SPOROZOITE TRANSMISSION BY *ANOPHELES FREEBORNI* AND *ANOPHELES GAMBIAE* EXPERIMENTALLY INFECTED WITH *PLASMODIUM FALCIPARUM*

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ABSTRACT. A micro-membrane feeding technique was used to evaluate sporozoite transmission for *Anopheles freeborni* and *An. gambiae* experimentally infected with *Plasmodium falciparum*. From cohorts of infected mosquitoes with equivalent sporozoite loads, 75.9% of 29 *An. freeborni* transmitted a geometric mean (GM) of 4.9 sporozoites and 80% of 30 *An. gambiae* transmitted a GM of 11.3 sporozoites. Ingested sporozoites, in the blood meal immediately after feeding, were detected in 86.2% of 29 *An. freeborni* (GM = 9.0) and in 70% of 30 *An. gambiae* (GM = 44.1). Overall, sporozoites were transmitted and/or ingested by 90% of both species. Most infective mosquitoes transmitted <1% of the total sporozoites in the salivary glands, and only up to 30% of the variation in transmission, ingestion, or total sporozoite output was related to sporozoite loads. The demonstration that *An. gambiae* transmitted more than twice as many sporozoites as *An. freeborni* is the first indication that vector species of anopheline mosquitoes differ in their innate potential for sporozoite transmission.

Malaria sporozoites are transmitted during mosquito blood-feeding. The actual number of sporozoites released by infective mosquitoes is probably impossible to determine by direct observation (Griffiths and Gordon 1952). Inoculative doses of sporozoites have been estimated by several methods, including capillary tube or membrane-feeding techniques (Rosenberg et al. 1990, Beier et al. 1991a, 1991b, 1992; Ponnudurai et al. 1991, Li et al. 1992), counts of exoerythrocytic forms (Vanderberg 1977), and mathematical models based on pre-patent periods (Davis et al. 1989). A central conclusion is that most infective mosquitoes transmit few sporozoites, generally less than 50. Extrapolations to epidemiologic situations are difficult because most studies have been conducted using *Anopheles stephensi* Liston mosquitoes. There have been few comparative studies, either in the laboratory (Beier et al. 1991a, 1992) or in the field (Beier et al. 1991b) to determine whether there are differences in sporozoite transmission potential among vector species of anophelines.

Ideally, sporozoite transmission should be evaluated by *in vitro* techniques which do not interfere with normal mosquito probing and blood-feeding behavior. Concerns regarding the excessive manipulation of mosquitoes, including anesthetization and/or dismemberment, have been addressed by Ponnudurai et al. (1991) in their demonstration that sporozoite transmission can be measured effectively by membrane-feeding techniques. Recent findings that most infective mosquitoes ingest sporozoites during natural blood-feeding on vertebrate hosts (Beier et al. 1992) emphasize that *in vitro* measures of the transmission process should partition sporozoite output according to the numbers of sporozoites which are "transmitted" and "ingested." These key parameters can be measured effectively only if mosquitoes are tested by methods which promote normal blood-feeding.

This study examines the sporozoite transmission potential of *An. freeborni* Aitken and *An. gambiae* Giles experimentally infected with *Plasmodium falciparum*. The main objective was to determine if anopheline species differ in their output of sporozoites.

MATERIALS AND METHODS

Laboratory-reared *An. freeborni* and *An. gambiae* (G-3 strain) were infected experimentally with the NF54 stain of *P. falciparum* (Beier et al. 1991a). Mosquitoes in screened, gallon-size paper containers were held in an insectary at 26°C and 70% RH with access to diluted Karo syrup solution on cotton and to water on a 6 × 12 cm strip of paper towel. Mosquitoes were held 19–21 days post-infection and before testing were deprived of nutrient solutions overnight to enhance blood-feeding. In preparation for feeding experiments, mosquitoes were placed individually in screened, pint-size containers and held for up to 1 hour.

