may reflect numerous intrinsic characteristics of each gametocyte isolate such as sex ratio, host response status, maturity or multiplicity of infection but also inaccuracy when assessing gametocyte density. Here,

by using dilutions of parasite isolates, we drastically reduced uncontrolled variability within dilution series and produced, to our knowledge, the most accurate existing data for measuring the effect of gametocyte density on mosquito infectivity in the natural *P. falciparum–An. coluzzii* combination. By creating an artificial range of gametocyte densities in a natural and epidemiologically relevant *Anopheles–Plasmodium* system, this dilution method appears particularly appealing for including infection intensity as a controlled variable when investigating TRI evaluations. As efficacy of TRIs has been suggested to be density dependent (Churcher et al., 2010), this will be of crucial importance for predicting the efficacy of TRI in different and changing patterns of malaria transmission.

In this study, the plasma of the *P. falciparum* blood donor was replaced by serum from a naïve donor preventing from naturally acquired transmission reducing factors in humans. In evaluations of candidate transmission blocking vaccines (TBV), this allows estimation of the efficacy without presuming on the patient's history of exposition to parasites. Replacing the serum of the gametocyte donor in TBV evaluation helps in the accurate primary assessment of efficacy with limited noise. However natural transmission reducing immunity would interact with the TBV delivered when

implemented in endemic areas and deserves attention. It would be then of particular interest to investigate the efficacy of naturally acquired transmission reducing immunity with regard to gametocyte density, how this will evolve in a context of malaria decline (WHO, 2011), and how this interacts with TBV efficacy to better estimate their potential efficacy in field.

Likely because we observed only comparatively high gametocyte densities, and because of the serum changes performed, we observed oocyst infection intensities that were for *P. falciparum*, to our knowledge, never reported before, with the highest mean oocyst number at approximately 1200 oocysts. Such infection intensities are not representative of loads observed in naturally infected mosquitoes (Billingsley et al., 1994; Gouagna et al., 2010; Medley et al., 1993). However it cannot be excluded that rare high infection levels happen in nature when mosquito vectors take a blood meal on exceptionally high density gametocyte carriers. Despite these unusually high infection intensities, prevalence was shown to plateau below 100% in laboratory infected mosquitoes. This is consistent with previous identification of resistance alleles in natural vector populations (Harris et al., 2010; Niare et al., 2002; Riehle et al., 2006).

P. berghei–An. stephensi is widely used as a model system for TRI evaluation. Typically, mouse blood is used for mosquito infection 3 days post-infection in phenyl-hydrazine infected mice; a time point selected for its high infectivity to the vector (Dearsly et al., 1990). For this reason, when performing gametocyte dilution in the MFA, infectious blood was diluted with fresh, non-infected blood from naïve mice or with heat inactivated infected blood. Dilution of *P. berghei* blood containing high densities of gametocytes with fresh, non-infected blood induced an often dramatic increase in infectivity to the mosquito host. This phenomenon is reminiscent of previous *in vitro* observations using rodent malaria parasites (Janse et al., 1985; Sinden et al., 1985), where serial dilution of *P. berghei* gametocytes in culture dramatically enhanced ookinete production in comparison with non-diluted controls. As far as the authors are aware, the study is the first time that the corresponding phenomenon has been investigated *in vivo*. Dilution with heat inactivated infected blood conversely demonstrated a positive relation between gametocyte density and infection load in mosquitoes, suggesting that dilution with non-infected blood enhances infection by diluting inhibitory factors in infected blood, such as metabolites (Fleck et al., 1994) or cytokines (Motard et al., 1993). Dilution with uninfected blood may then have reduced the toxicity of the environment for the remaining parasites and favored sporogony. Alternatively, addition of fresh blood from uninfected mice could be replenishing reduced blood moieties that aid transmission, previously exhausted by high parasitemias. From our experiments, it cannot be excluded whether a similar phenomenon exists for *P. falciparum*, although observed parasitemias are much lower in this system than for *P. berghei*. In our *P. falciparum* experiments, human serum was substituted so that it cannot be determined if we removed toxins at this step. If this is the case, it is possible that any transmission reducing factors have previously mistakenly been attributed to human immunity, although uncharacterized toxins may also be involved. Further experiments with serum substitution in *P. berghei* infected mice blood may decipher if such inhibiting factors are present in serum alone. Additionally, it would be advantageous to perform multiple complimentary experiments examining the impact of antibodies, cytokines and metabolites upon the transmission of *P. falciparum*, which clearly needs to be understood better than we do at present. Recently, Pollitt et al. (2013) reported a negative relationship between *P. berghei* oocyst and sporozoite densities at high oocyst loads in *An. stephensi*. Together with our findings, this strongly suggests that high infection densities impede parasite development at several stages of sporogony in this species combination. The density of the asexual parasite may also be a key component of the density effect.

