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Measuring the blockade of malaria transmission – An analysis of the Standard Membrane Feeding Assay

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ABSTRACT

The standard membrane feeding assay (SMFA) is currently considered to be the 'gold standard' for assessing the effectiveness of malaria transmission blocking interventions (TBIs) in vivo. The operation and analysis of SMFAs has varied between laboratories: field scientists often measure TBI efficacy as a reduction in the prevalence of infected mosquitoes whilst laboratory scientists are more likely to quote efficacy as a change in the number of oocysts within the mosquito. These metrics give outputs that differ widely, resulting in a need for greater understanding of how the SMFA informs TBI assessment. Using data from 536 different assays (conducted on *Plasmodium falciparum* and *Plasmodium berghei*, in either *Anopheles gambiae* or *Anopheles stephensi*) it is shown that the relationship between these metrics is complex, yet predictable. Results demonstrate that the distribution of oocysts between mosquitoes is highly aggregated, making efficacy estimates based on reductions in intensity highly uncertain. Analysis of 30 SMFAs carried out on the same TBI confirms that the observed reduction in prevalence depends upon the parasite exposure (as measured by oocyst intensity in the control group), with assays which have lower exposure appearing more effective. By contrast, if efficacy is estimated as a reduction in oocyst intensity, then this candidate demonstrated constant efficacy, irrespective of the exposure level. To report transmission-blockade efficacy accurately, the results of SMFAs should give both the prevalence and intensity of oocysts in both the control and intervention group. Candidates should be assessed against a range of parasite exposures to allow laboratory results to be extrapolated to different field situations. Currently, many studies assessing TBIs are underpowered and uncertainties in efficacy estimates rarely reported. Statistical techniques that account for oocyst over-dispersion can reduce the number of mosquitoes that need to be dissected and allow TBI candidates from different laboratories to be accurately compared.

1. Introduction

The intolerable health and economic burdens imposed by malaria have prompted an ambitious expansion of malaria control activities, recently including local elimination and future eradication as key objectives (Alonso et al., 2011). A key component of this endeavour is now recognised to be the interruption of transmission of the parasites through the mosquito vector (Smith et al., 2011), thereby reducing the number of secondary infections (Shaukat et al., 2010). The efficacy of newly developed transmission blocking interventions (TBIs) needs to be accurately assessed; for example a wide range of highly effective transmission blocking (TB) drugs and vaccines have been developed in the laboratory and these candidates now need to be selected and prioritised to transfer the best of these new tools to the field. This will require a standard method by which to assess the effectiveness of TBIs that will allow results from different laboratories to be compared directly.

The Standard Membrane Feeding Assay (SMFA) was developed from an experimental laboratory protocol devised to study transmission of malaria parasites to their vectors (Boyd, 1949; Ponnudurai et al., 1987). The presence/number of oocysts is used to report the success of infection, although it was quickly appreciated that the production of oocysts did not necessarily correlate with the successful transmission of the parasite from vector to vertebrate host (Boyd, 1949). In the 1970–80's the SMFA gained acceptance as a useful and standard method to determine,

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in vivo, the impact of TB antibodies targeting gametes and ookinetes, for which reporting oocyst production was the most direct readout. The ability to make multiple identical replicates with or without the intervention and to infect replicate groups of vectors in vivo was key to this application, and the assay rapidly acquired the accolade 'gold standard'. In this assay, potential TBI candidates are mixed with infectious gametocytes and fed to mosquitoes which are then dissected after a fixed time interval to determine how efficiently oocysts have developed on the midgut. Although this method is widely used across different laboratories, results are reported in different and often conflicting formats. Studies may report (i) the reduction in the proportion of infected mosquitoes (here defined as oocyst prevalence, Medley et al., 1993) or (ii) the reduction in the mean number of oocysts (here defined as oocyst intensity, see Medley et al., 1993). Experiments on TBIs have focused on the changes in the numbers of parasites in a mosquito as this is deemed the more discriminatory of the two metrics. Despite these two metrics being clearly related (Billingsley et al., 1994), the efficacy of an intervention will be described differently depending on which of the two measurements are used. This uncertainty in presentation reflects the deeper lack of understanding of how malaria transmission and the properties of mosquito infection are measured. Field malariologists tend to report malaria transmission as the Entomological Inoculation Rate (EIR), which is the number of potentially infectious bites received per person per time unit, in which a mosquito is deemed infectious if they have salivary gland sporozoites, regardless of their number (Smith et al., 2005). The apparent dichotomy between assay readouts needs to be resolved if appropriate decisions about which potential TBIs to take forward into field trials are to be made.

To allow different TBI candidates to be compared fully in the SMFA it is essential to understand the uncertainty around point estimates, an issue that is rarely discussed in the scientific literature. Transmission 'blockade' is conventionally measured by the change (fall or rise) in oocyst prevalence/intensity, induced by the intervention, by comparison with an appropriate, control replicate. The results are expressed as:

$$\text{Efficacy} = \frac{C - E}{C} \times 100 \quad (1)$$

where C is the mean prevalence/intensity in the control group and E is the mean prevalence/intensity in the intervention group (Lensen et al., 1999; Blagborough et al., 2012). The assay is repeated a number of times (typically three to five paired comparisons) and the mean efficacy reported. Results show here, that the accuracy of a result can vary dramatically depending on the number of mosquitoes dissected in both the control and intervention groups, and the number of replicate feeds carried out.

Perhaps the biggest practical drawback of the SMFA is the cost of the assay. Dissection of mosquito midguts and enumeration of oocysts, particularly when working with human pathogens, imposes exceptionally high manpower, time and infrastructure costs. Consequent financial and/or practical pressures lead many to reduce the number of feeds and mosquitoes dissected to a 'useful' minimum. The number of feeds conducted and mosquitoes dissected varies substantially between laboratories and power calculations are rarely determined, making comparison of different candidates even within the same laboratory potentially imprecise. An early critique of the assay used bootstrapping methodology to examine factors that influenced the reproducibility of the method and concluded that a study comparing oocyst intensity must have a control group oocyst mean intensity of $\geqslant 35$ (van der Kolk et al., 2005). Although this might be desirable in some cases, in reality oocyst counts in the control group are often significantly lower than this, and the results deemed to be less 'valuable'. As a result, more generalisable guidelines are required.