Mosquitoes were evaluated individually for sporozoite transmission using a micro-membrane feeding technique. The membrane feeder consisted of a 250 μl polypropylene tube, with an outer diameter of 5.8 mm and 30.6 mm in length (catalog no. 05-4078, Fisher Scientific). After placing 70 μl of a 10% suspension of washed human erythrocytes in medium 199 solution (Gibco Laboratories, Grand Island, NY)

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Table 1. Summary of sporozoite transmission experiments for *Anopheles freeborni* and *An. gambiae* experimentally infected with *Plasmodium falciparum*.

		Transmission			Ingestion			Sporozoite output*		
			Sporozoites			Sporozoites			Sporozoites	
Species	No. tested	% positive	G.M.	Range	% positive	G.M.	Range	% positive	G.M.	Range
An. freeborni	29	75.9	4.9	1–72	86.2	9.0	2–64	89.7	14.7	2–100
An. gambiae	30	80.0	11.3	1–50	70.0	44.1	4–394	90.0	36.5	1–420
Total	59	78.0	7.6	1–72	78.0	18.6	2–394	89.8	23.4	1–420

* Sporozoite output is defined as the total number of transmitted and ingested sporozoites per individual mosquito.

in each tube, the open end of the tube was covered with Baudruche membrane (Joseph Long, Inc., Belleville, NJ), secured with a strip of parafilm. Tubes were held upright in a water bath at 37°C just prior to use.

Individual mosquitoes were allowed to feed on the micro-membrane feeder, which was inverted to bring the blood solution in contact with the membrane, and held in place on the top of the screened carton. Holding the feeder close to the membrane and carefully moving the feeder toward the mosquito prompted probing and blood-feeding responses. The membrane feeder was removed when mosquitoes were engorged and had withdrawn their mouthparts. Most mosquitoes approached the membrane feeder, probed and fed to repletion in less than 5 minutes.

After feeding, membrane-feeding tubes were placed upright and tapped gently to move the blood to the bottom of the tube. The portion of the membrane in contact with the blood was removed, and mounted (inner side up) on a 7 mm diam spot of a 10-spot microslide coated with Poly-L-Lysine (Sigma Chemical Co., St. Louis, MO). Tubes were capped, held on ice and then centrifuged at 2,000 *g* for 15 min at 4°C; this removed 99% of the sporozoites from the supernatant. After removing the supernatant (60 μl), the pellet (ca. 10 μl of erythrocytes in medium 199 solution) was spotted on the same microslide.

Within 20 min, each blood-fed mosquito was aspirated, killed in 70% alcohol, rinsed in medium 199, and then dissected. Salivary glands were dissected in medium 199, transferred and ground gently in a small glass tissue homogenizer (Kontes, Vineland, NJ) containing 35 μl medium 199, and 5 μl were removed and spotted on the microslide. Next, midguts were removed, according to techniques described previously for determining numbers of ingested sporozoites (Beier et al. 1992), and the contents were spotted onto the microslide.

Sporozoites were counted on microslides by fluorescent microscopy after incubation for 30 min at room temperature with a 1:50 dilution of fluorescein-labeled 2A10 monoclonal antibody (0.1 mg/ml) (Zavala et al. 1983), and washing once with Dulbecco's phosphate buffered saline solution (pH 7.4) (Sigma Chemical Co., St. Louis, MO). For each blood-fed mosquito, the number of sporozoites in the salivary glands (i.e., the sporozoite load) was determined by multiplying the number of sporozoites counted in the 5 μl aliquot (spot #1) times 7, to account for the 35 μl initial volume (Beier et al. 1991a). The number of sporozoites transmitted (i.e., those sporozoites in the membrane feeder upon cessation of mosquito feeding) was determined as the number of sporozoites counted on the membrane (spot #2) plus those counted from the centrifuged pellet (spot #3). Corresponding information on the number of sporozoites ingested (i.e., sporozoites deposited in the membrane feeder but subsequently ingested during mosquito feeding) was obtained by sporozoite counts of midgut contents (spot #4) (Beier et al. 1992). The fifth spot on each microslide, containing sporozoites from pooled gland dissections, served as a positive control for each individual mosquito. In addition, "sporozoite output" was calculated as the sum total of those transmitted and ingested.

