SELECTED PUPAL PHENOTYPES OF *ANOPHELES FREEBORNI* AND THEIR SUSCEPTIBILITY TO *PLASMODIUM FALCIPARUM* AND *P. VIVAX*

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ABSTRACT. Naturally occurring and selected pupal color phenotypes of *Anopheles freeborni* were found to vary in their susceptibility to 2 different strains of *Plasmodium falciparum* and 1 strain of *P. vivax*. With the Santa Lucia strain of *P. falciparum* from El Salvador, 4 naturally occurring pupal phenotypes were less susceptible to infection than the base colony. After selection, there were no differences between the different phenotypes and the base colony. Brown and green nonstriped forms were more susceptible than brown striped forms. When the selected lines were fed upon monkeys infected with the West African I strain of *P. falciparum*, all of the selected lines were more susceptible than the base colony. With the Salvador II strain of *P. vivax*, naturally occurring green nonstriped forms were less susceptible than brown striped and nonstriped forms. Upon selection, the advantage shifted to the green nonstriped and brown striped forms. A comparison with previous studies with *An. albimanus* indicated that the relationship between susceptibility to infection and pupal phenotype varied not only between the species of mosquito but between the two malaria parasites, *P. vivax* and *P. falciparum*.

Warren et al. (1977) have reported differences in the susceptibility of pupal phenotypes of *Anopheles albimanus* to *Plasmodium vivax* and *P. falciparum*. Additional studies (Warren et al. 1979) indicated that naturally occurring (unselected) pupal phenotypes of *An. albimanus* varied in their susceptibility to these two human malarias, although not in the same order as with the selected lines.

Studies were made to determine if this phenomenon also occurred with other anophelines. An attempt was made (1) to select naturally occurring pupal phenotypes as they appeared in a colony

of *An. freeborni*, (2) to establish lines of this species with distinct pupal phenotypes, and (3) to determine their susceptibility to human malaria.

Reported here are the results of these studies with *An. freeborni* and the Santa Lucia and West African I strains of *P. falciparum* and the Salvador II strain of *P. vivax*.

MATERIALS AND METHODS

The F-1 strain of *An. freeborni* has been maintained as a highly inbred strain in the laboratory since 1944 (Hardman 1947). The colony has not been deliberately subjected to selection either for susceptibility to malaria or for pupal color. The "base" colony produces from 4,000 to 6,000 pupae per day. For the determination of the relative susceptibility of the naturally occurring pupal color phenotypes, an aliquot from each day's production was examined and separated on the basis of color and pattern into 4 categories—brown with a white dorsal stripe, brown without a stripe, green with a white stripe, and green without a white stripe. Those which did not fit distinctly into any of these categories were discarded. Pupae were placed in gallon ice cream carton cages and allowed to emerge.

From the base colony, pupae with 3 of the phenotypes, brown stripe, brown nonstripe, and green nonstripe were selected also, and allowed to inbreed following the procedures previously reported for *An. albimanus* (Warren et al. 1975). Once the colonies were shown to breed true for the specific pupal character, aliquots of pupae were removed daily from the colonies, placed in ice cream carton cages, and allowed to emerge.

For the comparative susceptibility feedings, mosquitoes of approximately the same age (3 to 6 days after emergence) were transferred to pint ice cream carton cages. The number of mosquitoes available varied but averaged ca. 35. These were allowed to feed on *Aotus trivirgatus* monkeys infected with either *P. falciparum* or *P. vivax*. Gametocytes were present on the peripheral blood films and were thought to be infectious at the time of feeding. All comparative feedings for a particular day were made during a period of 45 min or less, usually between 0830 and 0930 hr. Those mosquitoes which did not engorge on the monkey were removed from the cages and destroyed. Extrinsic incubation was at 25 ± 1° C. Five percent Karo® solution was provided daily on a cellulose sponge. From 7 to 10 days after the infective blood meal, the mosquitoes were dissected and the midguts examined for the presence of oocysts of *Plasmodium*.

Two different strains of *P. falciparum* were used in the study. The Santa Lucia strain was originally isolated from a 3-year-old Salvadoran female who presented at the Santa Lucia Clinic in La Paz Department, El Salvador (Collins et al. 1977). It has been maintained by blood and/or sporozoite passage in *A. trivirgatus* monkeys. The West African I strain of *P. falciparum* was isolated from the blood of an adult American male who had acquired the infection in Africa, probably Nigeria (Collins et al. 1979). It also had been maintained by blood and sporozoite passage in these monkeys. The Salvador II strain of *P. vivax* was isolated from a natural infection in the area of Las Guarumas, in the Department of La Paz, El Salvador, (Collins et al. 1973). Again, the parasite was maintained by blood and/or sporozoite passage in *A. trivirgatus* monkeys.

Data collected included the percent infected in each lot of mosquitoes and the level of infection obtained. The latter statistic was obtained by the dissection and examination of 20 to 25 mosquitoes from each lot (average of 22.6 mosquitoes per lot). The average number of oocysts per 100 guts for a particular lot is the gut infection index (Jeffery et al. 1954). Since a particularly heavily infected lot will skew the mean gut infection index, the logarithmic value was used and the geometric mean determined. The anti-

log is the geometric mean gut infection index. Statistical analysis of the results used a paired t-test in which

$$t = \bar{x}\sqrt{\frac{n(n-1)}{Sx^2}}$$

where $\bar{x}$ is the difference between the two groups and Sx^2 is the *pooled* sum of squares (Snedecor 1946).