Using data from 536 SMFA studies on both *Plasmodium falciparum* and *Plasmodium berghei* in *Anopheles gambiae* or *Anopheles stephensi*, the underlying relationship between oocyst prevalence and intensity is re-examined. It is shown how this relationship explains why the same TBI candidate may give very different estimates of TBI efficacy under different experimental conditions, discuss the consequent operational constraints of the SMFA, and suggest an improved method of interpreting the data.

2. Materials and methods

2.1. Parasites and mosquitoes

General parasite maintenance was carried out as described previously (Sinden, 2002; Blagborough et al., 2012). *Plasmodium berghei* strains ANKA 2.34, *Pb*GFPCON 259cl2 (Janse et al., 2006), or *Pb*GFPCON 507cl1, were maintained in 4–10 week old female Tuck's Ordinary (TO) or CD1 mice (Harlan, UK) by serial mechanical passage (up to a maximum of eight passages). Hyper-reticulocytosis was induced 2–3 days before infection with 200 µl i.p. phenylhydrazinium chloride (PH; 6 mg/ml in PBS; ProLabo, UK). Mice were then infected i.p. with 10^8 *P. berghei*-infected mouse blood cells and infections monitored with Giemsa-stained tail blood smears (Sinden, 2002). *Plasmodium falciparum* strains 3D7 and NF54 were maintained in asynchronous culture as described previously (Ifediba and Vanderberg, 1981; Graves et al., 1984). *Anopheles stephensi* mosquito strain SDA 500 and *A. gambiae* strains N'Gousso and G3 were produced as described previously (Sinden, 2002), and maintained on fructose (8% (w/v) fructose, 0.05% (w/v) *p*-aminobenzoic acid).

2.2. The Standard Membrane Feeding Assay

2.2.1. Plasmodium berghei

Batches of *Anopheles* housed in pots at 19 °C were starved for 24 h and then fed on heparinised *P. berghei*-infected blood using standard membrane feeding methods (Sinden, 1996). Briefly, infected mice were exsanguinated into heparinised syringes. Heparinised blood was then added to tubes (at 37 °C) containing the antibody or drug under investigation with appropriate controls (Normal/pre-immune serum with or without the drug (solvent only)). These mixed preparations were offered through Parafilm® membranes on feeders at 37 °C to replicate pots of 50–200 female mosquitoes obtained from the same generation/cohort. Twenty-four hours after feeding, mosquitoes were briefly anesthetised with CO_2 and unfed mosquitoes were removed. Mosquitoes were then maintained at 19–22 °C and 50–80% relative humidity. Recognising that midgut oocyst numbers vary over time (Dawes et al., 2009b,) midguts were dissected only on day 10 post-feeding. Mosquito midguts were dissected and cohort size, infection prevalence and the mean number of oocysts on the midgut (intensity), were measured for each pot of mosquitoes (Sinden, 1996; Blagborough et al., 2012).

2.2.2. Plasmodium falciparum

Mature gametocytes of *P. falciparum* (3D7 and NF54) were produced in vitro as described previously (Ifediba and Vanderberg, 1981; Graves et al., 1984). Briefly, mature gametocyte cultures (0.015–0.4% final gametocytaemia) were fed for 30 min at 37 °C to mosquitoes through a Parafilm® membrane. Engorged mosquitoes were housed in pots at 28 °C and 60–80% relative humidity. On days 8–10, midguts were dissected and the results analysed as in Section 2.2.1.

2.3. Mathematical analysis

A summary of the data used is given in Supplementary Table S1. The number of oocysts in a mosquito is highly over-dispersed (aggregated relative to the normal distribution) so here the statistical analysis was carried out using data from individual dissected mosquitoes (as opposed to mean oocyst estimates which is shown to be highly uncertain at low sample sizes). Among the 536 experiments analysed, individual mosquito count data were available for 189 different experimental feeds, in which a total of 9,119 mosquitoes were dissected (Supplementary Table S2). The relationship between oocyst prevalence and intensity was estimated by extending the methods of Medley et al. (1993) which fit a relationship between the mean parasite intensity and parasite prevalence using a binomial distribution. To fully capture the uncertainty in oocyst estimates, a full negative binomial distribution is used to allow the number of oocysts in infected mosquitoes to influence the distribution. To account for the abundance of uninfected mosquitoes seen, a zero-inflated negative binomial distribution was also tested. A range of different functional forms are used to describe how k (an inverse measure of aggregation; see Eq. (1) of Medley et al., 1993) changes with oocyst intensity. The most parsimonious models were chosen using the likelihood ratio test.

Estimates of the number of mosquitoes that need to be dissected (power calculations) were generated by sampling from the best fit distribution and functions according to the stated sample size and using the results from Eq. (1) (with simulations run a 10,000 times to generate 95% confidence intervals). Statistics were further refined using generalised linear mixed models (GLMMs), with binomial error structure for prevalence data and zero-inflated negative binomial for intensity data (Bolker et al., 2009). The uncertainty around point estimates was calculated by bootstrapping (for both mosquitoes and experimental feeds when estimates involving multiple assays were required).

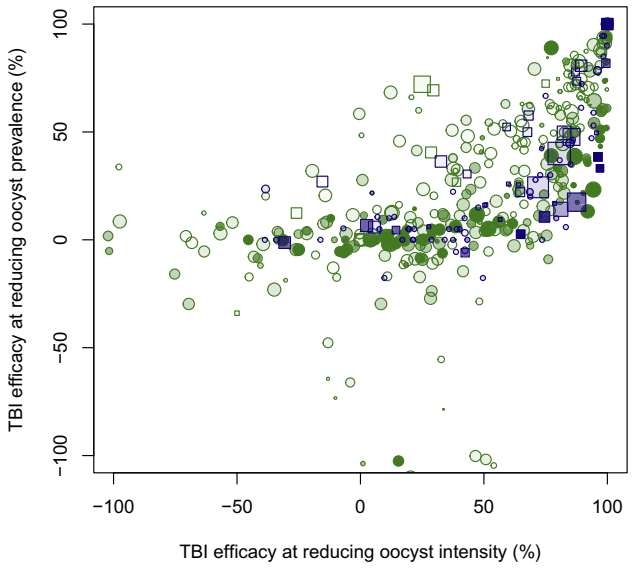

Fig. 1. Results of 536 Standard Membrane Feeding Assays (SMFAs) used to assess the effectiveness of 132 different transmission blocking intervention (TBI) candidate drugs and sera. Each point represents the efficacy of a single paired experiment as estimated using Eq. (1) (using the arithmetic mean). The colour of the point represents the parasite species used, be it *Plasmodium falciparum* (blue) or *Plasmodium berghei* (green) whilst the depth of the colour reflects the parasite exposure (darker shading reflecting higher mean intensity in the control group). The size of the point is relative to the number of mosquitoes dissected in each paired experiment whilst the shape of the point denotes the species of vector used, either *Anopheles gambiae* (square) or *Anopheles stephensi* (circle). Negative points on either axis indicate estimates where the TBI candidate appeared to increase either oocyst prevalence or intensity, although whether this is due to transmission enhancement or measurement error associated with the assay cannot be determined. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3. Results

