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SPOROGONIC DEVELOPMENT OF CULTURED *PLASMODIUM FALCIPARUM* IN SIX SPECIES OF LABORATORY-REARED *ANOPHELES* MOSQUITOES

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Abstract. Sporogonic development of cultured *Plasmodium falciparum* was compared in six species of *Anopheles* mosquitoes. A reference species, *A. gambiae*, was selected as the standard for comparison. Estimates of absolute densities were determined for each lifestage. From these data, four aspects of parasite population dynamics were analyzed quantitatively: 1) successive losses in abundance as parasites developed from gametocyte to ookinete to oocyst stages, 2) oocyst production of sporozoites, 3) correlation between various lifestage parameters, and 4) parasite distribution. Parasite populations in *A. gambiae* incurred a 316-fold loss in abundance during the transition from macrogametocyte to ookinete stage, a 100-fold loss from ookinete to oocyst stage, yielding a total loss of approximately 31,600-fold (i.e., losses are multiplicative). Comparative susceptibilities in order were *A. freeborni* ≫ *A. gambiae, A. arabiensis, A. dirus* > *A. stephensi, A. albimanus.* The key transition(s) determining overall susceptibility differed among species. Despite species differences in oocyst densities and infection rates, salivary gland sporozoite production per oocyst (approximately 640) was the same among species. The most consistent association among lifestage parameters was a positive correlation between densities and infection rates of homologous lifestages. A curvilinear relationship between ookinete and oocyst densities in *A. gambiae* indicated a threshold density was required for ookinete conversion to oocysts (approximately 30 ookinetes per mosquito). The same relationship in *A. freeborni* was linear, with no distinct threshold. Ookinete and oocyst populations were negative binomially distributed in all species. Indices of heterogeneity in mosquito susceptibility to infection indicated that gene frequencies determining susceptibility fluctuated with time in all species, except *A. freeborni* where susceptibility remained homogenous throughout the study. This approach provides a framework for identifying mechanisms of susceptibility and evaluating *Plasmodium* sporogonic development in naturally occurring vector species in nature.

The human malaria parasite *Plasmodium falciparum* can be transmitted only by mosquitoes of the genus *Anopheles*. However, not all species of *Anopheles* are equally susceptible to infection. In theory, vector susceptibility to infection can range from total susceptibility where all individuals support sporogonic development, to total refractoriness where no individuals support development. In reality, vector susceptibility is usually somewhere in between. Thus, susceptibility is a relative attribute ascribed to a species based upon comparison with other species. Several studies have defined the comparative susceptibilities of various *Anopheles* species to *P. falciparum* infection.[1–8] However, only one study has examined quantitatively the entire sporogonic cycle.[9] During sporogony, there is a progressive loss in parasite numbers from ingested gametocyte to mature oocyst. The magnitude and heterogeneity of these losses within a mosquito species ultimately determine its vector competence.

In this study, we examined the sporogonic development of cultured *P. falciparum* in six species of *Anopheles* mosquitoes, determining absolute densities of macrogametocytes, ookinetes, oocysts, and sporozoites. Transitional efficiencies between parasite lifestages were calculated using life table statistics and are used as the basis for comparison of vector competence. *Anopheles gambiae* G3 strain was chosen as the reference species on which to base species comparisons because of its ease in rearing, its readiness to feed from a membrane feeder, the importance of the species as a vector of falciparum malaria, and because *P. falciparum* sporogony has been best characterized in this species.[9] The primary goals of this study were two-fold: 1) to

identify for each *Anopheles* species, the key transition(s) determining its overall susceptibility as an initial step toward identifying specific mechanisms of susceptibility, and 2) to provide a simple methodology and analytical approach that can be used in the field to examine wild parasite populations developing within indigenous vector species.

MATERIALS AND METHODS

Mosquitoes. Six species of anopheline mosquitoes were examined: *A. gambiae* Giles (G-3), *A. arabiensis* Patton (GMAL), *A. dirus* Payton & Harrison, *A. freeborni* Aitken (MARYSVILLE), *A. albimanus* Weidemann, and *A. stephensi* Liston (PAKISTAN). Established colonies of *A. gambiae, A. arabiensis, A. dirus, A. stephensi,* and *A. freeborni* were obtained from the Laboratory for Parasitic Diseases, National Institutes of Health (Bethesda, MD). *Anopheles albimanus* was obtained from the Centers for Disease Control and Prevention (Atlanta, GA).

Parasites. Gametocytes of the NF54 strain and derived clones[10] of *P. falciparum* were cultured in vitro using standard techniques.[11]

Infectious feedings. Gametocytes were fed to mosquitoes within 15–19 days after culture initiation. Cultures were diluted approximately 1:10 with washed human erythrocytes mixed 1:1 with heat-inactivated human sera. Mosquitoes were allowed to feed for 15 min on the diluted cultures via water-jacketed membrane feeders (one per cage). Unfed mosquitoes were removed. Mosquitoes were maintained at 27°C and 70% relative humidity, with access to 5% Karo® (CPC, International, Inc., Englewood Cliffs, NJ) solution. Not all mosquito species were available every time parasite cultures were mature and as a result, different species often were infected on different days using different cultures. However, infectious feedings were always conducted with the reference species, *A. gambiae,* so that comparisons among different species could be made.

Macrogametocyte sampling. Mean macrogametocyte density per mosquito (n = six mosquitoes/species/trial) was estimated by multiplying mean blood meal volume times mean blood meal erythrocyte density times percent macrogametocytemia of the blood meal. Mean blood meal volume was determined by subtracting the average weight of unfed mosquitoes from that of engorged mosquitoes collected immediately after feeding and multiplying the difference by the specific gravity of blood (1.05). Blood meals were then excised, diluted 1:200 in physiological saline, and standard erythrocyte counts were performed with a hemacytometer. To estimate percent macrogametocytemia, two thin smears were prepared from the culture material from each feeder. Slides were fixed in methanol, stained with Giemsa solution, and examined under 1,000× oil immersion optics. The number of mature (stage V) macrogametocytes per 2–4 × 10^4 erythrocytes was determined and percentages for the two slides were averaged.