Our study was designed to investigate the relationship between gametocyte density, intensity of oocyst infection and the efficacy of TRIs. In this specific case, the use of the *P. berghei–An. stephensi* model system has highlighted a negative relationship between the efficacy of TRI and infection intensity, which may be highly relevant for interventions against human malaria. However, interventions which target pre-fertilization stages could potentially give misleading results when using the murine model if particularly high parasite density infections are used; indeed interactions between inhibiting factors and antibodies may happen and induce confounding factors. To avoid misleading experimental conditions, investigators using *P. berghei* may wish to perform MFAs at a range of gametocyte densities where gametocyte numbers and infectivity to mosquito can be positively related.

Using an antibody directed against the *P. berghei* Pbs28 antigen, we observed a constant transmission blocking activity measured as the reduction of oocyst intensity over all infection intensities, whereas a negative relationship between TRI efficacy at reducing prevalence and the degree of infection intensity in the control group was found. This is consistent with previous observations where the relationship between mean parasite load and prevalence in a mosquito population or sample is non-linear: at low and intermediate loads, prevalence increases with parasite load, but at high loads, prevalence reaches a threshold close to 100% and is no longer affected by parasite load. The consequence is that, at high infection levels, a TRI may decrease the parasite load without affecting the prevalence (Churcher et al., 2012). These results clearly demonstrate the need to quote both change in oocyst intensity and infection prevalence to allow different TRI candidates to be accurately compared (Churcher et al., 2012). While the reduction of prevalence of infection will be relevant for predicting the efficacy of the intervention for malaria control, only the efficacy at reducing the infection load will be informative to be compared in different conditions of infection intensities. Moreover, given that *P. berghei–An. stephensi* and *P. falciparum–An. coluzzii* systems revealed a similar relationship between mean parasite load and prevalence (Churcher et al., 2012), we can conjecture that the density dependent TRI efficacy may also occur in the natural system *P. falciparum–An. coluzzii*. This deserves particular attention to investigate if this phenomenon occurs in human malaria vectorial system and for all TRI candidates as it will have major implications for their evaluations: for example, if TRIs are evaluated in conditions where parasite load is very high in artificially infected mosquitoes the TRI efficacy on parasite prevalence is likely to be underestimated.

Our findings, combined with other recent studies suggest the importance of infectious parasite density on the measurement of efficacy of TRI. This highlights the fact that TRI candidates must be evaluated with regard to infection intensity. However it is surprising how little natural infection intensity in natural vectors is documented (but see Billingsley et al., 1994; Gouagna et al., 2010; Medley et al., 1993). This can be explained by difficulties in obtaining a large number of infected mosquitoes in nature. The few studies that reported oocyst loads from naturally infected mosquitoes indicate low numbers of parasites (mean number below 5), which is consistent with the important role of low density gametocyte carriage in humans (Schneider et al., 2007) for malaria transmission. However, more accurate estimates of effective parasite exposure in natural vector populations and subsequent infection intensities are needed in order to evaluate TBV candidates at epidemiologically relevant conditions and accurately predict efficacy of interventions.

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