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Quantitative Dynamics of *Plasmodium yoelii* Sporozoite Transmission by Infected Anopheline Mosquitoes

Darcy L. Medica† and Photini Sinnis*

Department of Medical and Molecular Parasitology, New York University School of Medicine, 341 E. 25th St., New York, New York 10010

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Malaria transmission begins with the injection of *Plasmodium* sporozoites into the skin of a vertebrate host by infected anopheline mosquitoes. Although the size of the sporozoite inoculum likely affects the course of the disease, the number of sporozoites injected by infected mosquitoes has not been determined in vivo. Using a quantitative PCR assay, we determined the number of sporozoites injected into mice by single mosquitoes. Analysis of 59 mosquito feedings showed that a single infected mosquito injected between 0 and 1,297 sporozoites, with a mean of 123 and a median of 18. Twenty-two percent of infected mosquitoes injected no sporozoites. The number of sporozoites injected was only weakly correlated to the salivary gland load. To better understand the large variability in sporozoite injection among mosquitoes, we quantified the sporozoites injected by individual mosquitoes on three different days. Approximately 20% of moderately to heavily infected mosquitoes injected few to no sporozoites on all 3 days, suggesting that some mosquitoes are poor transmitters of sporozoites. Other mosquitoes injected high numbers of sporozoites on at least one of the days observed and minimal numbers on the other day(s), supporting the hypothesis that sporozoite injection is discontinuous, a pattern that may aid in the establishment of malaria infection.

Malaria is transmitted by an infected anopheline mosquito which injects *Plasmodium* sporozoites into the skin of the vertebrate host as it is probing for blood. Sporozoites migrate from the site of injection, enter the bloodstream, and are carried to the liver, where they invade hepatocytes and develop into exoerythrocytic forms. Each mature exoerythrocytic form contains between 10,000 to 30,000 merozoites which emerge from the hepatocyte and invade erythrocytes. Although it is known that an infected mosquito injects only a small proportion of its salivary gland sporozoites during a single feeding (15), the exact number of sporozoites injected has not been determined in vivo.

Determination of the size of the sporozoite inoculum is of interest for several reasons. First, sporozoite dose is likely a critical factor in determining the severity of disease (4). Second, it is important to know the challenge dose received by volunteers in malaria vaccine trials. Currently, volunteers receive five infective mosquito bites, but the sporozoite dose and its effect on vaccine efficacy are not known (13). Finally, an understanding of the factors that determine the numbers of sporozoites transmitted during mosquito probing could enable us to refine our understanding of the entomological inoculation rate which in turn could lead to more precise mathematical models of malaria transmission.

The size of the sporozoite inoculum has been previously investigated in vitro using two different methodologies. For the first methodology, anesthetized and dismembered mosquitoes were forced to salivate into capillary tubes containing mineral oil, a sucrose solution, or blood, and the number of sporozoites ejected was quantified (1, 3, 14). In order to more closely mimic natural feeding conditions, other investigators used feeding chambers covered with membranes or mouse skin and counted sporozoites injected into the chamber cover (2, 10). The data from these in vitro studies were the first indication that the size of the sporozoite inoculum is usually small and that some infected mosquitoes may not inject sporozoites during probing. However, as the investigators themselves noted, these are not ideal experimental systems. It is not known whether forced salivation into capillary tubes or probing on artificial membranes is similar to the conditions that a mosquito encounters when it probes for blood on a live animal. Therefore, it cannot be concluded that the number of sporozoites ejected by mosquitoes in these systems reflects sporozoite injection into a vertebrate host. Here, we present quantitative data on the number of sporozoites injected when infected mosquitoes feed on live animal hosts and we analyze the relationship between gland load and sporozoite injection.

MATERIALS AND METHODS

Sporozoites. *Anopheles stephensi* mosquitoes were infected with the rodent malaria parasite *Plasmodium yoelii* as previously described (17), and mosquitoes were used on day 14 or 15 post-infective blood meal (p.i.) for the single-mosquito feeds and on the indicated days for the multiple-day feeds.

Single-mosquito feeds. Female Swiss Webster mice were anesthetized and maintained at 37°C on a slide warmer. Mosquitoes were starved for 12 to 18 h prior to feeding. Individual mosquitoes were isolated in a small feeding cage made of Plexiglas tubing (1-cm diameter) with fine mesh over one end and sealed with parafilm on the other end. The mosquito cage was placed mesh-side down on the ventral side of the mouse's ear which was taped so that the mosquito could probe only on a restricted portion. The mosquito probed for 3 minutes, during which time probing behavior was observed using a 2× hand-held lens. The mouse ear was then removed, weighed, snap-frozen in liquid nitrogen, and stored at −70°C until RNA extraction. Because of limitations in the RNA extraction procedure, the weight of the excised portion of the ear was not allowed to exceed 20 mg. After feeding, the salivary glands of each mosquito were placed onto

* Corresponding author. Mailing address: Department of Medical and Molecular Parasitology, New York University School of Medicine, 341 E. 25th St., New York, NY 10010. Phone: (212) 263-6818. Fax: (212) 263-8116. E-mail: photini.sinnis@med.nyu.edu.

