J. Parasitol., 80(5), 1994, p. 705–712

REINTERPRETATION OF THE GENETICS OF SUSCEPTIBILITY OF *AEDES AEGYPTI* TO *PLASMODIUM GALLINACEUM*

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ABSTRACT: Several studies have demonstrated a genetic basis for variation in susceptibility of *Aedes aegypti* to *Plasmodium gallinaceum*. Although 25 yr ago it was reported that *P. gallinaceum* susceptibility in *Ae. aegypti* is determined primarily by a single autosomal dominant gene, evidence for additional genetic factors has emerged. Two sublines, 1 refractory and 1 of intermediate susceptibility to *P. gallinaceum*, have been selected from the Moyo-In-Dry strain (MOYO) of *Ae. aegypti*. Prior to selection, the MOYO population was 20.3% refractory. Genetic crosses of the highly susceptible Rockefeller strain (ROCK) and the 2 selected sublines of the MOYO strain provide evidence for a complex mode of inheritance of *Plasmodium* susceptibility in *Ae. aegypti*.

The control of malaria remains a major problem with the World Health Organization estimating 280 million new cases and between 1 and 2 million deaths annually (Knudsen and Slooff, 1992). The need for the development of novel vector control strategies has become urgent due to the development of multiple pesticide-resistant vector populations as well as multiple drug-resistant *Plasmodium* strains (Spencer, 1985; Slooff, 1987; Schapira et al., 1993). Elucidation of genetic mechanisms of susceptibility and refractoriness of mosquito species to *Plasmodium* parasites is 1 approach that might provide innovative malaria control strategies based on genetically disrupting vector competence and increasing the frequency of refractory alleles in wild populations.

Numerous studies have shown that susceptibility of mosquitoes to *Plasmodium* infections is an inherited characteristic and varies among different mosquito species, even between different geographical strains of the same species. Early studies by Huff (1929, 1931) showed that susceptibility of *Culex pipiens* to *Plasmodium cathemerium* was subject to change by selection and seemed to behave as a simple recessive trait. Subsequent studies by Trager (1942) and Micks (1949) with other mosquito–*Plasmodium* models provided further evidence of the genetic basis of susceptibility. In a more thorough study made by Ward (1963), the susceptibility of *Aedes aegypti* to *Plasmodium gallinaceum* was reduced by 98% after 26 generations of selection; most of the genetic variation in susceptibility was attributed to the effect of a single genetic factor that lacked dominance.

In contrast with previous studies (Huff, 1931; Ward, 1963), Kilama and Craig (1969) provided genetic evidence that *P. gallinaceum* susceptibility in *Ae. aegypti* is determined by an autosomal dominant gene (*pls*) located on the second linkage group. However, this gene was not completely expressed, i.e., oocysts still appeared in their "refractory" line. This suggested the involvement of genetic factors in addition to *pls* as 1 explanation for the presence of these oocysts.

More recently, a strain of *Anopheles gambiae* was selected for resistance to a number of different *Plasmodium* species (Collins et al., 1986). Genetic analysis of *Plasmodium* resistance in *An. gambiae* has suggested that more than 1 gene controls the expression of resistant and susceptible phenotypes (Vernick and Collins, 1989; Vernick et al., 1989). The phenomenon of *Plasmodium* resistance in *An. gambiae* refers to an active destruction of ookinetes on the midgut surface via immune effector mechanisms, whereas *Plasmodium* refractoriness in *Ae. aegypti* refers to a physiological incompatibility between the parasite and the potential vector, resulting in the failure of parasite development.

Although a genetic basis for the variation in mosquito susceptibility to *Plasmodium* species has been recorded for several decades, nothing is known about the genes or gene products controlling these traits in any mosquito species. We report herein on the selection of *P. gallinaceum* refractory and intermediate susceptibility sublines from the Moyo-In-Dry strain of *Ae. aegypti* and on the genetic studies that were conducted to clarify further the mode of inheritance of *Plasmodium* susceptibility in *Ae. aegypti*.

