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# 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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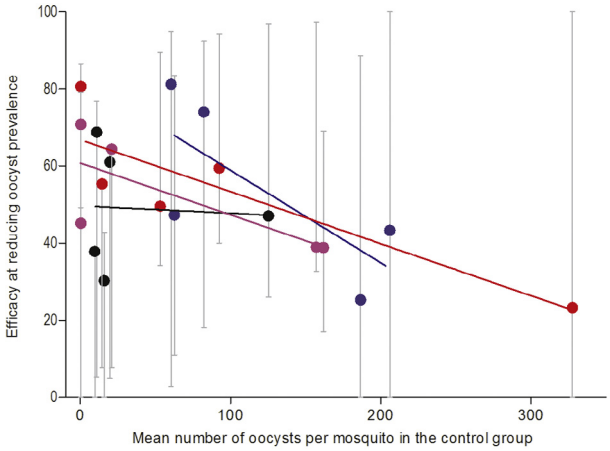
## HIGHLIGHTS

- 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