Data on sporozoite loads, sporozoite output, ingested and transmitted sporozoites were log transformed to normalize variance prior to testing by analysis of variance (ANOVA) and regression analysis.

RESULTS

Salivary gland sporozoite infections were detected in 29 of 31 (93.5%) *An. freeborni* and in 30 of 34 (88.2%) *An. gambiae* which were tested for sporozoite transmission. Geometric mean sporozoite loads were 4,841 (range: 77–29,904) for *An. freeborni* and 4,664 (range: 385–19,740) for *An. gambiae* ($F = 0.02$, df = 1,57, $P = 0.89$). Sporozoites were transmitted and/or ingested by 89.7% of 29 *An. freeborni* and by 90.0% of 30 *An. gambiae* (Table 1). *Anopheles gambiae* transmitted ($F = 7.62$, df = 1,44, $P = 0.008$) and

ingested ($F = 24.53$, df = 1,44, $P < 0.0001$) more sporozoites than *An. freeborni* (Fig. 1). Overall, the geometric mean sporozoite output for *An. gambiae* was 2.5 times greater than for *An. freeborni* ($F = 7.36$, df = 1,51, $P = 0.009$).

The subset of 26 *An. freeborni* and 27 *An. gambiae* which either transmitted or ingested sporozoites included 4 (15.4%) *An. freeborni* and 3 (11.1%) *An. gambiae* which ingested but did not transmit sporozoites, and one (3.8%) *An. freeborni* and 6 (22.2%) *An. gambiae* which transmitted but did not ingest sporozoites. There were no significant differences in sporozoite loads between mosquitoes showing concordance between transmission and ingestion, compared with those where there was discordance (for *An. freeborni*: $F = 2.92$, df = 1,27, $P = 0.1$; for *An. gambiae*: $F = 3.29$, df = 1,28, $P = 0.08$).

Species differences were observed in relationships between sporozoite transmission variables,

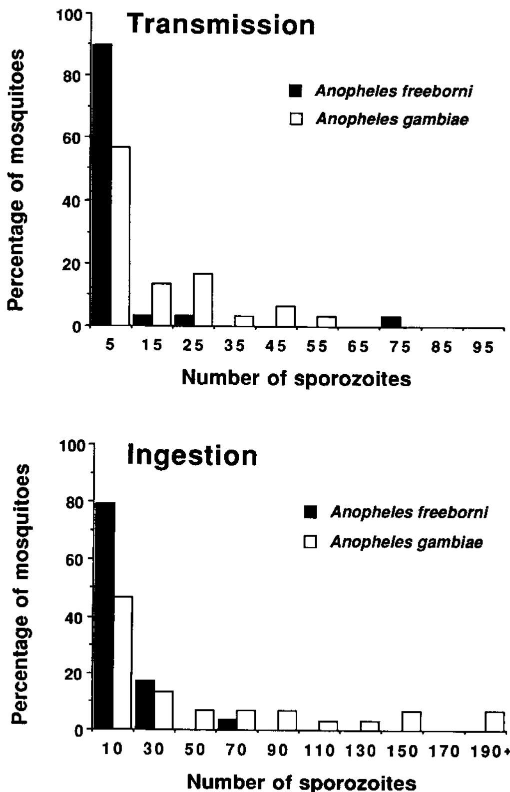

Fig. 1. Frequency distributions of *Plasmodium falciparum* sporozoites transmitted *in vitro* (top) or ingested (bottom) by experimentally infected *Anopheles freeborni* (n = 29) and *An. gambiae* (n = 30) during blood-feeding on micro-membrane feeders. Midpoint values for 10 sporozoite intervals are shown on the X-axis.