3.1. The relationship between oocyst prevalence and intensity

The results of 536 TBI assays on 132 different TBI candidates are shown in Fig. 1. This graph suggests that interventions can have widely different combinations of reported abilities to reduce oocyst prevalence and intensity. Superficial viewing might suggest that for both *P. falciparum* and *P. berghei* a substantial reduction in intensity (~70%), may be required before a noticeable (~10%) impact upon prevalence is achieved. However, further analysis suggests that this is not the case (see below).

The relationship between oocyst prevalence and intensity is shown in Fig. 2A. For what is believed to be the first time, these data show that the same relationship holds in both the control and the experimental groups. Fitting separate curves to the control and experimental groups does not significantly improve the fit of the model (likelihood ratio test P value = 0.060; see Supplementary Fig. S1). This suggests that if an intervention reduces oocyst intensity then the reduction seen in prevalence is entirely predicted by the course of the black line plotted in Fig. 2A. The shape of the best fit relationship can then be used to show what reduction in prevalence is expected for a given reduction in intensity in experiments in which the control intensity ranges from one to 100 oocysts (Fig. 2B), i.e. in an individual SMFA assay, if the control group has a mean oocyst intensity of 100 then one would only expect to see a reduction in oocyst prevalence of 20% from a TBI which reduced oocyst intensity by 90% (Fig. 2B). Upon closer inspection of Fig. 1 this can also be seen in the larger dataset of 536 paired experiments. Darker points signifying higher oocyst densities in the control group are more likely to be to the right of the x-axis, showing the strongly non-linear relationship between blockade of prevalence and blockade of intensity. The majority of data points clearly suggest a highly non-linear relationship between the reduction in prevalence and the reduction in intensity. This will be exacerbated by the current assumption that high control oocyst numbers equate to a 'successful' experiment within the majority of laboratory studies (Fig. 2B).

Whilst recognising that the majority of the *P. falciparum* data examined were gathered in different laboratories to the *P. berghei* data, it is interesting to note that the prevalence-intensity relationship appears relatively consistent between both parasite and vector combinations (Fig. 2A). Nevertheless statistical analysis shows that fitting different curves (Supplementary Fig. S2) to the different parasite species separately significantly improves the fit of the models ($P < 0.001$). Further analysis comparing data from *P. falciparum* and *P. berghei* from the same laboratory is needed to verify this result, although for the purposes of this study a single curve fitted to the whole dataset is used as it will make relatively little practical difference to power calculations. Laboratories may wish to improve their power estimates by fitting their own parasite-vector combination-specific prevalence-intensity relationships.

3.2. The aggregated distribution of oocysts

Medley et al. (1993) originally highlighted that the number of oocysts in each mosquito is far from normally distributed. They demonstrated that there are significantly more uninfected mosquitoes for a given mean oocyst intensity than you would expect with a Poisson (normal) distribution. This report goes further and

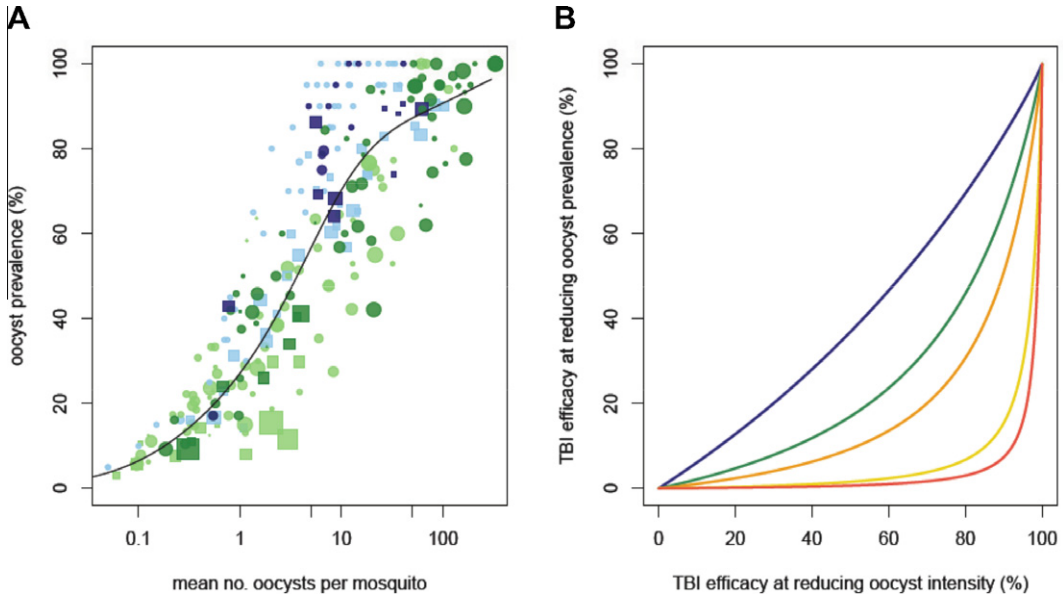

Fig. 2. The relationship between oocyst prevalence and intensity and how this relationship will determine the effectiveness of transmission blocking interventions (TBIs). (A) Oocyst prevalence – intensity relationship estimated from 189 Standard Membrane Feeding Assays (SMFAs). The colour of the point represents the parasite species used: *Plasmodium falciparum* (blue) or *Plasmodium berghei* (green). The dark shade denotes SMFA controls whilst the lighter shade signifies experiments with candidate TBIs. The size of the points indicates the number of mosquitoes dissected whist the shape denotes the species of vector used, either *Anopheles gambiae* (square) or *Anopheles stephensi* (circle). Oocyst intensity is shown as the arithmetic mean. The black line indicates the best fit model (see Supplementary Table S3 for the best fit parameters). (B) The expected relationships between efficacy and oocyst prevalence and intensity when using different parasite exposures according to the model presented in (A). The colours of the lines indicate the mean oocyst intensity in the control group, one (blue), five (green), 10 (orange), 50 (yellow) or 100 (red) oocysts. This can be compared with Fig. 1. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