† Present address: Biology Department, Pennsylvania State University Capital College, Schuylkill Campus, Schuylkill Haven, Pa.

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coverslips, squashed with another coverslip, and observed by phase microscopy to determine the intensity of infection. The glands were then stored at 4°C until RNA extraction. Each mouse was used for two mosquito feeds, one on each ear. Blood smears were made from the probed-upon mice 7 and 14 days after the experiment, stained with Giemsa stain, and checked for the presence of parasites. A smear was considered negative if no parasites were seen in 100 fields.

Single-mosquito feeds on multiple days. Individual mosquitoes were allowed to feed on three sequential (days 15, 16, and 17 p.i.) or alternate days (days 15, 17, and 19 p.i.). Between feedings, mosquitoes were kept in a humidified incubator at 24°C and given access to sugar water, which was removed 12 h before the next feeding. Salivary gland infection was determined on the last day of the experiment. Only data for mosquitoes that probed during all three feedings were included in the analysis.

Quantification of malaria infection in the liver. To determine whether significant numbers of sporozoites escaped from the injection site during probing, we quantified liver infection after five to eight mosquitoes probed on a mouse's ear for 3 min and then removed the ear or left it intact. Livers were harvested 40 h after mosquito probing, and total RNA was isolated using Tri-Reagent (Molecular Research Center, Cincinnati, OH) as per the manufacturer's instructions. The parasite burden in the liver was determined by reverse transcription (RT) followed by real-time PCR as outlined previously (5). RTs were performed with 0.5 μg of total RNA and random hexamers; and real-time PCR was performed using primers that recognize *P. yoelii*-specific sequences within the 18S rRNA (5). Liver infection was quantified by comparison to a standard curve which was made from mice that had been intravenously injected with 10, 100, or 1,000 sporozoites. Livers from these mice were harvested and processed in a manner identical to that for experimental livers.

Quantification of sporozoite number in the skin. Skin samples were homogenized in 1.6 ml of Tri-Reagent with a PowerGen 125 (Fisher Scientific) for 1 min on the highest speed and then extracted with 320 μl of chloroform. The aqueous phase was removed and mixed with an equal volume of 70% ethanol and added to an RNeasy Mini spin column (RNeasy Mini kit; QIAGEN Inc., Valencia, CA), and RNA was isolated according to the manufacturer's instructions. Total RNA was eluted in 40 μl of water, and RT–real-time PCR was performed with the one-step QuantiTect SYBR Green RT-PCR kit (QIAGEN) using primers specific for the *P. yoelii* 18S rRNA (100 pmol per 50-μl reaction). RT–real-time PCR was performed with a fixed proportion of the total RNA (1.5 μl of the 40-μl total volume) rather than a specific amount of RNA. Cycling parameters were as follows: 50°C for 30 min, 95°C for 15 min, and 40 cycles of 94°C for 15 seconds, 60°C for 30 seconds, 72°C for 30 seconds, and 76.3°C for 15 seconds, the final step being for data acquisition. A standard curve was prepared by adding known numbers of sporozoites to mouse ears before homogenization in Tri-Reagent.

Quantification of sporozoite number in mosquito salivary glands. Salivary gland load was determined both microscopically and by PCR. Salivary glands were squashed, sporozoites were counted using a phase-contrast microscope, and mosquitoes were placed into one of three groups: lightly infected (<100 sporozoites), moderately infected (100 to 5,000 sporozoites), or heavily infected (>5,000 sporozoites). For RNA extraction from salivary glands, 1.6 ml Tri-Reagent was added to coverslips containing the glands, samples were rocked on ice for 5 min, and RNA was isolated. One step RT–real-time PCRs were performed as for skin except that we used 25 pmol of primers per reaction. A standard curve was prepared by extracting RNA from known numbers of sporozoites. For 92% of the glands, both microscopic and PCR data were obtained. Comparison of the data indicated that glands classified as lightly infected by microscopy had <1,000 sporozoites by PCR, moderately infected glands had 1,000 to 10,000 sporozoites, and heavily infected glands contained >10,000 sporozoites.