Ookinete sampling. Estimates of ookinete density (n = 6–10) were obtained by emptying individual blood meals into 20 μl of a 3% acetic acid solution that lysed erythrocytes, leaving only parasites intact. Samples were mixed by pipetting, and ookinetes were counted with a hemacytometer under phase-contrast microscopy (400×). Ookinete residence in the blood meal is ephemeral. Therefore, three separate time-course trials were conducted to determine when ookinetes were most abundant in each mosquito species. At selected intervals postingestion, blood meals were excised from six to 10 mosquitoes of each species, and ookinetes were counted.

Oocyst sampling. Mosquito midguts were dissected, stained with mercurochrome, and examined (400×) for oocysts on days 9–12 postinfection.

Sporozoite sampling. Salivary gland sporozoite densities were determined by excising salivary glands from individual mosquitoes, placing the glands in small glass tissue grinders containing 35 μl of M199 medium, disrupting the glands, and counting sporozoites with a hemacytometer under phase-contrast microscopy (400×).

Analysis of parasite density. Cohort life tables[12, 13] were constructed to determine the overall level of species susceptibility to infection and to determine where in the life cycle population mortality was most intense. The decrease in density from one stage to the next (i.e., mortality) was expressed as a k value, which is simply the difference in population density, expressed as logarithms, between one lifestage and the next. Thus, $k - 1$ equals $\log_{10}$ (macrogametocyte) minus $\log_{10}$ (ookinete), $k - 2$ equals $\log_{10}$ (ookinete) minus $\log_{10}$ (oocyst), and K equals total mortality from macrogametocyte to oocyst (i.e.,

k − 1 plus k − 2). In biological terms, k − 1 represents the decrease in parasites due to macrogametocytes not being fertilized and/or failing to differentiate to ookinetes and k − 2 represents the decrease in parasite numbers due to ookinetes failing to penetrate the midgut and/or failing to develop to mature oocysts. Appropriate transformations (e.g., $\log_{10}x + 1$) were performed for statistical comparison of means (analyses of variance and paired t-tests, SAS version 6; SAS Institute, Inc., Cary, NC).

Lifestage correlations. Correlations within and among parasite lifestages were examined using simple linear regression and second-order polynomial regression (CRICKET GRAPH; Computer Associates Internationals, Inc., Islandia, NY). For each infection and vector species, stage-specific parameters (i.e., log-transformed mean densities or infection rates) were plotted against the parameters of successive lifestages. Correlation coefficients were tested for significance using a table of critical values.[14] In general, polynomial models yielded higher correlation coefficients (r), but many times the increase was marginal. Polynomial models (= curvilinear) were chosen over simple (= linear) models only if they gave a substantial improvement of fit as determined by their respective r values and visual inspection of scatter diagrams.

Analysis of parasite distribution. The goal was to examine changes in parasite distributions among mosquito species and parasite lifestages. The rationale was that the degree of clumping or overdispersion displayed by a parasite population is generally indicative of the heterogeneity in mosquito-to-mosquito susceptibility to infection. Frequency distributions were constructed for each parasite lifestage and mosquito species and departures of these frequency distributions from normality were measured with skewness moments. (PROC UNIVARIATE; SAS). Distributions were tested for fit to normal and negative binomial distributions by chi-square goodness of fit[15] (NEGBINOM.BASIC; John Wiley & Sons, New York, NY[16]). Green's index of dispersion[17] was used to compare dispersion patterns within individual infections. This index is based on the variance to mean ratio and was chosen because it is easy to calculate, and unlike the dispersion parameter k of the negative binomial distribution, it is independent of changes in the sample mean or sample size, making it appropriate for comparing different populations that vary in these parameters.[16]

Comparative susceptibility over time. Oocyst infection ratios[18] were calculated for each coinfection. An oocyst infection ratio is the ratio of the oocyst infection rate of a species divided by the oocyst infection rate in the reference species, *A. gambiae*. A ratio > 1.0 indicates that a species was more susceptible than *A. gambiae* and a ratio < 1.0 indicates that a species was less susceptible. Oocyst infection ratios were listed chronologically to detect changes in comparative susceptibilities over time.

RESULTS

Infectious feeding. All mosquito species fed from the membrane feeders, but feeding behaviors and engorgement rates differed among species. *Anopheles freeborni* and *A. albimanus* consistently had the best engorgement rates. They engorged quickly and left the feeder immediately. Other species, particularly *A. stephensi*, remained on the feeders for extended periods, filtering blood through the anus. Engorgement rates for *A. stephensi* and *A. gambiae* were generally good but rates for *A. arabiensis* and *A. dirus* were sometimes so low (< 10%) that it was not possible to obtain adequate sample sizes for the completion of life tables.

There were species differences in blood meal volumes ($F = 9.76$, degrees of freedom [df] = 5,56, $P < 0.0001$, by analysis of variance [ANOVA]) and erythrocyte densities ($F = 16.89$, df = 5,345, $P < 0.0001$, by ANOVA). Species taking the smallest blood meals (*A. gambiae, A. stephensi,* and *A. dirus*) concentrated erythrocytes during feeding while species taking the largest blood meals (*A. freeborni* and *A. albimanus*) did not (Table 1). These differences (i.e., a small volume of densely packed cells versus a large volume of loosely packed cells) counteracted each other so that the total numbers of erythrocytes per blood meal were the same among species. Because the macrogametocytemias of the infectious blood were also the same among species, the starting macrogametocyte densities per mosquito were the same among species for each experiment. However, the microenvironment in which sporogony began (i.e., the consistency of the blood meals) differed. Erythrocytes in *A. freeborni* and *A. albimanus* blood meals were less densely packed

TABLE 1

Mean ± SD blood meal volumes (μl), erythrocyte densities (cells per μl), and Plasmodium falciparum *macrogametocyte content (%) of blood fed to six species of* Anopheles *mosquitoes**