examines the overall distribution of parasites in infected mosquitoes and the number of uninfected mosquitoes observed. Results indicate that the degree of oocyst aggregation (over-dispersion) is even greater than previously described. Using a zero-inflated negative binomial distribution (Log-likelihood: −21686) instead of a normal negative binomial distribution (Log-likelihood: −21846; as used by Medley et al., 1993) significantly improved the fit of the best-fit model ($P < 0.001$). Across the whole dataset, 79% of oocysts were found in just 10% of all mosquitoes. The degree of oocyst aggregation (as measured by the variance to mean ratio) increases with increasing infection intensity (see Supplementary Fig. S3). This aggregation can substantially increase the uncertainty around study results, particularly in SMFAs with high parasite exposure.

3.3. Understanding the uncertainty around estimates generated using the current method of analysis

3.3.1. Number of mosquitoes dissected

Parasite aggregation makes estimates of oocyst intensity highly uncertain. This uncertainty is exacerbated when a relatively small number of mosquitoes are dissected. This is illustrated in Fig. 3A which is parameterised using the results of the model fitted in Fig. 2A and described in Supplementary Data S1. If only 20 mosquitoes were dissected, then a mean oocyst intensity of 20 would have 95% confidence interval estimates that ranged from ~10 to ~50. A worked interpretation and practical application of these findings is described within Supplementary Data S2. Dividing one uncertain result by another uncertain result (as done in Eq. 1) generates highly uncertain estimates of TBI efficacy. The uncertainty is worse if there is a low intensity of oocysts in the control group (i.e. low parasite exposure in the absence of a TBI), as small absolute changes in the denominator can have a big effect on the overall estimate of efficacy. This is illustrated in Fig. 3B for an intervention with a true efficacy of 80%. If, as commonly practised, 20 mosquitoes are dissected in both the control and the intervention group, the 95% confidence intervals for the efficacy could range between −10% (enhancement) to 100% depending on the oocyst intensity in the control group. Even if the control group had a mean oocyst intensity of 50, the confidence intervals would still be relatively large (~50% to ~95%). To have relative confidence in the precision of the efficacy estimates will require large numbers of mosquitoes to be dissected (Fig. 3C). There is considerably more certainty in the efficacy estimates of highly effective interventions because the degree of aggregation decreases with mean oocyst intensity. This means that interventions with a lower efficacy will typically show greater variability in the efficacy observed when using the SMFA. Similar uncertain predictions were generated for efficacy estimates that rely on prevalence (Fig. 3D and E). The same problems arise when using Eq. (1), making SMFA results highly uncertain.

3.3.2. Number of experimental feeds

Decisions about potential TBIs are currently being made using relatively small numbers of experimental replicates (often as low as one). The results of 30 different paired assays using the same TBI candidate (anti *Pbs28* monoclonal antibody (mAb) 13.1) are shown in Fig. 4A and B. There is considerable variation in assay readout, between experimental feeds which could be explained by heterogeneity between hosts (mice in this case), feeding apparatus, experimental technique or cage effects. This uncertainty in efficacy should be taken into consideration when designing experiments but also in the statistical analysis. Mosquitoes from the same cage will not be statistically independent as they are more likely to have similar levels of infection than mosquitoes fed and reared for a replicate experiment. An example of this "cage effect" could be the degree with which the parafilm is stretched over the feeding device as that might influence mosquito infectivity for all mosquitoes within that experimental feed, or the microbiota present in the gut of mosquitoes reared in a single cohort. Studies