Received 1 December 1993; revised 25 April 1994; accepted 25 April 1994.

MATERIALS AND METHODS

Mosquitoes and parasites

Three strains of *Ae. aegypti* were used in our study. The Rockefeller strain (ROCK) is an efficient vector of *P. gallinaceum*. The Moyo-In-Dry strain (MOYO) was originally collected from Shauri Moyo Village, Mombasa, Kenya, in 1974. The RED strain was used in a preliminary study conducted to determine the relative susceptibility of different strains of *Ae. aegypti* to infection with *P. gallinaceum*. Mosquitoes were reared as previously described (Christensen and Sutherland, 1984). Female mosquitoes, 5–9 days old, were deprived of sucrose for 24 hr before receiving an infective bloodmeal.

The *P. gallinaceum* strain used in this study is routinely maintained in our laboratory by natural transmission between the ROCK strain and inbred White Leghorn chicks obtained from the Poultry Research Lab at the University of Wisconsin–Madison. All chicks were infected by sporozoite transmission by feeding infected mosquitoes on restrained birds. The parasitemia, percentage of gametocytes, and exposure index (parasitemia × percentage of gametocytes) were monitored daily beginning 1 wk postinfection (PI) by thin blood smears stained with VWR® Stat Stain. When a parasitemia ranging from 10 to 20% and a gametocytemia of at least 2% were reached, usually 9–10 days PI, the ability of the microgametocytes to undergo exflagellation was tested. Naive mosquitoes were fed on infective chicks for 15–20 min. No single infective chick was exposed to mosquito feeding for more than 3–4 hr. The susceptible ROCK strain was interposed as a control in each feeding experiment to assess the infectivity of the bloodmeal. Following exposure to the infective bloodmeal, fully engorged females were separated, supplied with sucrose solution, and held in the environmental chamber until dissected 6–7 days post-exposure (PE).

Selection methods and crosses

All selective breeding experiments were conducted with the MOYO strain using isofemale lines. Ninety randomly mated female MOYO mosquitoes were allowed to engorge on restrained infective chicks. Three days PE, bloodfed females were allowed to oviposit individually on a 2 × 9-cm strip of paper toweling placed in a dish containing distilled water to a depth of 1 cm. Oviposition papers containing the eggs were removed 2–3 days later, dried at room temperature, and stored in separate envelopes. Those females that had oviposited were subsequently dissected in a drop of *Aedes* saline (Hayes, 1953) and their midguts examined using Nomarski optics to determine the number of developing oocysts. Susceptibility to infection with *P. gallinaceum* was assessed solely on the basis of the number of oocysts seen developing on the midgut. The progenies of those females determined to be totally refractory, i.e., no oocyst development, were selected for rearing. Initially, refractory individuals descending from a single refractory female in the parental population were subjected to 2 generations of mass selection with an intervening generation of amplification. Adult females representing refractory isofemale lines then were subjected to pairwise matings and assessed for their level of refractoriness for 3 generations. Again, only progeny of fully refractory females were selected for rearing. Families of individual refractory females were kept separate at each generation of isofemale line selection. To improve fecundity, female mosquitoes that had received infective bloodmeals were fed on uninfected blood 1 day after the infective feed. Subsequent selection procedures leading to the establishment of refractory and intermediate susceptibility sublines used mass selection schemes where progenies of individual females expressing the desired characteristic (low oocyst numbers [0–10] for selection of intermediate susceptibility and no oocysts for selection of refractory sublines) were pooled and allowed to mate freely. A representative subset of females from each generation was tested for susceptibility.