Species	No. of infections	Blood meal volume (μl)	RBC density per μl (× 10^{-4})	Macrogametocytemia (%)	Macrogametocyte density per mosquito
A. gambiae	14	1.87 ± 0.24† (n = 14)	565 ± 134‡§ (n = 84)	0.19 ± 0.11‡ (n = 28)	20,651 ± 12,977‡ (n = 14)
A. arabiensis	7	2.35 ± 0.53§¶ (n = 7)	532 ± 185‡§¶ (n = 30)	0.17 ± 0.08‡ (n = 14)	21,304 ± 13,183‡ (n = 7)
A. dirus	4	2.16 ± 0.22¶† (n = 4)	577 ± 187‡§ (n = 22)	0.24 ± 0.13‡ (n = 8)	28,074 ± 13,505‡ (n = 4)
A. stephensi	12	2.13 ± 0.27¶† (n = 12)	590 ± 146‡ (n = 70)	0.20 ± 0.13‡ (n = 24)	25,586 ± 16,163‡ (n = 12)
A. albimanus	11	2.64 ± 0.34‡§ (n = 11)	414 ± 159¶ (n = 61)	0.17 ± 0.09‡ (n = 22)	19,521 ± 11,915‡ (n = 11)
A. freeborni	14	2.92 ± 0.69‡ (n = 14)	428 ± 120§¶ (n = 84)	0.20 ± 0.12‡ (n = 28)	25,411 ± 16,179‡ (n = 14)

* These values were used to calculate the estimated numbers of macrogametocytes ingested per mosquito for each infection. Samples sizes (n) indicate total number of mosquito pools (blood meal volumes), individual mosquitoes (red blood cell [RBC] densities), or culture slides (macrogametocytemia) examined. Means within columns followed by the same symbol do not differ significantly from each other ($P < 0.05$, by Duncan's multiple range test).

than in other species. For most species, blood meal erythrocytes appeared normal but in *A. albimanus*, erythrocytes consistently appeared damaged and agglutinated.

Ookinete kinetics. Development of ookinetes was monitored in conjunction with gonatrophic development in host mosquitoes. In three separate time-course trials, mature ookinetes were most abundant when mosquito ovarian development reached Christopher stage III (i.e., period of increasing yolk deposition). For most species, this occurred at 30–32 hr postinfection. Ovarian development and blood meal digestion were substantially slower in *A. dirus*. Likewise, ookinete abundance in *A. dirus* peaked later (48–50 hr postinfection) than in the other five mosquito species.

Parasite density. Stage-specific parasite densities are presented in life table format as paired comparisons (Table 2). This was to indicate more accurately the comparative susceptibility of each species relative to that of the reference species, *A. gambiae*. Mean starting densities of macrogametocytes were the same among species (Tables 1 and 2). The overall susceptibility to infection can be ascertained by examining the last two columns of Table 2 (oocyst density and total mortality, K). In the reference species, *A. gambiae*, oocyst densities and total mortalities were approximately 0.7 and 4.5, respectively, except in the *A. dirus*/*A. gambiae* coinfections, where the number of replicates were small (n = 4). A K value of 4.5 represents a 31,623-fold (antilog of 4.5) decrease in parasite numbers from macrogametocyte to oocyst stage. Thus, a high K value indicates low susceptibility to infection and vice versus. In most species, K values exceeded the starting macrogametocyte densities because calculation of mean densities for subsequent lifestages included zero counts (i.e., mosquitoes in which parasites failed to develop).

Overall susceptibilities of *A. arabiensis* (K = 4.8) and *A. dirus* (K = 5.4) were the same as that of coinfected *A. gambiae* (K = 4.5, 5.0, respectively, Table 2). There were no significant differences in parasite lifestage densities in either *A. arabiensis* and *A. dirus* compared with those in coinfected *A. gambiae*. The *A. dirus* coinfections illustrate the necessity of paired comparisons. If the overall susceptibility of *A. dirus* (K = 5.4) based on four infections were compared with that of all 14 *A. gambiae* infections (K = 4.5), then *A. dirus* would appear to be significantly less susceptible (a log-fold difference) to infection than *A. gambiae*. However, this would be misleading since it happened that the gametocyte cultures used in the four *A. dirus* coinfections were not particularly fertile cultures compared with other cultures used in infectious feeds where *A. dirus* were not available.

Overall susceptibilities of *A. stephensi* (K = 5.1) and *A. albimanus* (K = 5.3) were less than those of coinfected *A. gambiae* (K = 4.5 and 4.4, respectively), resulting in significantly lower oocyst densities. Overall susceptibility in *A. freeborni* (K = 3.6) was greater than that of

TABLE 2

Stage-specific mean densities (95% confidence limits) and mortalities (k values) of Plasmodium falciparum *developing within six species of* Anopheles *mosquitoes**

Species	No. of coinfections	Macrogametocytes	k − 1	Ookinetes	k − 2	Oocysts	K
A. arabiensis	7	21,304	2.79	34.58	1.98	0.36	4.77
		(9,502–33,088)	616-fold	(6.09–177.43)	95-fold	(0–1.35)	58,884-fold
A. gambiae		18,869	2.36	81.80	2.11	0.63	4.47
		(8,825–28,912)	231-fold	(40.39–164.64)	129-fold	(0.03–1.57)	29,716-fold
A. dirus	4	28,074	3.09	22.95	2.28	0.12	5.37
		(9,329–46,819)	1,223-fold	(0–796.00)	192-fold	(0–0.36)	233,089-fold
A. gambiae		27,829	2.85	39.59	2.11	0.31	4.96
		(14,956–40,702)	708-fold	(16.83–91.40)	129-fold	(0–0.90)	91,201-fold
A. stephensi	12	25,586	2.69	51.85	2.40	0.21	5.09
		(15,419–32,753)	490-fold	(31.00–86.28)	250-fold	(0.06–0.37)	123,027-fold
A. gambiae		22,269	2.40	89.53	2.06	0.77†	4.46
		(13,878–30,660)	252-fold	(51.72–154.45)	116-fold	(0.26–1.49)	28,840-fold
A. albimanus	11	19,521	2.46	68.27	2.82	0.10	5.28
		(11,613–27,428)	288-fold	(35.54–130.30)	660-fold	(0–0.22)	190,546-fold
A. gambiae		20,744	2.52	61.92	1.89	0.78†	4.41
		(10,641–30,847)	331-fold	(20.58–188.37)	79-fold	(0.22–1.59)	25,704-fold
A. freeborni	14	25,411	2.26	138.53†	1.32	6.68‡	3.58
		(16,136–34,686)	182-fold	(65.61–291.30)	21-fold	(3.49–12.13)	3,802-fold
A. gambiae		20,651	2.53	60.23	1.97	0.65	4.50
		(13,212–28,091)	339-fold	(25.62–139.83)	93-fold	(0.22–1.23)	31,623-fold