Statistical analyses. Parasite loads in mouse livers were analyzed by *t* test for nonpaired data. The relationship between sporozoite load and the number injected into skin was analyzed by linear regression analysis. The correlation between mosquito feeding behaviors and sporozoites injected, transmission efficiency, and gland load was determined using two different methods. For those behaviors that were either exhibited or not (e.g., taking a blood meal or probing at multiple sites), data from the mosquitoes that exhibited the behavior were compared to those that did not by *t* test of nonpaired data. For behaviors that were quantified, such as the number of probes and the time spent probing, we used linear regression analysis. All analyses were performed using SPSS software (SPSS Inc., Chicago, IL) with significance set at the 5% level. The number of sporozoites injected into skin was nonnormal, i.e., aggregated towards 0 in all data sets and so was transformed to log(number injected into skin + 1) prior to analysis. Transmission efficiency is defined as follows: (number of sporozoites injected into skin/number of salivary gland sporozoites) × 100.

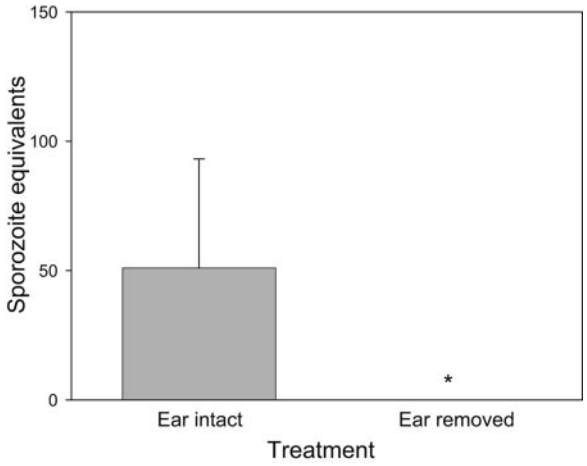

FIG. 1. Mice in which the injection site is removed do not develop malaria infection. Five to eight infected mosquitoes were allowed to probe on a mouse's ear, and then the ear was either left intact or removed. Forty hours later, malaria infection in the livers of these mice was determined by RT–real-time PCR. Infection was quantified using a standard curve made from results for the livers of mice injected with known numbers of sporozoites. There were three mice per group, and the means with standard deviations are shown. The asterisk indicates that there was no detectable signal in this group.

RESULTS

Previous studies have demonstrated that sporozoites are deposited into the avascular portion of the dermis and begin to leave the skin within 5 min (9, 16). These studies suggest that if probing is allowed to proceed for only 3 min, the fed-upon portion of skin should contain most or all of the sporozoites injected and therefore could be used to quantify the number of sporozoites injected by a single mosquito. To test if this was the case, we allowed mosquitoes to probe on a mouse's ear for 3 min, removed the ear or left it intact, and subsequently determined the parasite load in the liver. As shown in Fig. 1, the mice in which the ears were removed immediately after probing did not have detectable levels of malaria infection in the liver, indicating that most or all of the sporozoites remained at the injection site.

To quantify the sporozoites injected into the ear, we developed a quantitative RT–real-time PCR assay. Using skin samples spiked with known numbers of sporozoites, this assay could detect fewer than 10 sporozoites, while skin without sporozoites gave no signal (Fig. 2). We then used this assay to determine the number of sporozoites injected into the ear of a live mouse by single mosquitoes infected with the rodent malaria parasite *P. yoelii*. After the feeding experiments, mice were kept and checked for the development of blood-stage infection. Only two mice developed patent parasitemias, suggesting that in most cases, the ear sample we removed contained all of the sporozoites injected.

The results of 59 single-mosquito feeds, using four different batches of mosquitoes, are shown in Fig. 3. The number of sporozoites injected by a single mosquito was highly variable, and 22% of infected mosquitoes injected no sporozoites. The mean number of sporozoites injected was 123, with a median of 18 and a range of 0 to 1,297. The number of sporozoites

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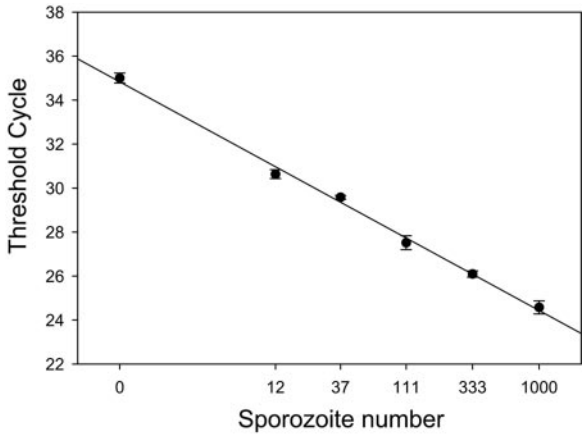