as determined by regression analysis (Table 2). For *An. freeborni*, there were no relationships between sporozoite loads and the number of sporozoites transmitted, the number ingested, or the total sporozoite output. In contrast, there were significant, positive relationships between sporozoite loads and each of these variables for *An. gambiae* but the amount of variation explained by sporozoite loads, as indicated by coefficients of determination (r^2), did not exceed 30%. Further, there were no significant relationships between the numbers of transmitted and ingested sporozoites for either species.

Total sporozoite output was highly variable, representing 0.03–19.5% and 0.03–7.5% of the total sporozoites in the salivary glands for *An. freeborni* (n = 29) and *An. gambiae* (n = 30), respectively. For example, some mosquitoes with fewer than 100 salivary gland sporozoites had an output of >10 sporozoites and some with sporozoite loads of >15,000 did not transmit. Relationships between the probability of sporozoite output and sporozoite loads were not determined because sporozoite output was observed for all but 3 gland positive mosquitoes of each species. Additionally, sporozoite loads of the 7 *An. gambiae* with total outputs of >100 sporozoites were not significantly higher than those determined for the 20 individuals with outputs of <100 sporozoites ($F = 3.34$, df = 1,25, $P = 0.08$); the single *An. freeborni* with an output of 100 sporozoites had a sporozoite load of 5,432.

DISCUSSION

Key parameters of sporozoite transmission were evaluated effectively using the simple micro-membrane feeding technique, as demonstrated by high rates of sporozoite transmission (>75%), sporozoite ingestion (≥70%), and sporozoite output (90%). Findings that >95% of the mosquitoes transmitted <50 sporozoites are consistent with previous studies using a variety of *in vitro* methods (Rosenberg et al. 1990, Beier et al. 1991a, 1991b, 1992; Li et al. 1992), including the studies of Ponnudurai et al. (1991) which demonstrated the utility of membrane feeders covered by mouse skin. Similarly, the numbers of sporozoites ingested by *An. gambiae* were comparable to estimates for this species obtained from direct feeds on live animals and *in vitro* tests (Beier et al. 1992). Most mosquitoes responded quickly to the feeders, >95% fed to repletion, and sporozoites were easily detected. Importantly, the technique involved minimal handling and no manipulation of mosquitoes prior to testing, major advantages over most other *in vitro* methods.

This study provides the first evidence that vector species of *Anopheles* mosquitoes differ in

Table 2. Linear regression analysis of sporozoite transmission parameters for *Anopheles freeborni* and *An. gambiae* infected with *Plasmodium falciparum*.

Number of sporozoites		Regression equation statistics*											
		Anopheles freeborni						*Anopheles gambiae*					
Variable 1	Variable 2	b	m	SE	r	*P*	*n*	b	m	SE	r	*P*	*n*
Sporozoite load	Transmission	−0.15	3.87	0.14	−0.24	0.281	22	0.39	3.42	0.13	0.54	0.007	24
Sporozoite load	Ingestion	−0.13	3.80	0.24	−0.11	0.594	25	0.37	3.13	0.18	0.43	0.050	21
Sporozoite load	Sporozoite output**	−0.12	3.83	0.26	−0.09	0.650	26	0.28	3.29	0.12	0.43	0.025	27
Transmission	Ingestion	−0.01	0.72	0.26	0.00	0.984	21	0.03	1.12	0.15	0.04	0.863	18

* Statistics for the equation (Variable 1 = [b * Variable 2] + m) are b = slope; m = constant; SE = standard error; r = correlation coefficient; *P* = significance; *n* = number of observations. Note: Log_{10} values for each variable were used in the regression analysis.