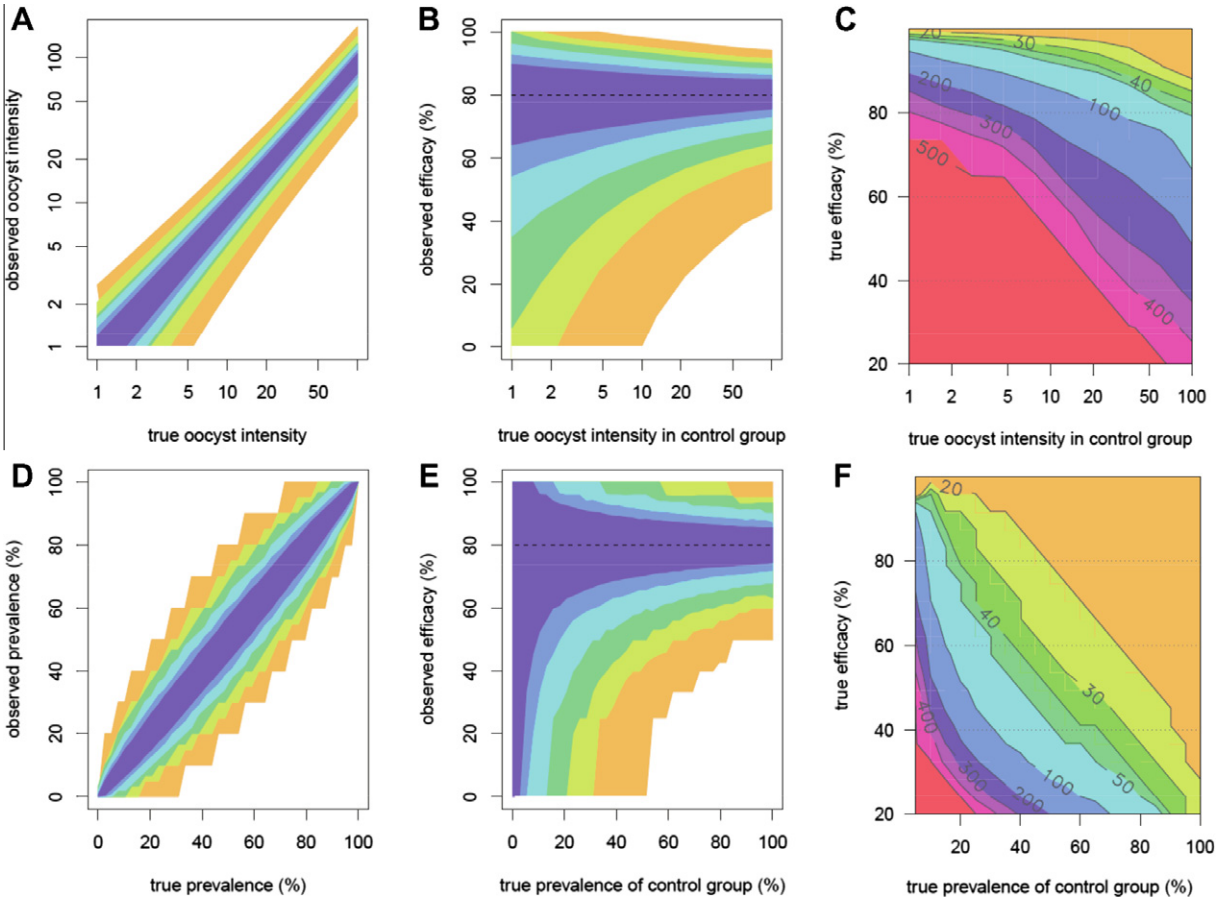

Fig. 3. Indications of the uncertainties in efficacy estimates generated by 30 repeat Standard Membrane Feeding Assays (SMFAs) conducted on the same transmission blocking intervention (TBI) candidate in the same laboratory using the standard method. Efficacy is measured as either a reduction in the mean number of *Plasmodium berghei* oocysts developing within *Anopheles stephensi* (A–C) or oocyst prevalence (D–F). (A) and (D) show the range of oocyst intensity estimates that could be observed for a given true oocyst intensity according to the number of mosquitoes dissected, be it 10 (orange), 20 (yellow), 30 (green), 50 (light blue), 100 (dark blue), 200 (purple). (B) and (E) show the range of efficacies that could be generated by a TBI candidate with a true efficacy of 80% (denoted by the black dashed line). Shaded colours represent different sample sizes (as above). (C) and (F) show the minimum number of mosquitoes that need to be dissected to ensure that reported efficacy is within 10% of the true efficacy. If the number of mosquitoes required is >500 then a value of 500 is given. All figures show 95% confidence interval estimates. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

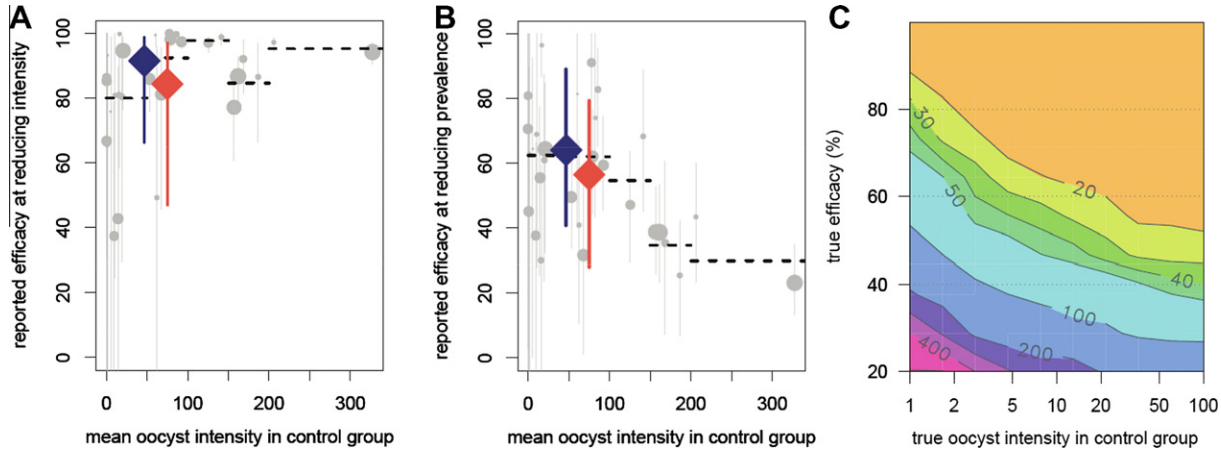

Fig. 4. The range of efficacies seen for a single transmission blocking intervention (TBI) candidate calculated using oocyst intensity (A) or prevalence (B). Each grey point indicates a paired *Plasmodium berghei – Anopheles stephensi* assay with the size of the point representing the mean number of mosquitoes dissected in the experiment. Vertical lines indicate 95% confidence interval estimates. Black dotted lines show the average result pooling experiments of similar oocyst intensities in the control group together (bin range, 0–50, 50–100, 100–150, 150–200, 200+). The large red point indicates the average efficacy of the candidate (estimated using Eq. (1)) whilst the blue point shows estimates derived using a mixed-effects model. (C) The minimum number of mosquitoes that need to be dissected to ensure that observed TBI efficacy is within 10% of the true efficacy using the mixed-effects model. This method substantially reduces the number of mosquitoes that need to be dissected compared with the traditional method (Fig. 3C). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

which fail to account for this variability risk describing inaccurate TBI efficacies and, subsequently, differences between different TBI candidates when they do not exist. Ideally multiple feeders and cages should be used for each feed and it is suggested the results be analysed using GLMMs. This multi-level analysis allows a single estimate of the TBI efficacy to be estimated (the fixed effect) whilst allowing the level of infection in the different assays (i.e. using different sources of blood, feeders or cages) to vary at random (the random effect, see Bolker et al., 2009). GLMMs can allow the error structure of the data to be described by a variety of different statistical distributions. This is particularly appealing when dealing with intensity data since it will reduce the variability associated with the overdispersed data. These models also allow the overall estimate to be weighted by the certainty in the individual feeding estimates (determined by the number of mosquitoes dissected), something which is ignored using the current standard method. The advantages of using a GLMM as opposed to Eq. (1) are illustrated in Fig. 4C (in comparison with Fig. 3F). Using the GLMM estimates of efficacy are higher for both oocyst prevalence (64.0% versus 56.5%) and intensity (91.5% versus 84.3%), and for the later the 95% confidence region is reduced by 43%.