* Infections are presented as paired comparisons to indicate susceptibility of a species relative to that of the reference species, *A. gambiae*.
† Significantly greater at the 0.05 level (paired *t*-test on $\log_{10}$ transformed means).
‡ Significantly greater at the 0.001 level (paired *t*-test on $\log_{10}$ transformed means).

coinfected *A. gambiae* (K = 4.5), resulting in a significantly greater oocyst density (Table 2, $P < 0.001$, by paired *t*-tests).

Dynamics contributing to species differences in overall susceptibilities can be assessed by examining k − 1 and k − 2. In *A. stephensi*, lower comparative susceptibility was due to the combined effects of both k − 1 and k − 2. Although neither mortality factor was statistically significant by itself, their multiplicative effect resulted in lower overall susceptibility (high K) and significantly lower oocyst densities (Table 2). In *A. albimanus*, lower overall susceptibility was due solely to a high k − 2 value (i.e., mortality during the ookinete to oocyst transition). The greater comparative susceptibility of *A. freeborni* was due to increased efficiency at both life cycle transitions (i.e., low k − 1 and k − 2).

Sporozoites were quantified in the salivary glands of *A. gambiae*, *A. dirus*, *A. stephensi*, and *A. freeborni* (Table 3). Sporozoites were not observed in the other species due to insufficient numbers of 14-day old mosquitoes (*A. arabiensis*) and species refractoriness (*A. albimanus*). *Anopheles freeborni* had significantly greater gland infection rates and oocyst density per infected midgut than did other species ($P < 0.05$, Table 3). There were no significant differences among species in mean sporozoite density within infected glands (overall mean = 2,546, range = 835–3,724 sporozoites) or estimated gland sporozoite production per oocyst (overall mean = 640, range = 540–1,361 sporozoites).

Lifestage correlations. Relationships between population parameters were examined among macrogametocytes, ookinetes and oocysts developing in the six species of *Anopheles* (Table 4). There was no relationship between macrogametocyte and ookinete densities for any mosquito species. Instead, there were significant effects ($P < 0.0001$, by ANOVA) on ookinete production according to the date on which an infection was conducted (i.e., the particular culture used) for every mosquito species. This indicated that certain gametocyte cultures were simply more fertile than others regardless of gametocyte density.

There were significant relationships between ookinete and oocyst densities in *A. gambiae* (r = 0.741), *A. arabiensis* (r = 0.644), and *A. free-*

TABLE 3

Plasmodium falciparum *sporozoite density and production in the salivary glands of four species of* Anopheles *mosquitoes**

Species	No. of infections	Overall percentage of gland sporozoite positive mosquitoes	Mean gland sporozoite density per infected mosquito†	Mean oocyst density per infected mosquito†	Estimated gland sporozoite production per oocyst†‡
A. gambiae	5	30.1% (22/73)	1,259 (367–4,323)	2.51 (1.51–4.19)	502 (217–1,160)
A. dirus	1	22.2% (4/18)	835	1.17	713
A. stephensi	2	42.4% (14/33)	1,910 (7–545,846)	1.40 (0.02–102.49)	1,361 (348–5,322)
A. freeborni	8	78.2%§ (86/110)	3,724 (2,414–5,744)	6.91§ (4.16–11.48)	540 (389–748)

* Means were transformed to logarithms and analyzed by Duncan's multiple range test; infection rates were analyzed by Fisher's exact test.
† Values in parentheses are 95% confidence limits.
‡ Sporozoite production per oocyst was calculated by dividing the mean log sporozoite density of each infection by the corresponding mean log oocyst density, summing the values, and dividing by the number of infections.
§ $P = 0.05$ versus the other three species.

borni (r = 0.836). The nature of these relationship differed among species. In *A. gambiae* and *A. arabiensis*, ookinete and oocyst densities were related curvilinearly, whereas in *A. freeborni*, the relationship was linear. This is illustrated more clearly in Figure 1 where ookinete and oocyst densities within *A. gambiae* were plotted using our complete database accumulated over several years (n = 76). In *A. gambiae*, the relationship was curvilinear and conversion of ookinetes to oocysts was negligible below a threshold of approximately 30 ookinetes per mosquito (= log 1.5). Above this threshold, conversion of ookinetes to oocysts increased exponentially. In contrast, conversion of ookinetes to oocysts within *A. freeborni* (n = 19, Figure 1) was linear with no distinct threshold requirement.

There were significant correlations between all other ookinete/oocyst parameters within *A. gambiae* and *A. freeborni* (Table 4). Lack of significant correlations between ookinete and oo-

TABLE 4

Correlation between lifestage parameters of Plasmodium falciparum *macrogametocytes, ookinetes, and oocysts developing in six species of* Anopheles *mosquitoes**