FIG. 2. Quantification of *P. yoelii* sporozoites in mouse skin. RNA was extracted from mouse ear homogenates containing the indicated number of sporozoites, and RT followed by real-time PCR was performed using parasite-specific primers. Shown is the PCR cycle number at which each sample became positive over background fluorescence.

injected was only weakly correlated to salivary gland load (Pearson correlation coefficient = 0.35; $R^2 = 0.12$; $P < 0.01$) (Fig. 3A). Regression analysis of the data indicated that gland infection was responsible for 12% of the variation observed among individual mosquitoes. However, when the analysis was limited to those mosquitoes with gland loads of 12,000 or greater, there was no relationship between gland load and sporozoite injection (Pearson correlation coefficient = 0.27; $R^2 = 0.07$; $P = 0.09$; $n = 40$).

The distribution frequency of the numbers of sporozoites injected is shown in Fig. 3B. Almost 50% of mosquitoes injected fewer than 10 sporozoites, 35% of mosquitoes injected between 11 and 200 sporozoites, and a small proportion of the mosquitoes injected very large numbers of sporozoites. We also calculated the transmission efficiency for these mosquitoes, and the majority (85%) injected less than 1% of their salivary gland load (Fig. 3C).

To determine whether the variation in the number of sporozoites injected was the result of differences in mosquito feeding behavior, we monitored several different behaviors and determined whether any of these correlated to infectivity (Table 1). We compared the number of sporozoites injected, the transmission efficiency, and the gland load for those mosquitoes that exhibited each behavior versus those that did not, and we found no differences between the two groups ($P > 0.05$). In the case of behaviors that could be quantified, i.e., the number of probes and the total time spent probing, we performed a regression analysis and found that neither parameter was significantly correlated to sporozoites injected, transmission efficiency, or gland load ($P > 0.05$).

The large variation in the number of sporozoites injected by mosquitoes with similar gland infections led us to hypothesize that perhaps sporozoite injection was a property of the mosquito. To test this, we performed a series of multiple-day feeds. First, we examined sporozoite injection by single mosquitoes on three consecutive days, and the pooled results from two experiments are shown in Fig. 4A. Many individuals consistently injected few sporozoites, even if they were heavily infected, while most of the other individuals injected large numbers on at least one of the three days. The large number of mosquitoes that consistently injected few to no sporozoites