Genetic crosses involved the highly susceptible ROCK strain, and the refractory (MOYO R) and intermediate susceptibility (MOYO IS) sublines of the MOYO strain. Virgin females from selected sublines were separated from males as pupae and were allowed to pairwise mate with males from the appropriate subline/strain. MOYO R female mosquitoes were pair-mated with a male from the susceptible ROCK strain (cross 1). Likewise, MOYO R females were crossed with a MOYO IS male mosquito (cross 2). Finally, MOYO IS females were crossed with a ROCK male mosquito (cross 3). Adult mosquitoes were approximately 1 wk old when females were provided an infective bloodmeal. Bloodfed females oviposited individually and were assessed for susceptibility by counting the number of oocysts seen at 6–7 days PE. Eggs were hatched 1 wk after oviposition papers were dried and resulting female progeny were separated from males. The F_1 female hybrids from crosses 1 and 2 were backcrossed to the refractory parent. These females then were fed on infected blood, allowed to oviposit, and tested for infection. Finally, backcross progeny of individual females within particular groups in each cross were selected for rearing. Adult female mosquitoes within each group were exposed to an infective bloodmeal and evaluated for *Plasmodium* susceptibility. If susceptibility was determined solely by a single-locus autosomal dominant gene, then the expected segregation ratio of refractory-to-susceptible phenotypes is 1:1. Chi-square goodness-of-fit values for segregation ratios among backcross (BC) progeny in cross 1 were calculated based on an expected 1:1 ratio for refractory-to-susceptible phenotypes. In 1 case, mosquitoes were considered refractory by the criterion of Kilama and Craig (1969), i.e., 0–10 oocysts, and in another example the refractory condition was defined by our criterion, i.e., no oocysts on their midguts.

RESULTS

Variability between strains

The relative susceptibility of the MOYO, ROCK, and RED strains of *Ae. aegypti* to *P. gallinaceum* is shown in Figure 1. High intraspecific variability in susceptibility was observed. A sample of 79 MOYO, 30 ROCK, and 31 RED females carried 8.9 ± 12.2 oocysts (mean