The data in Fig. 4 also illustrates how the efficacy of a TBI candidate, calculated by reductions in oocyst prevalence, decreases the higher the oocyst intensity in the control group (Fig. 4B). Interestingly, if efficacy is estimated as changes in oocyst intensity then the candidate has approximately the same efficacy irrespective of the oocyst intensity in the control group (Fig. 4A) – this can be shown statistically by including control oocyst intensity as a covariate in the GLMM; reported efficacy was significantly lower in experiments with control group intensities of >100 (Fig. 4B), where efficacy was reported by reduction in infection prevalence ($P < 0.001$) but not in Fig. 4A, where efficacy was reported by reduction in infection intensity ($P = 0.11$).

4. Discussion

Recognising the central importance of developing effective TBIs to achieving the recently rediscovered objectives of local elimination and global eradication of malaria, and the established current central role of the SMFA in evaluating such interventions, the technique has been re-analysed using a substantial body of data gathered by this method on both model rodent (*P. berghei*) and human malaria (*P. falciparum*). Results show that: (i) reductions in oocyst prevalence and intensity follow predictable relationships, (ii) a single TBI investigated in great detail (anti *Pbs28* mAb 13.1) appears, on average, to be equally effective irrespective of the number of parasites ingested within the range tested, (iii) it is suggested that previously published TBI efficacy estimates are highly uncertain due to aggregation of infections in mosquito cohorts and small sample sizes, (iv) different statistical techniques should be used to refine efficacy estimates and optimise power calculations, and (v) it is strongly recommend that for every SMFA carried out authors should report changes in oocyst prevalence and intensity together with the mean control oocyst number and the number of mosquitoes dissected. These points shall be discussed in turn in relation to some of the technical questions challenging TBI assessment.

Currently the efficacy of transmission blocking vaccines (Carter, 2001), drugs (Delves et al., 2012) and other interventions (Cirimotich et al., 2011) are most commonly estimated as a reduction in oocyst prevalence, oocyst intensity or both. Simply giving point estimates of efficacy fails to understand that these results, and their associated uncertainty, will depend on the specific variables of the experiment from which they were derived. Key variables that are rarely made explicit include the fact that blockade was achieved at a particular intensity of exposure (as measured by the mean oocyst number in the control group), and with an accuracy determined by the number of experimental infections and the number of mosquitoes dissected in each experiment. This paper has outlined how these variables have a substantial and predictable impact upon the results reported.

It could be argued that the prevalence of mosquito infection is more important in epidemiological terms than intensity. It is therefore tempting to estimate TBI efficacy as a reduction in oocyst prevalence since detecting significant changes in the number of infected/uninfected is not complicated by highly infected mosquitoes. This method has the advantage in that it is quicker (a microscopist does not need to count oocysts, just detect their presence/absence) and is easily mechanised using oocyst-specific markers (e.g. *P. berghei* PbGFPCON 259cl2 (Vlachou et al., 2004), which produces ookinetes and oocysts expressing GFP in vivo). However, using oocyst prevalence alone as a measure for efficacy has a number of pitfalls. Firstly evidence indicates that the reduction in the number of infected mosquitoes will depend on the parasite exposure, with it being much easier to show reductions in oocyst prevalence if there is a low or intermediate (<50%) oocyst prevalence in the control group. Secondly, in highly infected mosquitoes small changes in oocyst prevalence can mask large differences in oocyst intensity. This can be seen in Fig. 2A where increasing the oocyst prevalence from 90% to 95% would increase oocyst intensity by more than 2.6-fold (from a mean of 87–230). Ultimately, investigators will need all information to assess different TBI candidates so that observed inhibition should be described for each pair of assays, citing both oocyst intensity and prevalence, and the experimental constraints of sample size and control oocyst number.

The clear relationship between oocyst prevalence and intensity, and their respective reductions, will allow epidemiologists to predict how effective an intervention will be at reducing both the number of infected bites and perhaps the number of parasites within these bites. This will allow the results from laboratory assays to be extrapolated to the field, allowing scientists who use different metrics (such as EIR) to communicate effectively. The most effective reduction in the prevalence of oocysts will be when the mean oocyst intensity in wild caught mosquitoes is at its lowest. This is encouraging, recognising that oocyst counts from wild caught mosquitoes are typically very low (Rosenberg, 2008), and that in the majority of malaria-infected areas of the globe, the EIR is reported to be less than one (Gething et al., 2010). Our ability to predict what impact a TBI will have in the field depends on whether TBI candidates have approximately the same efficacy at reducing oocyst intensity, irrespective of intensity of infection in the control group (Fig. 4A). In this study there were only sufficient repeat assays of a single intervention to do this statistical analysis once; we would therefore strongly advise other candidates to be tested in this thorough way (i.e. using gametocyte exposures that generate a wide range of oocyst intensity/prevalence estimates in the control group).

Over the years the SMFA has been refined to maximise transmission. This has been so successful that some laboratories can repeatedly achieve levels of infection with very high oocyst densities, both in laboratory models (Farrance et al., 2011) and using field populations of human parasites (Harris et al., 2012). With such high control oocyst intensities, and recognising the prevalence/intensity relationship described in Fig. 2, it is not surprising that SMFAs have been used to estimate changes in oocyst intensity in order to evaluate TBIs and understand the dynamics of the mosquito immune system. The ability of a TBI candidate to reduce oocyst prevalence has been shown to depend on parasite exposure (as measured by the number of oocysts in the control group, Fig. 4B). As shown in Fig. 2B, just because an intervention fails to achieve a sufficient reduction in oocyst prevalence at high parasite

exposures does not mean that it will not achieve this in field settings. To fully evaluate the effectiveness of a TBI, mosquitoes should be fed on hosts with a wide range of gametocyte densities and not just those with the highest counts. This may involve feeding mosquitoes on hosts with sub-microscopic gametocytemia (Schneider et al., 2007).

There are a number of different reasons why the results of separate SMFAs might provide different readouts for a single intervention. These can be broadly categorised as either 'host' effects (for example differences in immunological responses) or due to intra-laboratory experimental variation when performing the assay (different conditions in the cage or feeding apparatus). Both of these sources of variability need to be embraced to enable different TBI candidates to be compared accurately. Ideally, multiple experimental replicates and mosquito populations should be used with a number of different parasite preparations and the results analysed in a GLMM. This is potentially impractical to perform for each individual assay. We suggest laboratories should estimate their individual SMFA-variability once and use this information to adjust the precision of subsequent feeding experiments. Our data suggests that the number of oocysts in mosquitoes feeding on the same blood sample is best described using a zero-inflated negative binomial distribution. Using a statistical method (such as GLMMs) that takes this distribution into account when analysing oocyst intensity data will reduce the number of mosquitoes that need to be dissected in order to achieve a statistically significant result. To carry out this type of statistical analysis requires individual oocyst counts from all dissected mosquitoes, something that has rarely been made available in the past once mean estimates have been reported.