Lifestage parameters		*Anopheles* species					
		gambiae	*arabiensis*	*dirus*	*stephensi*	*albimanus*	*freeborni*
Macrogametocyte density	Ookinete density	r = 0.402 (n = 14) NS	r = 0.045 (n = 8) NS	r = 0.024 (n = 4) NS	r = 0.210 (n = 13) NS	r = 0.089 (n = 12) NS	r = 0.185 (n = 14) NS
Ookinete density	Oocyst density	r = 0.741 (n = 17) Curvilinear	r = 0.644 (n = 9) Curvilinear	r = 0.335 (n = 5) NS	r = 0.378 (n = 13) NS	r = 0.137 (n = 14) NS	r = 0.836 (n = 19) Linear
Ookinete density	Ookinete infection rate	r = 0.995 (n = 17) Curvilinear	r = 0.982 (n = 9) Curvilinear	r = 0.996 (n = 5) Curvilinear	r = 0.997 (n = 13) Curvilinear	r = 0.995 (n = 14) Curvilinear	r = 0.984 (n = 19) Curvilinear
Oocyst density	Oocyst infection rate	r = 0.981 (n = 17) Curvilinear	r = 0.998 (n = 10) Linear	r = 0.589 (n = 5) NS	r = 0.997 (n = 13) Curvilinear	r = 0.993 (n = 14) Linear	r = 0.977 (n = 19) Curvilinear
Ookinete density	Oocyst infection rate	r = 0.683 (n = 17) Curvilinear	r = 0.469 (n = 9) NS	r = 0.758 (n = 5) NS	r = 0.346 (n = 13) NS	r = 0.137 (n = 14) NS	r = 0.825 (n = 19) Curvilinear
Ookinete infection rate	Oocyst infection rate	r = 0.642 (n = 17) Linear	r = 0.472 (n = 9) NS	r = 0.784 (n = 5) NS	r = 0.375 (n = 13) NS	r = 0.171 (n = 14) NS	r = 0.801 (n = 19) Linear

* Means include zero counts (i.e., uninfected mosquitoes) and were transformed to $\log_{10}(x + 1)$ for analysis. r = correlation coefficient; n = no. of infections; NS = not significant at the 0.05 level.

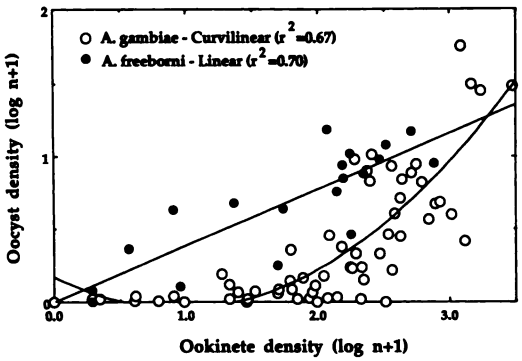

FIGURE 1. Relationship between ookinete and oocyst densities of *Plasmodium falciparum*. Each data point indicates mean values for ookinete and oocyst densities within a single infection. This demonstrates that the relationship of ookinetes to oocysts is curvilinear in *Anopheles gambiae* **(open circles)** and linear in *A. freeborni* **(closed circles)**.

cyst parameters within the other mosquito species are indicative of small sample sizes (e.g., *A. dirus*) or low oocyst densities/infection rates due to poor species susceptibility.

Only *A. gambiae* and *A. freeborni* had sufficient replicate sporozoite infections (n = 5 and 8, respectively) to conduct regression analyses on parameters contributing to sporozoite production. In *A. gambiae*, there were highly significant correlations among all lifestage parameters. In *A. freeborni*, there were significant correlations among most interactions, but not between ookinete parameters and sporozoite infection rates, nor between sporozoite density and sporozoite rate.

The most consistently robust associations between lifestage parameters among all species were the positive correlations between densities and infection rates of homologous lifestages, with the only exceptions being the density/rate correlations of oocysts in *A. dirus* (Table 4) and of sporozoites in *A. freeborni*. In most cases, relationships between density and rate were curvilinear because rates tended to flatten out near 100% with increasing density.

Parasite distribution. Parasite distributions within vector species were examined from two perspectives; the overall distribution of all infections combined (i.e., the forest) and the distribution within individual infections (i.e., the trees). With regard to the former, stage-specific frequency distributions were constructed. Macrogametocyte distributions were not included because they are established artificially by culture dilution and, more importantly, macrogametocyte density has no relationship to successive lifestages (Table 4). In general, macrogametocyte distributions were either normally distributed, polymodal, or platykurtic (flattened).

Frequency distributions for ookinetes and oocysts were not normal but were skewed to the right and fitted reasonably well to negative binomial types of distributions ($P < 0.05$, by chi-square goodness of fit). Frequency distributions for ookinetes were similar among mosquito species. Frequency distributions for oocysts within most species were considerably more skewed than their corresponding ookinete distributions. The notable exception was the frequency distribution of oocysts within *A. freeborni* where the increase in skewness from ookinete to oocyst was much less pronounced. Differences in skewness between lifestages was attributed to the dramatic differences in lifestage densities (Table 2) and overall infection rates. Skewness moments (g) and overall infection rates (%) for ookinetes were *A. gambiae* (g = 1.6, 62%), *A. arabiensis* (g = 1.7, 50%), *A. dirus* (g = 1.6, 52%), *A. stephensi* (g = 2.2, 55%), *A. albimanus* (g = 2.5, 68%), and *A. freeborni* (g = 1.8, 79%). Skewness moments and overall infection rates for oocysts were *A. gambiae* (g = 6.9, 24%), *A. arabiensis* (g = 8.6, 15%), *A. dirus* (g = 8.6, 11%), *A. stephensi* (g = 6.0, 15%), *A. albimanus* (g = 7.2, 6%), and *A. freeborni* (g = 2.4, 73%).

The distribution within individual infections were examined by calculating and plotting Green's index of dispersion for each infection where more than one infected mosquito was found (Figure 2). A high index of dispersion indicates heterogeneity in mosquito susceptibility to infection. A low index indicates relative homogeneity. Indices for *A. arabiensis* and *A. dirus* are not shown because the numbers of infections were small. For most species (including *A. arabiensis* and *A. dirus*), dispersion indices varied from infection to infection (note ranges and 95% confidence intervals in Figure 2). The variation was irrespective of parasite densities (linear regression r^2 values < 0.5). The notable exception was *A. freeborni* where in all but a few ookinete infections, indices of dispersion were consistently low (= low heterogeneity). For all species, dispersion tended to be somewhat higher in ookinetes than in oocysts.