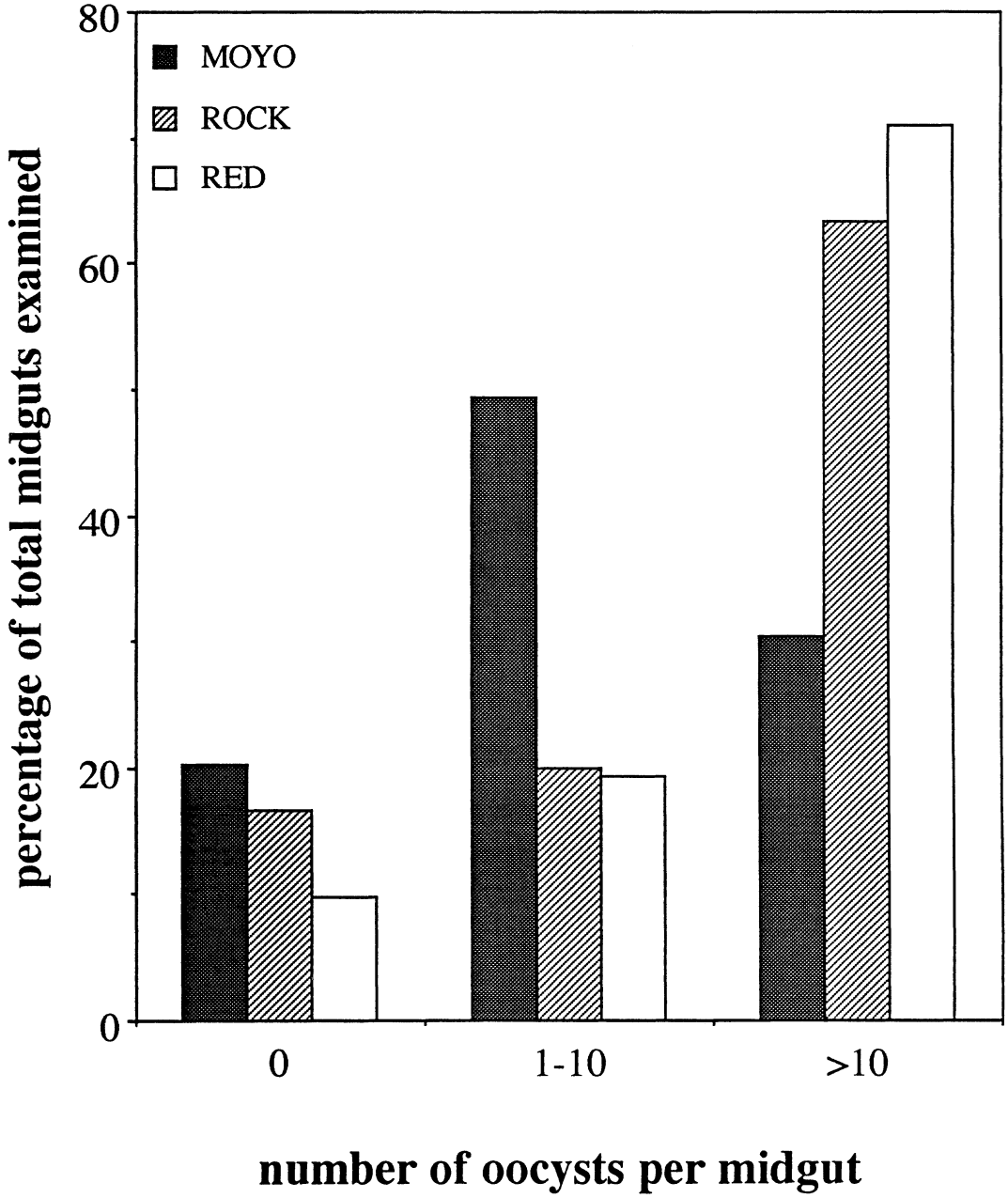

FIGURE 1. Oocyst distribution in the Moyo-In-Dry (MOYO), Rockefeller (ROCK), and RED strains of *Aedes aegypti* exposed to *Plasmodium gallinaceum*; 79, 30, and 31 female mosquitoes from each strain, respectively, were tested.

± SD; range = 0–55 oocysts), 22.9 ± 23.0 oocysts (range = 0–85 oocysts), and 44.1 ± 45.7 oocysts (range = 0–200 oocysts), respectively. The MOYO strain showed the lowest susceptibility and sufficient heterogeneity on which to base selection schemes.

Selection for refractoriness and intermediate susceptibility

The MOYO strain used for selection was 20.3% refractory (16 of 79) to *P. gallinaceum* infection. Initial attempts at selecting a 100% refractory line failed when a mass selection scheme was