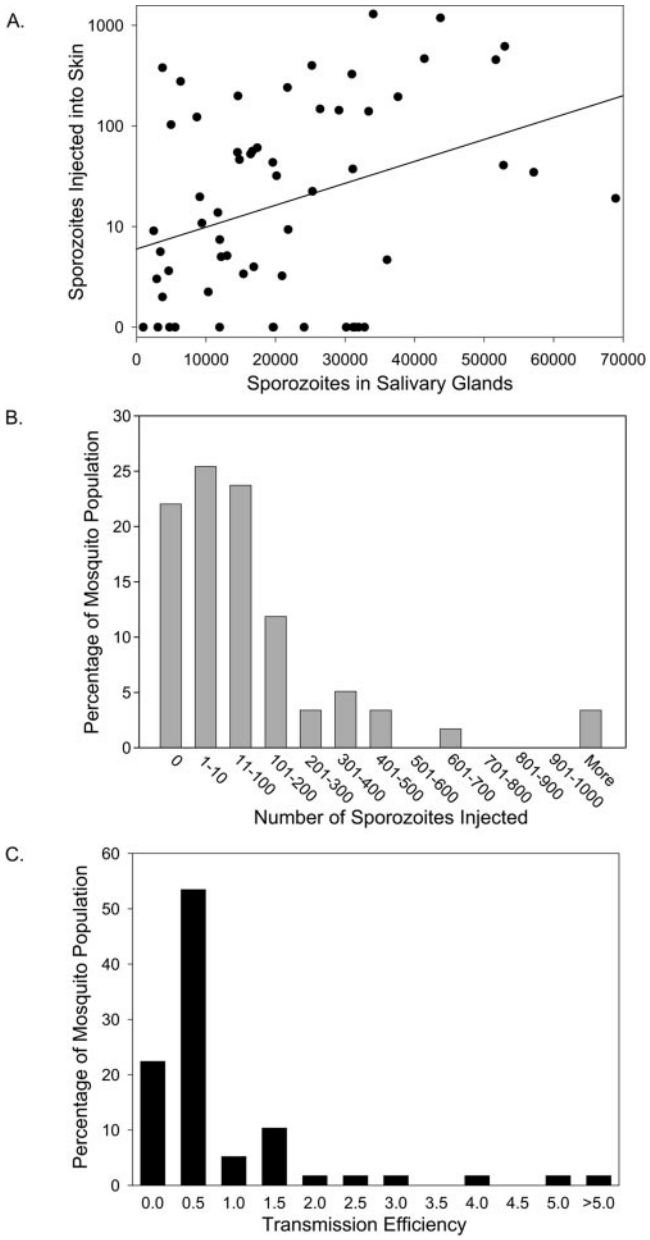

FIG. 3. Quantification of sporozoite injection by individual mosquitoes. (A) Shown is the relationship between sporozoite injection and salivary gland load. Individual mosquitoes were allowed to probe for 3 min on the ear of an anesthetized mouse. The ear of the mouse and the salivary glands of the mosquito were removed, and the number of sporozoites in each was quantified by RT–real-time PCR. The regression line for the data is shown. (B) The distribution frequency of the data shown in panel A is plotted to show the percentage of mosquitoes that injected a given range of sporozoites. (C) For each of the mosquitoes shown in panel A, the percentage of the salivary gland load that was injected during probing (transmission efficiency) was determined, and shown is the percentage of mosquitoes which corresponds to each of these values.

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TABLE 1. Mosquito feeding behavior

Behavior or parameter	% Exhibiting behavior	Population avg[a]
Probed at multiple sites	66.1	
Probed near tape	59.7	
Took a blood meal	17.7	
No. of probes		7.9 ± 4.0
Time (s)		159.9 ± 30.9

[a] Values are means ± standard deviations for the mosquitoes in the single-feed experiment ($n = 59$).

could be due to the developmental state of the sporozoites. If this were the case, however, we would expect to see an increase in the number of sporozoites injected over time. Although there were two such mosquitoes (Fig. 4A, first and sixth individuals from the right), there was no overall pattern suggestive of this phenomenon. Nonetheless, to ensure that this was not the case, we also performed alternate-day feeds in order to give the sporozoites more time to mature. As shown in Fig. 4B, the injection pattern of the mosquitoes which probed on three alternate days was similar to that for the consecutive-day feeds.

Analysis of the multiple-feed data sets showed a significant correlation between gland load and sporozoite injection (data in Fig. 4A, Pearson correlation coefficient = 0.59, $R^2 = 0.34$, $P = 0.01$; data in Fig. 4B, Pearson correlation coefficient = 0.50, $R^2 = 0.25$, $P = 0.02$). For the data shown in Fig. 4A and B, salivary gland load could explain 34% and 25%, respectively, of the variation observed. When the analysis was limited to heavily infected mosquitoes (salivary gland loads >12,000), however, there was no relationship between gland load and sporozoites injected (data in Fig. 4A, Pearson correlation coefficient = 0.16, $R^2 = 0.03$, $P = 0.62$, $n = 12$; data in Fig. 4B, Pearson correlation coefficient = 0.29, $R^2 = 0.09$, $P = 0.47$, $n = 8$).

DISCUSSION

Determination of the sporozoite inoculum under natural mosquito feeding conditions is critical to understanding malaria transmission biology. In this study, we quantified sporozoite injection by infected *A. stephensi* mosquitoes probing on live animals. Analysis of data from 59 individual feeds shows that the mean number of sporozoites injected was 123, the median was 18, and the range was between 0 and 1,297. Previous studies which quantified sporozoites ejected by mosquitoes salivating into mineral oil or probing through membrane feeders observed lower numbers (1–3, 10, 14). In these cases, the median values ranged from 8 to 15, and in the one study reporting an arithmetic mean, it was 47.

One reason our numbers are higher is likely technical. Previous investigators quantified ejected sporozoites by microscopy, a method that likely underestimated sporozoite numbers. There are many reasons for this; first, sporozoites may be washed off during processing of the slide; second, in thick specimens such as skin, it can be difficult to find all of the sporozoites; and lastly, sporozoites can be ejected in clumps (8, 10), and it is difficult to accurately count the individuals in these clumps. Interestingly, a recent study which directly counted green fluorescent protein-expressing sporozoites as they were being ejected during salivation in vitro reported numbers similar to ours (mean, 114; median, 39 [6]). Although one cannot directly compare our data to data from that study because these mosquitoes salivated for a longer time (10 min), it is likely that direct observation eliminates losses that occur during processing of samples. To overcome the limitations of microscopy for quantitation of injected sporozoites, we used quantitative PCR. In general, the problem with PCR is not sensitivity but specificity, and false positives are commonly observed. However, despite the fact that we were amplifying rare *Plasmodium* sequences from a solution that contained primarily host DNA, we never observed amplification of host sequences. This may be due to the AT-rich genome of *Plasmodium* which makes it less likely that parasite-specific primers will anneal to host sequences.