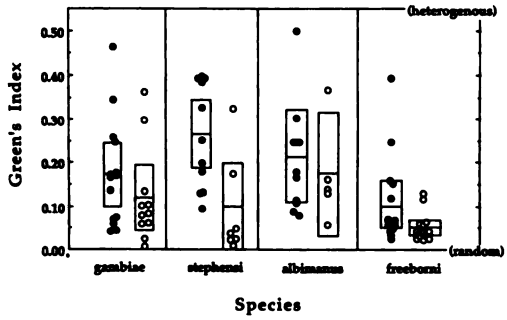

FIGURE 2. Indices of dispersion for *Plasmodium falciparum* ookinete **(closed circles)** and oocyst **(open circles)** infections in four species of *Anopheles* mosquitoes. Box plots indicate the mean and 95% confidence limits for values of Green's Index, which range from 0 (random distribution) to 1 (maximum clumping).

Comparative susceptibility over time. Temporal changes in comparative susceptibilities were examined by listing oocyst infection ratios chronologically. The comparative susceptibilities of *A. albimanus* and *A. stephensi* remained fairly constant with respect to one another, but near the end of the study, both changed relative to the susceptibility of the reference species, *A. gambiae* (Table 5).

TABLE 5

Susceptibilities with time of Anopheles stephensi *and* A. albimanus *to infection with* Plasmodium falciparum *relative to the susceptibility in the coinfected reference species,* A. gambiae*

Days elapsed since initiation of study	Oocyst infection ratio†		Oocyst infection rate in reference species (*A. gambiae*)
	A. stephensi	*A. albimanus*	
1	0.95 (n = 21)	0.00 (n = 37)	10% (2/20)
2	0.23 (n = 30)	0.18 (n = 39)	14% (3/21)
25	0.44 (n = 73)	—	3% (3/95)
41	0.69 (n = 27)	0.04 (n = 71)	37% (15/40)
42	0.28 (n = 44)	0.29 (n = 21)	65% (13/20)
64	0.32 (n = 50)	—	50% (20/40)
183	0.09 (n = 49)	0.39 (n = 97)	24% (10/42)
225	0.00 (n = 31)	0.46 (n = 39)	11% (3/27)
230	0.05 (n = 76)	0.00 (n = 29)	50% (16/32)
237	0.81 (n = 36)	0.05 (n = 65)	58% (18/31)
305	2.57 (n = 33)	2.16 (n = 55)	6% (2/34)
328	1.40 (n = 19)	1.21 (n = 55)	7% (3/40)
349	3.83 (n = 35)	7.14 (n = 8)	7% (2/30)

* Relative susceptibilities are expressed as oocyst infection ratios. n = no. of mosquitoes examined.

† Oocyst infection ratio = oocyst infection rate of test species ÷ oocyst infection rate of reference species.

DISCUSSION

Species differed in their susceptibility to infection. This is not surprising for it has long been known that such differences exist among mosquito species. However, most studies have used oocyst infection rates or densities as a measure of the comparative susceptibility of a species or group of species.[1-8] Such data reveal little about the mechanisms occurring within the mosquito responsible for susceptibility. Here, we have not specifically identified mechanisms of susceptibility. Rather as a first step, we have provided a quantitative description of the sporogonic cycle within six vector species. In so doing, we have identified for each species critical transitions in the parasite life cycle where mechanisms responsible for susceptibility may exert the strongest influence.

There are three major transitions during sporogony: gametocyte to ookinete, ookinete to oocyst, and oocyst to salivary gland sporozoite. The first two transitions result in net losses in parasite numbers; the third results in an increase in parasite numbers. In some parasite-vector systems, the third transition can be an important factor in limiting successful sporogony.[19] This was not the case in our studies (Table 3). The first two transitions were the most important in determining susceptibility.

We found that the critical transition determining susceptibility was different for different species. For example in *A. albimanus,* susceptibility was most severely constrained by ookinete losses (k − 2, Table 2). Most likely, these losses were due to ookinetes failing to traverse the midgut or atrepic degeneration (i.e., lack of essential nutrients) of early oocysts. Antiblastic (antagonist) mechanisms known to occur in *Anopheles,* such as melanotic encapsulation,[20] were not observed.

In contrast, susceptibility in *A. freeborni* was enhanced at both transitions (Table 2). Increased ookinete density in *A. freeborni* indicated that fertilization and/or zygote transformation was more efficient than in other vector species. Several distinctive features about *A. freeborni* may account for this. First, *A. freeborni* does not concentrate or agglutinate ingested erythrocytes as in the other species. Blood meal compaction or agglutination may restrict the motility of exflagellating male gametes and impede fertilization.[21] Second, *A. freeborni* display lower levels

of midgut aminopeptidase activity than *A. gambiae* (Chege G, unpublished data). Aminopeptidase activity is correlated with refractoriness to *P. falciparum*.[22] Midgut homogenates of *A. freeborni* contain substance(s) known as exflagellation factor that stimulate the exflagellation of male gametocytes in vitro.[23] However, exflagellation factor cannot account for the increased ookinete production by *A. freeborni* since the level of exflagellation enhancement induced in vitro by midgut homogenates of *A. freeborni* did not differ from the exflagellation induced by midgut homogenates of the other five vector species.[24]

In addition to their enhanced production, ookinetes in *A. freeborni* passed through the midgut with greater efficiency than in other species. Reasons for this are not known but may involve species differences in ookinete-midgut receptor interactions and/or peritrophic matrix, thought to be a physical barrier to ookinete passage.[25,26] Macroscopically, the peritrophic matrix in *A. freeborni* appeared to be as robust and the kinetics of its formation was similar to that of other species. However, the transition of ookinetes to oocysts was linear in *A. freeborni* but clearly curvilinear in *A. gambiae* (Figure 1). The curvilinear relationship observed in *A. gambiae* indicates the existence of a barrier (peritrophic matrix?) that is insurmountable until a critical mass of ookinetes is achieved. No threshold requirement was observed in *A. freeborni*. This suggests that peritrophic matrix in *A. freeborni*, although present, is not a significant obstacle to ookinete passage, and as such, may have a different microscopic structure or chemical composition than that of other species. The linear conversion of ookinetes to oocysts in *A. freeborni* is more consistent with a lectin-ligand recognition mechanism of ookinete passage.