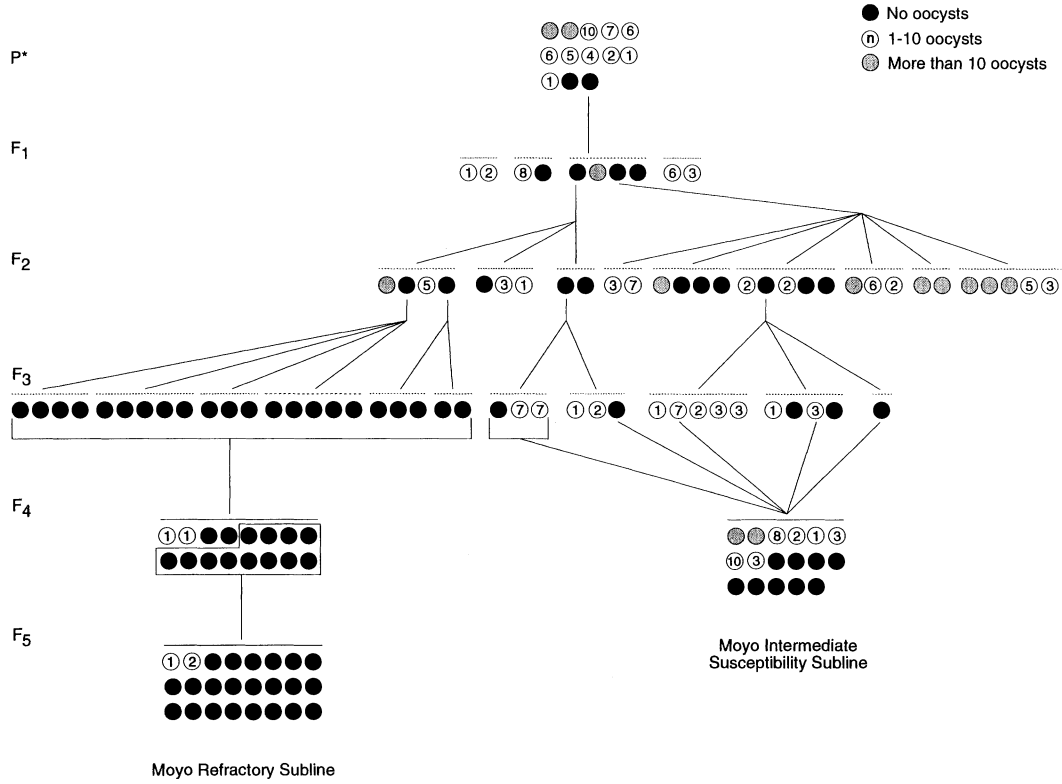

FIGURE 2. Scheme of selection in the Moyo-In-Dry (MOYO) strain of *Aedes aegypti* for refractoriness and intermediate susceptibility to *Plasmodium gallinaceum*. The parental population (asterisk) was derived from a family descending from a single female from the MOYO stock that carried no oocysts, after 2 generations of mass selection for refractoriness with an intervening generation of amplification without selection; horizontal dotted bars indicate progeny of individual females that were subjected to pairwise (single male sib) matings; horizontal solid bars indicate progeny of individual females that were pooled and allowed to mate freely (mass selection).

used. Two generations of mass selection with an intervening generation of amplification without selection yielded a population of 13 females, 11 of which were susceptible (Fig. 2). The intensity of infection was considerably reduced, however, in that 81.8% (9 of 11) of infected females carried only 1–10 oocysts compared to 61.9% (39 of 63) in the parental population.

Figure 2 shows the pedigrees of the MOYO R and MOYO IS sublines that were established. Eggs were hatched from a refractory MOYO female and the resulting sibs allowed to mate in a pairwise fashion. The susceptibility of 10 females distributed in 4 pair mating groups was assessed. Although population size was small, the response to selection after 1 generation was obvious because susceptibility decreased to 60%, with 5 of the 6 infected females having 1–10 oocysts. After a second generation of selection, susceptibility decreased to 44% in a family of 9 females. A completely refractory population was obtained after the third generation of selection. These individuals were the progeny of 2 refractory females that had been crossed with the same male sib. Three of the females in this population had abortive infections, each with 1 completely degenerated oocyst on the wall of the midgut. Eggs from all females of the third generation were pooled in order to minimize the effects of selective inbreeding and rapidly build up a population of refractory individuals. While 14 of the 16 females tested at the fourth generation were completely refractory, 2 had 1 oocyst each. An additional round of mass selection was performed on this generation by pooling progenies of 12 completely refractory females. Subsequent generations (F_5–F_{15}) maintained an average refractory rate of 91.9%. No female from the refractory

TABLE I. The susceptibility of the parental Rockefeller (ROCK) strain and the refractory subline of the Moyo-In-Dry (MOYO R) strain, F_1 hybrids, and backcross progeny of *Aedes aegypti* to infection with *Plasmodium gallinaceum.*

Category	Parents		Female progeny						
			Number tested	No. of oocysts					
	Female	Male		0	1–10	>10	Range	χ^2†	χ^2‡
Parental	MOYO R	MOYO R	24	22	2	0	0–2		
	ROCK	ROCK	21	2	2	17	0–84		
F_1	MOYO R	ROCK	22	0	7	15	2–89		
Backcross	(MOYO R × ROCK)F_1	MOYO R							
	1 (32)*		25	15	2	8	0–103		
	2 (89)		21	12	4	5	0–130		
	3 (9)		32	20	6	6	0–117		
			78	47	12	19	0–130	20.52	3.28

* Number of oocysts per F_1 female.