Another factor that may have contributed to the higher numbers of injected sporozoites that we observed is the mosquito-parasite species combination that we used. Previous in vitro studies were performed primarily with *A. stephensi* or *A. gambiae* infected with *P. falciparum* (1, 2, 10, 14). Here, we studied *A. stephensi* infected with the rodent malaria parasite *P. yoelii*, a combination that has not been previously used in transmission studies. Previous in vitro studies did not show a significant difference in *P. falciparum* sporozoite ejection between *A. stephensi* and *A. gambiae* mosquitoes. However, more mosquito-parasite combinations need to be investigated, preferably using an in vivo system such as the one we have developed, in order to better understand the role of different mosquito-parasite combinations in transmission efficiency.

Our ability to follow the transmission pattern of a single mosquito over time has led to several interesting observations. First, we found that about 20% of moderately to heavily infected mosquitoes consistently injected few (<10) to no sporozoites. Although we found high rates of noninjectors in our single-feed experiments, as did previous investigators using in vitro systems (1, 3, 10), since sporozoite injection was measured only at a single time point, it was possible that these mosquitoes might inject larger numbers of sporozoites during a subsequent feed. However, we found that some mosquitoes remained poor transmitters of sporozoites over time, despite the fact that they had adequate numbers of parasites in their

FIG. 4. The ejection pattern of individual mosquitoes over time. Individual mosquitoes were allowed to feed on the ears of anesthetized mice for either three consecutive days (days 15, 16, and 17 p.i.) (A) or three alternate days (days 15, 17, and 19 p.i.) (B). After each feeding, the number of sporozoites injected into the ear was determined by RT–real-time PCR. After the last feed, the salivary gland load of the mosquito was determined by visual inspection and in most cases also by RT–real-time PCR. Shown for each mosquito are the numbers of sporozoites injected during each of three feedings plotted against the salivary gland load/100 of that mosquito. The designations light, moderate, and heavy indicate the level of gland infection as determined by microscopy. Mosquitoes for which only visual assessment of gland infection was available are indicated with an asterisk. Zeros denote no detectable sporozoites in the skin on all three days.

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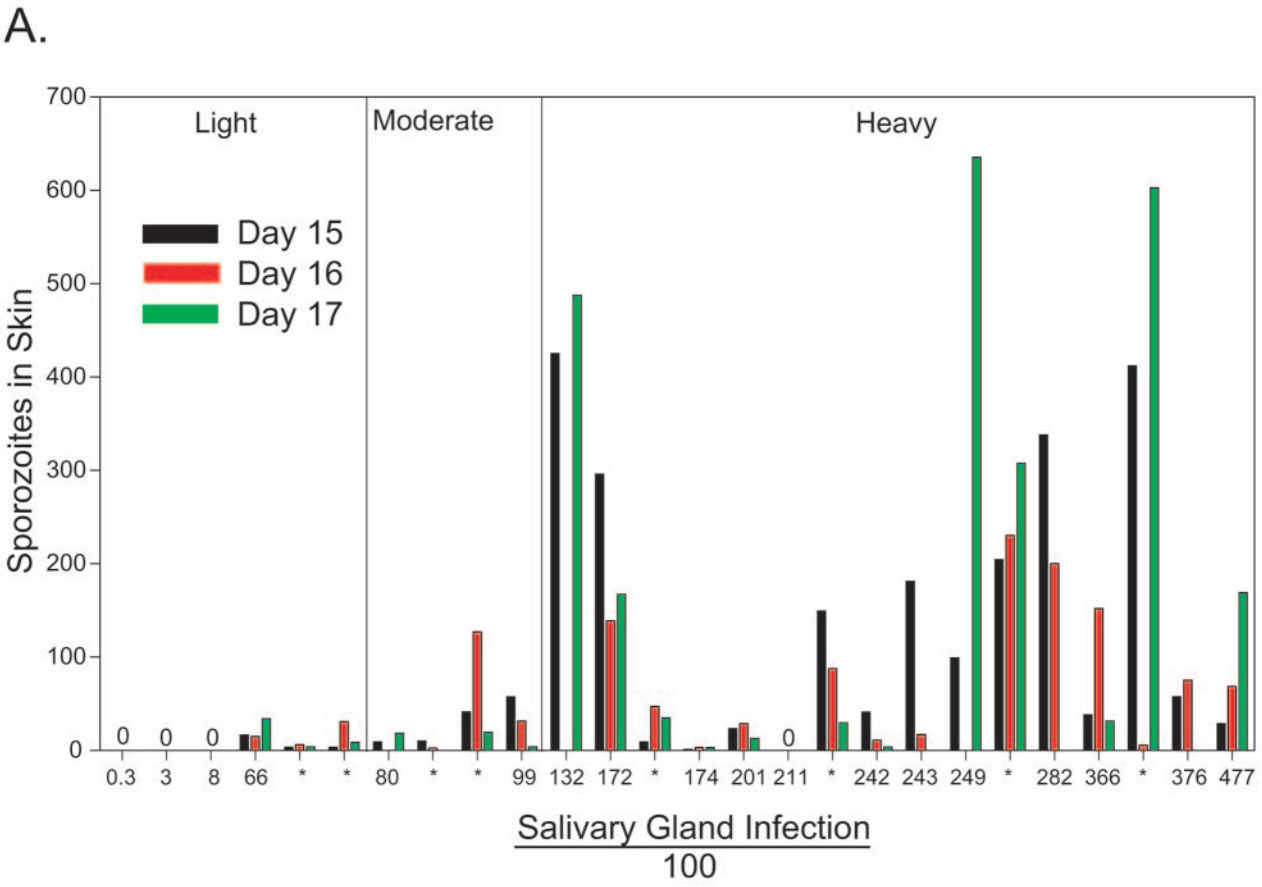