Swellengrebel (as reported in Boyd and Stratman-Thomas, 1933) advocated that field studies examining malaria infections in mosquitoes should examine a minimum of 100 mosquitoes, and if at that point the estimate of mean oocyst intensity remains variable, that the sample size be continuously expanded until stable values are reached. This commendable ideal is unfortunately not a practical approach to the SMFA. The number of mosquitoes that needs to be dissected will depend on the question under investigation, the certainty with which the null hypothesis is rejected (the study's power) and the type of statistical analysis that will be carried out. It will also depend on the number of experimental feeds to be carried out and the degree of intra-assay variability (as there is no benefit in having highly precise estimates from a single assay if there is considerable intra-assay variability). This paper clearly illustrates that the number of mosquitoes to be dissected will also depend on parasite exposure. Generally, the higher the intensity in the control group, the fewer mosquitoes that need to be dissected. Should the scientific community continue estimating efficacy as reductions in oocyst intensity using the standard method (Eq. 1), it must be acknowledged that a single SMFA will require >100 mosquitoes to be dissected to ensure that the recorded efficacy is within 10% of the true efficacy, if that efficacy is 80% or less (Fig. 3C). Fig. 3 can help inform this decision (a worked example of this is given in Supplementary Data S2). Ultimately no TBI efficacy estimate should be given without an expression of the uncertainty around the point estimate. This can be simply calculated using the GLMM or generated using bootstrapping methodology using the traditional method (Eq. 1).

Perhaps the most frustrating aspect of this discussion is our need to conclude the analysis at the point of oocyst burden. Surprisingly, despite extensive studies (Boyd and Stratman-Thomas, 1933; Vaughan et al., 1992, 1999; Pumpuni et al., 1997; Beier, 1998; Frischknecht et al., 2004; Medica and Sinnis, 2005; Zollner et al., 2006; Kebaier et al., 2009; Conteh et al., 2010) it is only in *P. berghei* that the relationship between oocyst number and salivary gland sporozoite burden has been described (Dawes et al., 2009a), and for no species is the relationship between the gross salivary gland sporozoite load and the contribution of the mosquito to overall transmission known (the mosquito to human basic reproduction number). This needs urgent resolution as without this knowledge the impact that partially effective TBIs can have on person-to-person transmission cannot easily be assessed.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ijpara.2012.09.002.

References

Alonso, P.L., Brown, G., Arevalo-Herrera, M., Binka, F., Chitnis, C., Collins, F., Doumbo, O.K., Greenwood, B., Hall, B.F., Levine, M.M., Mendis, K., Newman, R.D., Plowe, C.V., Rodriguez, M.H., Sinden, R., Slutsker, L., Tanner, M., 2011. A research agenda to underpin malaria eradication. PLoS Med. 8, e1000406.

Beier, J.C., 1998. Malaria parasite development in mosquitoes. Annu. Rev. Entomol. 43, 519–543.

Billingsley, P.F., Medley, G.F., Charlwood, D., Sinden, R.E., 1994. Relationship between prevalence and intensity of *Plasmodium falciparum* infection in natural populations of *Anopheles* mosquitoes. Am. J. Trop. Med. Hyg. 51, 260–270.

Blagborough, A.M., Delves, M.J., Ramakrishnan, C., Lal, K., Butcher, G.A., Sinden, R.E., 2012. Assessing transmission-blockade in *Plasmodium* spp. In: Malaria Methods and Protocols. Humana Press Inc., Totowa, NJ (Chapter 7.6).

Bolker, B.M., Brooks, M.E., Clark, C.J., Geange, S.W., Poulsen, J.R., Stevens, M.H., White, J.S., 2009. Generalized linear mixed models: a practical guide for ecology and evolution. Trends Ecol. Evol. 24, 127–135.

Boyd, M.F., 1949. Epidemiology: factors related to the definitive host. In: Boyd, M.F. (Ed.), Malariology. W.B. Saunders, Philadelphia, pp. 608–697.

Boyd, M.F., Stratman-Thomas, W.K., 1933. Studies on benign tertian malaria. 2. The clinical characteristics of the disease in relation to the dosage of sporozoites. Am. J. Hyg. 17, 666–685.

Carter, R., 2001. Transmission blocking malaria vaccines. Vaccine 19, 2309–2314.

Cirimotich, C.M., Clayton, A.M., Dimopoulos, G., 2011. Low- and high-tech approaches to control *Plasmodium* parasite transmission by *Anopheles* mosquitoes. J. Trop. Med. 2011, 891342.

Conteh, S., Chattopadhyay, R., Anderson, C., Hoffman, S.L., 2010. *Plasmodium yoelii*-infected *A. stephensi* inefficiently transmit malaria compared to intravenous route. PLoS ONE 5, e8947.

Dawes, E.J., Churcher, T.S., Zhuang, S., Sinden, R.E., Basáñez, M.G., 2009a. *Anopheles* mortality is both age- and *Plasmodium*-density dependent: implications for malaria transmission. Malar. J. 8, 228.

Dawes, E.J., Zhuang, S., Sinden, R.E., Basáñez, M.G., 2009b. The temporal dynamics of Plasmodium density through the sporogonic cycle within *Anopheles* mosquitoes. Trans. R. Soc. Trop. Med. Hyg. 103, 1197–1198.

Delves, M., Plouffe, D., Scheurer, C., Meister, S., Wittlin, S., Winzeler, E.A., Sinden, R.E., Leroy, D., 2012. The activities of current antimalarial drugs on the life cycle stages of *Plasmodium*: a comparative study with human and rodent parasites. PLoS Med. 9, e1001169.

Farrance, C.E., Rhee, A., Jones, R.M., Musiychuk, K., Shamloul, M., Sharma, S., Mett, V., Chichester, J.A., Streatfield, S.J., Roeffen, W., van de Vegte-Bolmer, M., Sauerwein, R.W., Tsuboi, T., Muratova, O.V., Wu, Y., Yusibov, V., 2011. A plant-produced Pfs230 vaccine candidate blocks transmission of *Plasmodium falciparum*. Clin. Vaccine Immunol. 18, 1351–1357.