Not only did the transitional efficiencies of sporogony differ among species but the biological processes themselves were influenced by the vector species. For example, *A. dirus* took a relatively long time (4–5 days) to digest a blood meal and produce eggs. There was also a concurrent lag in ookinete formation, with the peak occurring at 48–50 hr postingestion instead of the usual 30–32 hr. This indicates that the timing of fertilization and ookinete formation was somehow coordinated with the digestive processes occurring within the vector. The developmental clues triggering transformation of zygotes to ookinetes within the mosquito midgut are unknown. However, it is interesting to note that in *A. stephensi*, peak ookinete abundance coincides with peak digestive enzyme activity within the midgut,[27] suggesting that an increasing protease level in the blood meal may signal zygotes to initiate the transformation process.

While the analysis of parasite abundance (e.g., life tables) provides insights into the mechanism of vector susceptibility, analysis of parasite distribution provides insights into the population genetics of vector susceptibility. Ookinete and oocyst populations were not distributed randomly among mosquitoes, but like most parasite populations,[28–30] they were overdispersed or clumped (skewed) in distribution. It is generally assumed that overdispersal in parasite populations is generated by the heterogeneity within the host population's susceptibility to parasite infection. That is, most mosquitoes in a population are either not susceptible or only weakly so, while a few mosquitoes are very susceptible. Our results confirm that the observed heterogeneity in sporogonic lifestages was due primarily to mosquito heterogeneity, not parasite heterogeneity. Evidence for this comes from the differences in dispersion indices observed for *A. freeborni* versus other species (Figure 2). In *A. freeborni*, sporogonic processes occurred fairly homogeneously throughout the course of the study. Dispersion indices for both ookinetes and oocysts clustered near zero (i.e., low heterogeneity). Thus, within a suitable mosquito species (in this case *A. freeborni*), populations of fertile gametocytes developed in a homogenous manner infection after infection. Yet in another mosquito species (e.g., *A. gambiae*), the very same gametocyte populations developed homogeneously in one mosquito cohort and heterogeneously in the next. This indicates that it was not the parasite populations that were responsible for fluctuating heterogeneity, but instead fluctuations were the result of shifting heterogeneity within mosquito populations.

Even within our inbred colonies, the gene frequencies determining mosquito susceptibility were not stable over time. This was evident not only from the infection-to-infection variation in dispersion indices (Figure 2), but also in the reversal in relative susceptibilities near the end of our study (Table 5). This reversal was most likely due to a decrease in the frequency of susceptible phenotypes in *A. gambiae* rather than to

simultaneous increases in the frequencies of susceptible phenotypes in *A. albimanus* and *A. stephensi*. Temporal shifts in relative susceptibility have been reported previously in laboratory colonies of *A. stephensi* infected with *P. cynomolgi*.[31]

Genetic drift in our *A. gambiae* colony was apparently random. However, in areas of west Africa, shifting gene frequencies in *A. gambiae* populations, as measured by chromosomal inversion forms, may occur on a more predictable, seasonal basis.[32, 33] It remains to be determined whether heterogeneity in mosquito susceptibility to *Plasmodium* infection fluctuates temporally within wild anopheline populations (as it does in laboratory colonies), particularly in regions of seasonal environmental instability.

In conclusion, we have described quantitatively the sporogonic cycle of *P. falciparum* in six species of *Anopheles* and have identified the critical transitions in the cycle responsible for overall species susceptibilities. However, one should not interpret our results to represent what is occurring in the field. There are several distinct differences. For example, the gametocyte densities in this study far exceed those normally found in nature.[34–36] But perhaps the biggest precautionary note is our use of laboratory-adapted populations of both mosquitoes and parasites. The population dynamics of sporogony in wild parasites, coadapted to their local vector species, is probably very different from the population dynamics of laboratory-adapted strains. Although the transitional efficiencies of *P. falciparum* sporogony in naturally-infected mosquitoes are not known, it may be inferred from recent studies in western Kenya on the reservoir of infection[36] and ookinete densities[37] that transitional efficiencies in nature far exceed those observed in the laboratory.

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REFERENCES

1. Collins WE, Warren McW, Skinner JC, Richardson BB, Kearse TS, 1977. Infectivity of the Santa Lucia (El Salvador) strain of *Plasmodium falciparum* to different anophelines. *J Parasitol 63:* 57–61.
2. Warren McW, Collins WE, Richardson BB, Skinner JC, 1977. Morphologic variants of *Anopheles albimanus* and their susceptibility to *Plasmodium vivax* and *P. falciparum*. *Am J Trop Med Hyg 26:* 607–611.
3. Collins WE, Skinner JC, Stanfill PS, Richardson BB, 1978. Studies on the Burma (Thau.) strain of *Plasmodium falciparum* in *Aotus trivigatus* monkeys. *J Parasitol 63:* 497–500.
4. Collins WE, Warren McW, Richardson BB, Skinner JC, Kearse TS, 1979. Selected pupal phenotypes of *Anopheles freeborni* and their susceptibility to *Plasmodium falciparum* and *P. vivax*. *Mosq News 39:* 466–472.
5. Collins WE, Warren McW, Skinner JC, Richardson BB, Chin W, 1979. Studies on the West African I strain of *Plasmodium falciparum* in *Aotus trivigatus* monkeys. *J Parasitol 65:* 763–767.
6. Warren McW, Collins WE, Richardson BB, Skinner JC, 1979. Naturally occurring pupal phenotypes of *Anopheles albimanus* and their susceptibility to *Plasmodium vivax* and *P. falciparum*. *Mosq News 39:* 472–477.
7. Collins WE, Nguyen-Dinh P, Skinner JC, Sutton BB, 1981. Infectivity of a strain of *Plasmodium falciparum* from Hainan, People's Republic of China, to different anophelines. *Am J Trop Med Hyg 30:* 538–543.
8. Klein TA, Lima JBP, Tada MS, 1991. Comparative susceptibility of anopheline mosquitoes to *Plasmodium falciparum* in Rondonia, Brazil. *Am J Trop Med Hyg 44:* 598–603.
9. Vaughan JA, Noden BH, Beier JC, 1992. Population dynamics of *Plasmodium falciparum* sporogony in laboratory-infected *Anopheles gambiae*. *J Parasitol 78:* 716–724.
10. Davis JE, Cortese JF, Herrington DA, Murphy JR, Clyde DF, Thomas AW, Baqar S, Cochran MA, Thanassi J, Levine MM, 1992. *Plasmodium falciparum:* in vitro characterization and human infectivity of a cloned line. *Exp Parasitol 74:* 159–168.
11. Ponnudurai T, Lensen AD, Leeuwenberg ADE, Meuwissen JHET, 1982. Cultivation of *Plasmodium falciparum* gametocytes in semi-automatic systems. 1. Static cultures. *Trans R Soc Trop Med Hyg 76:* 812–818.
12. Varley GC, Gradwell GR, Hassell MP 1973. *Insect Population Ecology: An Analytical Approach.* Berkeley: University of California Press.