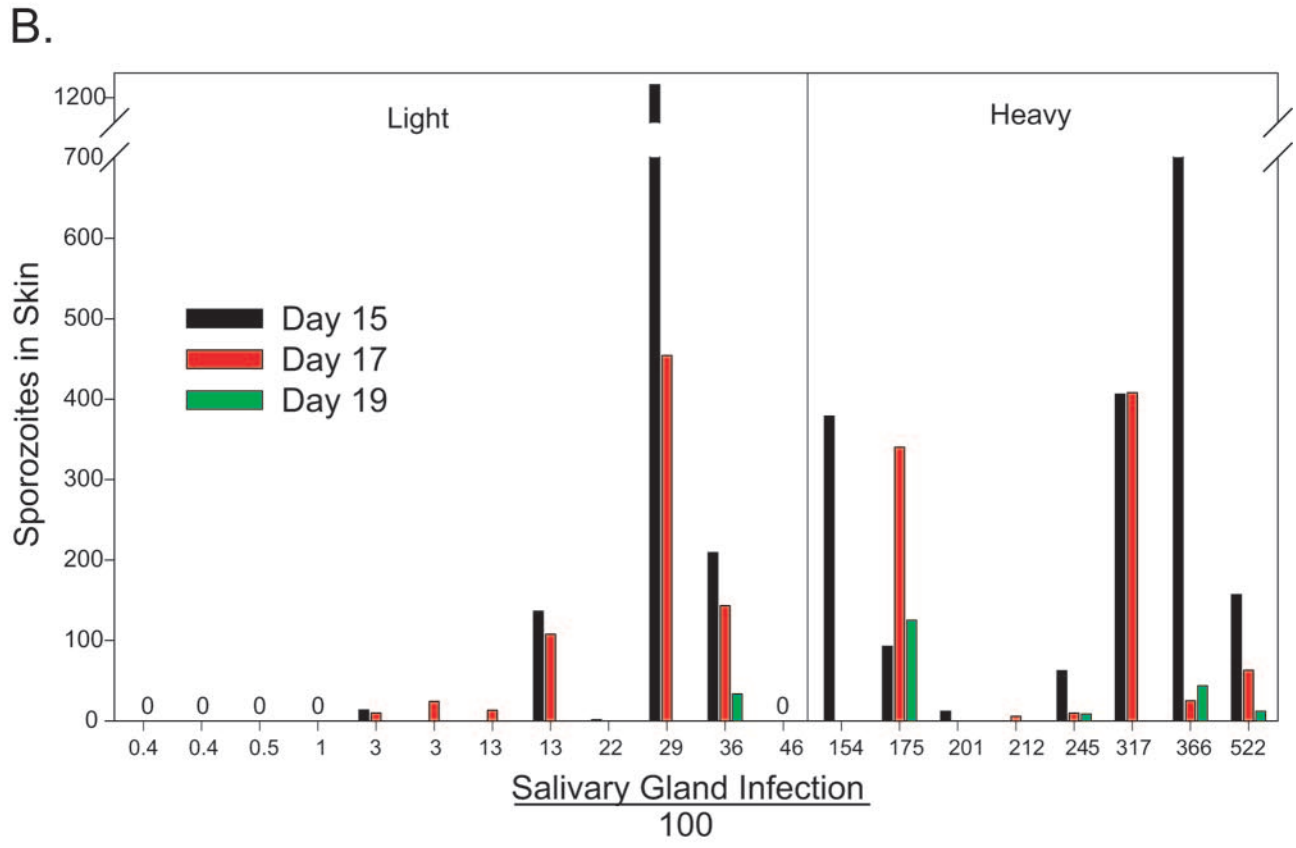

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glands. Although we do not understand the factors responsible for such poor transmission dynamics, some studies have suggested that salivation rate and volume may be important determinants of sporozoite delivery (12, 14). Another possibility is that differences among sporozoites affect their transmission potential. Recent data demonstrating that sporozoite motility is required for movement from the acinar cell to the salivary duct raise the possibility that parasite motility may also affect transmission dynamics (6). More work is needed to better understand the physiology of sporozoite ejection as well as the parasite and host factors that regulate this process.