Frischknecht, F., Baldacci, P., Martin, B., Zimmer, C., Thiberge, S., Olivo-Marin, J.C., Shorte, S.L., Menard, R., 2004. Imaging movement of malaria parasites during transmission by *Anopheles* mosquitoes. Cell. Microbiol. 6, 687–694.

Gething, P.W., Patil, A.P., Smith, D.L., Guerra, C.A., Elyazar, I.R., Johnston, G.L., Tatem, A.J., Hay, S.I., 2010. A new world malaria map: *Plasmodium falciparum* endemicity in 2010. Malar. J. 10, 378.

Graves, P.M., Carter, R., McNeill, K.M., 1984. Gametocyte production in cloned lines of *Plasmodium falciparum*. Am. J. Trop. Med. Hyg. 33, 1045–1050.

Harris, C., Morlais, I., Churcher, T.S., Awono-Ambene, P., Gouagna, L.C., Dabire, R.K., Fontenille, D., Cohuet, A., 2012. *Plasmodium falciparum* produce lower infection intensities in local versus foreign *Anopheles gambiae* populations. PLoS ONE 7, e30849.

Ifediba, T., Vanderberg, J.P., 1981. Complete in vitro maturation of *Plasmodium falciparum* gametocytes. Nature 294, 364–366.

Janse, C.J., Franke-Fayard, B., Mair, G.R., Ramesar, J., Thiel, C., Engelmann, S., Matuschewski, K., van Gemert, G.J., Sauerwein, R.W., Waters, A.P., 2006. High efficiency transfection of *Plasmodium berghei* facilitates novel selection procedures. Mol. Biochem. Parasitol. 145, 60–70.

Kebaier, C., Voza, T., Vanderberg, J., 2009. Kinetics of mosquito-injected *Plasmodium* sporozoites in mice. Fewer sporozoites are injected into sporozoite-immunized mice. PLoS Pathog. 5, e1000399.

Lensen, A., Bril, A., van de Vegte, M., van Gemert, G.J., Eling, W., Sauerwein, R., 1999. *Plasmodium falciparum*: infectivity of cultured, synchronized gametocytes to mosquitoes. Exp. Parasitol. 91, 101–103.

Medica, D.L., Sinnis, P., 2005. Quantitative dynamics of *Plasmodium yoelii* sporozoite transmission by infected anopheline mosquitoes. Infect. Immun. 73, 4363–4369.

Medley, G.F., Sinden, R.E., Fleck, S., Billingsley, P.F., Tirawanchai, N., Rodriguez, M.H., 1993. Heterogeneity in patterns of malarial oocyst infections in the mosquito vector. Parasitology 106, 441–449.

Ponnudurai, T., van Gemert, G.J., Bensink, T., Lensen, A.H., Meuwissen, J.H., 1987. Transmission blockade of *Plasmodium falciparum*: its variability with gametocyte numbers and concentration of antibody. Trans. R. Soc. Trop. Med. Hyg. 81, 491–493.

Pumpuni, C.B., Mendis, C., Beier, J.C., 1997. *Plasmodium yoelii* sporozoite infectivity varies as a function of sporozoite loads in *Anopheles stephensi* mosquitoes. J. Parasitol. 83, 652–655.

Rosenberg, R., 2008. Malaria: some considerations regarding parasite productivity. Trends Parasitol. 24, 487–491.

Schneider, P., Bousema, J.T., Gouagna, L.C., Otieno, S., van de Vegte-Bolmer, M., Omar, S.A., Sauerwein, R.W., 2007. Submicroscopic *Plasmodium falciparum* gametocyte densities frequently result in mosquito infection. Am. J. Trop. Med. Hyg. 76, 470–474.

Shaukat, A.M., Breman, J.G., McKenzie, F.E., 2010. Using the entomological inoculation rate to assess the impact of vector control on malaria parasite transmission and elimination. Malar. J. 9, 122.

Sinden, R.E., 1996. Infection of mosquitoes with rodent malaria. In: Crampton, J.M., Beard, C.B., Louis, C. (Eds.), Molecular Biology of Insect Disease Vectors: A Methods Manual. Chapman & Hall, London, UK, pp. 67–91.

Sinden, R.E., 2002. Molecular interactions between *Plasmodium* and its insect vectors. Cell. Microbiol. 4, 713–724.

Smith, D.L., Dushoff, J., Snow, R.W., Hay, S.I., 2005. The entomological inoculation rate and *Plasmodium falciparum* infection in African children. Nature 438, 492–495.

Smith, T.A., Chitnis, N., Briet, O.J., Tanner, M., 2011. Uses of mosquito-stage transmission-blocking vaccines against *Plasmodium falciparum*. Trends Parasitol. 27, 190–196.

van der Kolk, M., De Vlas, S.J., Saul, A., van de Vegte-Bolmer, M., Eling, W.M., Sauerwein, R.W., 2005. Evaluation of the Standard Membrane Feeding Assay (SMFA) for the determination of malaria transmission-reducing activity using empirical data. Parasitology 130, 13–22.

Vaughan, J.A., Noden, B.H., Beier, J.C., 1992. Population dynamics of *Plasmodium falciparum* sporogony in laboratory-infected *Anopheles gambiae*. J. Parasitol. 78, 716–724.

Vaughan, J.A., Scheller, L.F., Wirtz, R.A., Azad, A.F., 1999. Infectivity of *Plasmodium berghei* sporozoites delivered by intravenous inoculation versus mosquito bite: implications for sporozoite vaccine trials. Infect. Immun. 67, 4285–4289.

Vlachou, D., Zimmermann, T., Cantera, R., Janse, C.J., Waters, A.P., Kafatos, F.C., 2004. Real-time, in vivo analysis of malaria ookinete locomotion and mosquito midgut invasion. Cell. Microbiol. 6, 671–685.

Zollner, G.E., Ponsa, N., Garman, G.W., Poudel, S., Bell, J.A., Sattabongkot, J., Coleman, R.E., Vaughan, J.A., 2006. Population dynamics of sporogony for *Plasmodium vivax* parasites from western Thailand developing within three species of colonized *Anopheles* mosquitoes. Malar. J. 5, 68.