† The chi-square value obtained for an expected 1:1 ratio of refractory: susceptible phenotypes where mosquitoes with 0–10 oocysts are considered refractory ($P < 0.01$).

‡ The chi-square value obtained for an expected 1:1 ratio where only mosquitoes that carried no oocysts are considered refractory ($P > 0.05$).

subline ever had more than 3 oocysts when challenged with an infective bloodmeal. The generations (F_5–F_6) used in subsequent genetic studies exhibited refractory rates of 91.7% and 100%, respectively.

The MOYO IS subline was derived from offspring descending from 2 refractory female F_1 sibs (Fig. 2). After the second round of pair matings, the F_3 progeny from these females were found to be 67% susceptible and 70% susceptible, respectively, where all susceptible individuals carried only 1–7 oocysts. Thus, by the fourth generation, an "intermediate susceptibility" subline was established that reflected a reduced susceptibility rate (47.1%) and, most importantly, where the majority of the infected females carried only 1–10 oocysts. In subsequent generations (F_5–F_{12}), the average susceptibility was 42.9% and ranged from 0% to 75%, where 46.2–100% of infected females carried 1–10 oocysts. Although susceptibility of this subline fluctuated considerably in generations following active selection, we observed that susceptibility remained greatly reduced both in prevalence and intensity of infection.

Study of the mode of inheritance of susceptibility and refractoriness

Table I shows that 100% of the progeny from the cross between fully refractory MOYO females and a ROCK male (cross 1) were susceptible to infection. The mean number of oocysts ± SD within the F_1 population (19.2 ± 18.7) was intermediate between the mean number of oocysts within the 2 parental populations (0.1 ± 0.4 and 30.4 ± 25.5 oocysts for the MOYO R and ROCK strains, respectively), suggesting that incomplete dominance is involved. In the backcross to the refractory parent, segregation of the individual progeny (mean oocyst number = 15.0 ± 30.0) fitted an expected 1:1 ratio for refractory-to-susceptible phenotypes ($\chi^2 = 3.28$, $P > 0.05$), only if mosquitoes that carried no oocysts on their midguts were considered refractory. These data suggest that susceptibility of our *Ae. aegypti* strains to infection with *P. gallinaceum* is determined in part by a major gene exhibiting at least partial dominant inheritance. While these findings support previous studies (Kilama and Craig, 1969), the rationales upon which they are based conflict. That is, if a baseline of 10 developing oocysts is selected as the point of demarcation between refractory and susceptible individuals as utilized by Kilama and Craig (1969), 31.8% of the F_1 progeny observed in this cross would be considered refractory, and the observed segregation ratio among the BC progeny would exhibit a significant deviation from an expected 1:1 (Table I).

Table II shows the results of crosses between the selected MOYO R and MOYO IS sublines (cross 2) and subsequent backcrosses of F_1 hybrid females to a male of the refractory parental type. The mean number of oocysts in the MOYO IS subline was 8.1 ± 9.2. Parental female mosquitoes selected for these crosses harbored no oo-

TABLE II. The susceptibility of the parental refractory (MOYO R) and intermediate susceptibility (MOYO IS) sublines of the Moyo-In-Dry strain, F_1 hybrids, and backcross progeny of *Aedes aegypti* to infection with *Plasmodium gallinaceum*.

Category	Parents		Female progeny				
			Number tested	No. of oocysts			
	Female	Male		0	1–10	>10	Range
Parental	MOYO R	MOYO R	24	22	2	0	0–2
	MOYO IS	MOYO IS	18	5	6	7	0–30
F_1	MOYO R	MOYO IS	23	17	6	0	0–7
Backcross	(MOYO R × MOYO IS)F_1	MOYO R					
	1 (0)*		5	2	3	0	0–1
	2 (1)		4	4	0	0	0
	3 (0)		4	4	0	0	0
	4 (0)		11	11	0	0	0
	5 (6)		19	14	5	0	0–6
	6 (6)		9	9	0	0	0
			52	44	8	0	0–6

* Number of oocysts per F_1 female.

cysts on their midguts. The mean number of oocysts within the F_1 population was 1.0 ± 2.2 oocysts with all individuals carrying between 0 and 7 oocysts. In the backcross to the refractory parent nearly all progeny were refractory (mean oocyst number = 0.3 ± 1.0). We identified 8 individuals out of a total of 52 (15.4%) that carried 1–6 oocysts. While a specific mechanism of inheritance is unclear from this cross in itself, these results provide further evidence of partial dominance at 1 major locus with its phenotypes modified by either allelic variation at that locus, or by additional loci of smaller effect.