Another interesting finding from the multiple-feed data set was the significant amount of intraindividual variability in the number of sporozoites injected. Of the 46 mosquitoes that successfully fed for three sequential or alternate days, 40% injected high numbers (usually over 100 sporozoites) on at least one of the three days and 0 or minimal numbers of sporozoites (<20) on one or both of the other days. Many investigators have noted that sporozoites are frequently injected in clumps and have hypothesized that sporozoite injection is not uniform (3, 8, 10). Our data provide strong experimental evidence that sporozoite ejection from the glands is discontinuous. Using mathematical models to pattern different ejection behaviors, Li et al. predicted that discontinuous ejection of sporozoites would optimize transmission under conditions in which a threshold of infection exists (8), a scenario that is likely true for malaria infection.

The question of whether there is a correlation between gland load and sporozoite injection is an important one and has been the topic of some debate. In our single-feed data set (Fig. 3), we found a weak relationship between gland load and infectivity, with only 12% of the variation in sporozoite injection being attributed to gland load. This is in agreement with most previous studies which used in vitro systems to measure sporozoite ejection (1, 10). This poor correlation, however, is likely to be due to the confounding effects of two variables: (i) the quantal nature of sporozoite ejection and (ii) the large group of noninjectors. When we analyzed the data from the multiple-feed data set (Fig. 4), where we summed three data points for each mosquito, we found a stronger correlation between gland infection and sporozoite ejection, suggesting that the injection pattern of a mosquito over time *is* correlated to gland load. Interestingly, one in vitro study found a strong correlation between gland load and sporozoite ejection (14). In this study, investigators measured salivation volume and only in the highest salivators was there a strong correlation between gland load and sporozoite ejection. One explanation for this is that the mosquitoes that inject few to no sporozoites may be low salivators and so their removal from the analysis strengthens the correlation between gland load and infectivity. We would predict that if we had been able to measure the salivation volume of the mosquitoes used in the single-mosquito feeds (Fig. 3), we too would have seen a correlation between gland load and sporozoite injection in the highest salivators.

Unfortunately, the additional information needed to correlate sporozoite injection with gland load (i.e., sporozoite injection over time or salivation volume) is not readily available in the field or in vaccine trials where parasite load in the salivary glands is usually the only parameter measured. As we and others have shown, this parameter on its own is not predictive of sporozoite transmission. However, our data indicate that there is one situation in which gland load can be used to predict sporozoite transmission, and that is in mosquitoes with low parasite loads. Our data clearly show that mosquitoes with gland loads of fewer than 5,000 sporozoites are likely to inject few sporozoites. This is supported by data from an in vitro study in which it was shown that mosquitoes with low gland infections were much less likely to transmit sporozoites (3) and by studies in humans which have shown a clear correlation between the ability to produce a malaria infection and the intensity of gland infection (4, 7). In contrast to this, one cannot predict, based on gland load data alone, how many sporozoites a heavily infected mosquito will inject, a finding that is supported by human vaccine trials in which volunteers must be challenged with four to five bites of heavily infected mosquitoes in order to insure that all unvaccinated controls are infected (13).

In summary, we have shown that mosquitoes allowed to probe on a live host inject higher numbers of sporozoites than previously observed in studies using artificial feeding strategies. Like other investigators using in vitro systems, we found a large variation in the numbers of sporozoites injected by individual mosquitoes, and regression analysis indicated that the level of gland infection could explain only a small amount of this variation. This poor correlation, however, is likely to be due to the confounding effects of the quantal nature of sporozoite ejection and the large group of noninjectors. We found a great deal of intraindividual variation in the number of sporozoites injected over time, indicating that sporozoite ejection is discontinuous and providing support for the hypothesis that clumping of sporozoites optimizes the parasites' chances for survival. Both the quantal nature of sporozoite ejection and the large group of mosquitoes that consistently inject few to no sporozoites provide some rationale for the observation, in areas where malaria is endemic, that the majority of infective bites do not result in malaria infection (11).

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All animals used in this study were handled in accordance with federal guidelines and institutional policies of the New York University School of Medicine.

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