13. Southwood TRE, 1978. *Ecological Methods.* New York: John Wiley & Sons, 356–387.
14. Rohlf FJ, Sokal RR, 1969. *Statistical Tables.* San Francisco: W. H. Freeman & Co.
15. Bliss CI, Fisher RA, 1953. Fitting the negative binomial distribution to biological data. *Biometrics 9:* 176–196.
16. Ludwig JA, Reynolds JF, 1988. *Statistical Ecology.* New York: John Wiley & Sons.
17. Green RH, 1966. Measurement of non-randomness in spatial distributions. *Res Pop Ecol 8:* 1–7.
18. Collins WE, Roberts JM, 1991. *Anopheles gambiae* as a host for geographic isolates of *Plasmodium vivax. J Am Mosq Control Assoc 7:* 569–573.
19. Rosenberg R, 1985. Inability of *Anopheles freeborni* to transmit *Plasmodium knowlesi* sporozoites. *Am J Trop Med Hyg 34:* 687–691.
20. Collins FH, Sakai RK, Vernick KD, Paskewitz S, Seeley DC, Miller LH, Collins WE, Campbell CC, Gwadz RW, 1986. Genetic selection of a *Plasmodium*-refractory strain of the malaria vector, *Anopheles gambiae. Science 234:* 607–610.
21. Janse CJ, Mons B, Rouwenhorst RJ, van der Klooster PFJ, Overdulve JP, van der Kaay HJ, 1985. In vitro formation of ookinetes and functional maturity of *Plasmodium berghei* gametocytes. *Parasitology 91:* 19–29.
22. Feldemann AM, Billingsley PF, Savelkoul E, 1990. Bloodmeal digestion by strains of *Anopheles stephensi* Liston (Diptera: Culicidae) of differing susceptibility to *Plasmodium falciparum. Parasitology 101:* 193–200.
23. Nijhout MM, 1979. *Plasmodium gallinaceum:* exflagellation stimulated by a mosquito factor. *Exp Parasitol 48:* 75–80.
24. Mendis C, Noden BH, Beier JC, 1994. Exflagellation responses of cultural *Plasmodium falciparum* (Haemosporida: Plasmodiidae) gametocytes to human sera and midguts of anopheline mosquitoes (Diptera: Culcidae). *J Med Entomol 31:* (in press).
25. Sieber K, Huber M, Kaslow D, Banks SM, Torii M, Aikawa M, Miller LH, 1991. The peritrophic membrane as a barrier: its penetration by *Plasmodium gallinaceum* and the effect of a monoclonal antibody to ookinetes. *Exp Parasitol 72:* 145–156.
26. Billingsley PF, Rudin W, 1992. The role of the mosquito peritrophic membrane in bloodmeal digestion and infectivity of *Plasmodium* species. *J Parasitol 78:* 430–440.
27. Billingsley PF, Hecker H, 1991. Blood digestion in the mosquito, *Anopheles stephensi*/Liston (Diptera: Culcidae): activity and distribution of trypsin, aminopeptidase and α-glucosidase in the midgut. *J Med Entomol 28:* 865–871.
28. Eyles DE, 1951. Studies on *Plasmodium gallinaceum.* I. Characteristics of the infection in the mosquito, *Aedes aegypti. Am J Hyg 54:* 101–112.
29. Crofton HD, 1971. A quantitative approach to parasitism. *Parasitology 62:* 179–193.
30. Anderson RM, Gordon DM, 1982. Processes influencing the distribution of parasite numbers within host populations with special emphasis on parasite-induced mortalities. *Parasitology 85:* 373–398.
31. Rutledge LC, Hayes DE, Ward RA, 1970. *Plasmodium cynomolgi:* sources of variation in susceptibility of *Anopheles quadrimaculatus, A. balabacensis,* and *A. stephensi. Exp Parasitol 27:* 53–59.
32. Rishikesh N, DiDeco MA, Petraca V, Coluzzi M, 1985. Seasonal variations in indoor resting *Anopheles gambiae* and *Anopheles arabiensis* in Kaduna, Nigeria. *Acta Trop 42:* 165–170.
33. Toure YT, 1991. The *Anopheles gambiae* complex in West Africa. *Science in Africa: Achievements and Prospects.* Washington, DC: American Association for the Advancement of Science, 55–75.
34. Jeffery GM, Eyles DE, 1955. Infectivity to mosquitoes of *Plasmodium falciparum* as related to gametocyte density and duration of infection. *Am J Trop Med Hyg 4:* 781–789.
35. Muirhead-Thomson RC, 1957. The malarial infectivity of an African village population to mosquitoes (*Anopheles gambiae*). A random xenodiagnosis survey. *Am J Trop Med Hyg 6:* 971–979.
36. Githeko AK, Brandling-Bennett AD, Beier M, Atieli F, Owaga M, Collins FH, 1992. The reservoir of *Plasmodium falciparum* malaria in a holoendemic area of western Kenya. *Trans R Soc Trop Med Hyg 86:* 355–358.
37. Beier JC, Copeland RS, Mtalib R, Vaughan JA, 1992. Ookinete rates in Afrotropical anopheline mosquitoes as a measure of human malaria infectiousness. *Am J Trop Med Hyg 47:* 41–46.