Table III shows the susceptibility of F_1 progeny produced by matings between the selected MOYO IS subline and the susceptible ROCK strain (cross 3). Two MOYO IS females, carrying 1 and 6 oocysts, respectively, were involved in the parental cross. According to criteria of Kilama and Craig (1969), these 2 MOYO IS females were refractory to *P. gallinaceum*. Thus, cross 2 would be considered 1 between refractory and susceptible strains, i.e., identical to cross 1. However, our results clearly do not fit this model. All but 1 of the F_1 hybrids were susceptible to *P. gallinaceum* but carried a mean of only 9.8 ± 7.8 oocysts. In comparison, the F_1 hybrids resulting from cross 1 (Table I) showed a higher susceptibility, with a mean of 19.2 ± 18.7. Cross 3 demonstrates that the genetics of susceptibility involved in the MOYO IS line are different than in the ROCK strain.

DISCUSSION

Our results describe the identification of a strain of *Ae. aegypti*, Moyo-In-Dry, that carries a major gene controlling *Plasmodium* refractoriness and, most importantly, describe the successful establishment of *P. gallinaceum*-refractory and intermediate-susceptibility sublines by selective inbreeding from this strain. This is the first description of selection for *Plasmodium* refractoriness in *Ae. aegypti* in 25 yr (Kilama and Craig, 1969). Initial attempts at isolating a refractory line by mass selection, i.e., pooling the progenies of selected females that carried no oocysts, were unsuccessful. This approach produced only a general reduction in intensity of infection in a predominately susceptible population. Successful selection for refractoriness was obtained after 2 generations of isofemale line selection. Isofemale line selection schemes have often been reported to produce rapid responses to selection for parasite susceptibility or refractoriness in insect vectors (Trager, 1942; Kilama and Craig, 1969; Collins et al., 1986; Feldmann and Ponnudurai, 1989; Tabachnick, 1991).

Our *Plasmodium*-refractory line of *Ae. aegypti* did permit some oocyst production, maintaining an average refractory rate of 91.9% for 11 generations after the withdrawal of selection pressure; however, infected females carried only 1–3 developing oocysts. In this respect it resembles lines of mosquitoes selected for *Plasmodium* re-

fractoriness by other workers (Ward, 1963; Kilama and Craig, 1969; Feldmann and Ponnudurai, 1989). For example, Kilama and Craig (1969) considered their selected line as refractory when female mosquitoes harbored up to 10 developing oocysts on their midguts. However, we maintained the criterion of no oocyst development for refractoriness because a mosquito with even a single viable oocyst is likely to transmit sporozoites (Huff, 1954; Pringle, 1965). To minimize environmental effects on observed levels of *Plasmodium* infection, all mosquitoes were exposed to similar levels of parasitemia and gametocytemia. Additionally, all bloodfeedings of experimental mosquitoes were coupled with bloodfeedings on a highly susceptible control group of mosquitoes to monitor any changes in the infectivity of the parasite. However, it is possible that factors associated with the parasite itself influence the susceptibility of mosquitoes to infection. Although a single strain of *P. gallinaceum* was used in these studies, threshold effects or genetic variability in the parasite's ability to influence mosquito susceptibility may also influence the phenotypic expression of oocyst number.