** Sporozoite output is defined as the total number of transmitted and ingested sporozoites per individual mosquito.

their sporozoite transmission potential. Findings that *An. gambiae* transmitted, on average, more than twice as many sporozoites as *An. freeborni* show that it is not realistic to generalize that all vectors have an equivalent potential for sporozoite transmission. Although previous studies comparing experimentally infected *An. gambiae* and *An. stephensi* (Beier et al. 1991a) or naturally infected *An. gambiae* and *An. funestus* Giles (Beier et al. 1991b) found no differences in transmission potential, clearly not enough species have been examined. In fact, *An. freeborni* (subgenus *Anopheles*) is the first anopheline species outside the subgenus *Cellia* to be tested for *P. falciparum* transmission.

The biological significance of a two-fold difference in sporozoite transmission by 2 competent vector species is unknown, considering that over 95% of all individuals transmitted <50 sporozoites per feeding. Findings that nearly all infective mosquitoes transmitted few sporozoites, irrespective of sporozoite loads, again stress the importance of salivary gland and duct barriers for sporozoite output (Sterling et al. 1973). We could not account for species differences in sporozoite output by measuring salivary duct diameters for 22 *An. freeborni* and 55 *An. gambiae*. For both species, mean diameters for proximal regions of the medial and both lateral lobes of the salivary glands were ca. 1 ± 0.5 μm, nearly the same diameter as a *P. falciparum* sporozoite (1×11 μm, *n* = 45) (J. Vaughan and C. Mendis, unpublished data).

The most reasonable explanation for the observed species differences in sporozoite transmission potential is that there are major behavioral and physiological differences between *An. freeborni* and *An. gambiae*. We have recently shown that the mean duration of blood-feeding for *An. gambiae* (232 ± 48 sec) is more than twice as long as that of *An. freeborni* (80 ± 40 sec), and have found that *An. gambiae* concentrates erythrocytes while *An. freeborni* does not (Vaughan et al. 1991). Just as vectors with longer feeding periods should be capable of greater sporozoite output, species which concentrate erythrocytes should be capable of ingesting correspondingly greater numbers "egested" sporozoites. Indeed, *An. gambiae* ingested about 5 times as many sporozoites as *An. freeborni* and the total sporozoite output (i.e., transmission and ingestion) of *An. gambiae* was more than twice that of *An. freeborni*.

Ingested sporozoites accounted for 67 and 69% of the total sporozoites output by *An. freeborni* and *An. gambiae*, respectively. This is consistent with initial observations, using other *in vitro* methods and corresponding counts of ingested sporozoites from mosquitoes fed on live hosts, that about two-thirds of the sporozoites are ingested (Beier et al. 1992). Considering the early work of Yorke and Macfie (1924), *An. freeborni* is the fourth vector species where sporozoite ingestion has been documented. By all accounts, sporozoite ingestion is a key parameter of the transmission process, and one that should be considered further in studies of sporozoite transmission.

Relationships between sporozoite loads and parameters of sporozoite transmission indicate further that the number of sporozoites in the salivary glands is a poor indicator of sporozoite transmission potential (Beier et al. 1991a, 1991b; Ponnudurai et al. 1991). Although there was a significant relation between sporozoite loads and sporozoite transmission for *An. gambiae*, 70% of the variation in this relationship was not explained. Similar trends were found between sporozoite loads and sporozoite ingestion, and sporozoite transmission and ingestion. This is not surprising because sporozoite output is highly variable, and most infective mosquitoes transmitted <1% of the total sporozoites resident in the salivary glands.

In conclusion, this study provides simple approaches for measuring the sporozoite transmission potential of infective mosquitoes. Clearly, anopheline species differ in their output of sporozoites and there is a need to investigate further the transmission potential of major vectors from different geographic areas of malaria endemicity. Clues to species-specific differences in sporozoite transmission potential may lie in the inherent differences in blood-feeding behavior and digestive physiology among vector species.

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