Our intermediate susceptibility subline has maintained the characteristics of reduced prevalence and intensity of infection through 8 generations after withdrawal of selection pressure. The isolation and maintenance of this population suggest that allelic variation at a single locus or additional minor modifier loci contributes to the expression of the susceptible or refractory phenotypes. Furthermore, in addition to the *Plasmodium*-refractory and intermediate-susceptibility sublines, we have isolated a highly susceptible subline of the MOYO strain from the same stock population after 5 generations of selection, using a mixture of isofemale line and mass selection systems. Female mosquitoes in this subline display increased prevalence and especially increased intensity of infection with *P. gallinaceum.*

Encapsulated oocysts, such as those seen in a *Plasmodium*-resistant *An. gambiae* strain (Collins et al., 1986), were never found in our refractory subline of *Ae. aegypti.* However, in a few instances refractory females carrying 1 or 2 completely degenerated oocysts were seen. Similarly, female mosquitoes with abortive *Plasmodium* infections but no encapsulated oocysts were seen in a line of *An. gambiae* selected for refractoriness to *P. berghei* (Al-Mashhadani et al., 1980).

TABLE III. The susceptibility of parental and F_1 *Aedes aegypti* to infection with *Plasmodium gallinaceum* (MOYO IS = intermediate susceptibilty subline of the Moyo-In-Dry strain, ROCK = Rockefeller strain).

			Female progeny				
	Parents		Number tested	No. of oocysts			
Category	Female	Male		0	1–10	>10	Range
Parental	MOYO IS	MOYO IS	18	5	6	7	0–30
	ROCK	ROCK	21	2	2	17	0–84
F_1	MOYO IS	ROCK	20	1	11	8	2–26

It has been suggested that the biological basis for the refractory phenotype in *Ae. aegypti* is either the absence of an essential growth factor (Kilama and Craig, 1969) or the presence of an inhibitory substance that prevents normal parasite development. However, no gene product has been identified that confers susceptibility or refractoriness of any mosquito species to a specific parasite.

Our results suggest that susceptibility of our strains of *Ae. aegypti* to *P. gallinaceum* is not controlled solely by a simple monofactorial character. While a major gene exhibiting partial dominance apparently provides primary genetic control of susceptibility, crosses involving our intermediate-susceptibility subline suggest that this locus either exhibits allelic variability in its effect or that *Plasmodium* susceptibility is a multigenic trait and additional minor genes are involved in determining the susceptible phenotype. Even the simple observation that approximately twice as many oocysts develop in the RED strain than do in the ROCK strain, both considered highly susceptible to *P. gallinaceum,* is indicative of the more complex nature of this trait. Indeed, several studies have suggested a multigenic mode of inheritance of *Plasmodium* susceptibility in mosquitoes (Ward, 1963; Al-Mashhadani et al., 1980; Collins et al., 1986; Vernick et al., 1989). Successful development of a saturated restriction fragment length polymorphism (RFLP) genetic linkage map for *Ae. aegypti* (Severson et al., 1993), in conjunction with our selected mosquito strains, should provide the necessary tools to define more clearly the relationship between phenotype and genotype in determining *Plasmodium* susceptibility.

The *Ae. aegypti–P. gallinaceum* model system is therefore a good choice for studies designed to investigate the genetic basis for vector compe-

tency because of the wealth of genetic linkage data available for this vector (Munstermann and Craig, 1979; Munstermann, 1990; Severson et al., 1993) and because sequence analysis studies have suggested that the most virulent of *Plasmodium* species in humans, *P. falciparum,* shares a closer phylogenetic relationship with *P. gallinaceum* than with other human, murine, or primate *Plasmodium* species (Waters et al., 1991, 1993). Consequently, *P. gallinaceum* and *P. falciparum* also might share biochemical requirements for development within their respective vectors.

ACKNOWLEDGMENTS

The authors thank L. A. Christensen and A. Mori for technical assistance. We thank George B. Craig at the University of Notre Dame for supplying the 3 strains of mosquitoes used in the present study. The *P. gallinaceum* strain was obtained from Robert Gwadz of the NIH. This study was supported by the National Institutes of Health grant AI33127 to D.W.S.

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