RESULTS

The four different pupal color phenotypes extracted directly from the base colony were each compared with the base colony and with each other for differences in mean percent infection, and geometric mean, gut infection index. The results of the feedings with the Santa Lucia strain of *P. falciparum* are presented in Table 1. The probability of the percentage infection and gut infection index of the base colony and the brown striped pupal phenotype for infection with the Santa Lucia strain of *P. falciparum* as representing the same population is less than 1%. Based on the percentage infection, statistical differences were apparent only for the base colony versus the brown striped comparisons. However, there was a significant difference between the base and all the phenotypes with regard to the gut infection index. Further analysis of all possible pairs indicated that even though the brown nonstriped and green nonstriped phenotypes had higher values than did the brown striped, the differences were not statistically significant at the 5% level. The same was true for the green versus green nonstriped comparison. Apparently, the unselected fraction of the pupal production which did not fit into any of the 4 categories contained the mosquitoes most susceptible to infection. An examination of the results of the feeding of naturally occurring pupal color phenotypes in the base colony on infections of the Salvador II strain of *P. vivax* (Table 2) indicated there was a difference between the susceptibility of the base colony and the brown striped, brown nonstriped, and green striped phenotypes. However, the base had a higher percentage infection and higher mean gut infection index than the green nonstriped phenotype. Further analysis of all possible pairs indicated significant advantages for the parasite of only the brown nonstriped and green striped over the green nonstriped.

Paired comparisons indicated no significant difference between the derived lines with the different pupal phenotypes and the base colony with regard to infection with the Santa Lucia strain of *P. falciparum* (Table 3). Based on the gut infection index, however, the brown nonstriped and green nonstriped lines

Table 1. Relative susceptibility of different naturally occurring pupal color phenotypes of *Anopheles freeborni* to the Santa Lucia (El Salvador) strain of *Plasmodium falciparum*.

Comparison	No. of comparisons	Mosquitoes examined	Mean percent infection	Geo. mean Gut infection index
Base : Brown striped	45	1,229 : 1,003	69.9 : 59.9**	389 : 240**
Base : Brown nonstriped	41	1,122 : 897	72.5 : 68.1	447 : 339*
Base : Green striped	29	807 : 549	71.7 : 64.5	380 : 275*
Base : Green nonstriped	43	1,175 : 1,003	71.7 : 68.3	437 : 324*
Brown striped : Brown nonstriped	41	921 : 897	62.6 : 68.1*	282 : 339
Brown Striped : Green striped	29	663 : 549	62.3 : 64.5	275 : 275
Brown striped : Green nonstriped	43	953 : 1,003	62.3 : 68.3	269 : 324
Brown nonstriped : Green striped	28	632 : 530	68.1 : 65.7	339 : 295
Brown nonstriped : Green nonstriped	39	851 : 739	69.9 : 72.4	372 : 389
Green striped : Green nonstriped	29	549 : 552	64.5 : 68.6	275 : 324

* Significant difference at 5 percent level.
** Significant difference at 1 percent level.

Table 2. Relative susceptibility of different naturally occurring pupal color phenotypes of *Anopheles freeborni* to the Salvador II strain of *Plasmodium vivax*.

Comparison	No. of comparisons	Mosquitoes examined	Mean percent infection	Geo. mean Gut infection index
Base : Brown striped	38	1,003 : 734	74.4 : 72.2	603 : 501
Base : Brown nonstriped	39	1,030 : 762	72.1 : 74.5	562 : 550
Base : Green striped	18	436 : 302	65.8 : 63.8	251 : 224
Base : Green nonstriped	27	660 : 409	74.2 : 65.8*	501 : 331**
Brown striped : Brown nonstriped	37	724 : 705	73.1 : 76.6	537 : 631
Brown striped : Green striped	16	263 : 249	73.0 : 69.5	288 : 275
Brown striped : Green nonstriped	27	470 : 409	72.5 : 65.8	437 : 331
Brown nonstriped : Green striped	17	308 : 302	67.2 : 64.8	245 : 229
Brown nonstriped : Green nonstriped	26	431 : 391	77.8 : 65.3**	575 : 331**
Green striped : Green nonstriped	15	234 : 201	73.2 : 67.1	324 : 245*

* Significant difference at 5 percent level.
** Significant difference at 1 percent level.

had statistically significant higher values than did the brown striped. Paired comparisons between these derived lines with the different pupal phenotypes and the Salvador II strain of *P. vivax* indicated statistically significant advantages of both the brown striped and green nonstriped over the base colony and the brown nonstriped (Table 4).

In order to determine if the pattern of susceptibility was oriented to the strain of *P. falciparum*, additional feedings were made on animals infected with the African strain of the parasite (Table 5). The differences were much more pronounced with the West African I strain than with the Santa Lucia. Based on both the mean percent infection and the gut infection indices, all of the 3 pupal phenotypes were more heavily infected than the base colony. The brown nonstriped continued to be more heavily infected than the brown striped.

A comparison between the results of these susceptibility studies are presented in Table 6. It is obvious that there were changes in the relationships between the different pupal phenotypes as a result of selection.

DISCUSSION

The results of the feedings of the unselected phenotypes indicated that there were differences in the level of susceptibility to the Santa Lucia strain of *P. falciparum* within the F-1 strain of *An. freeborni*. All the pupal phenotypes examined had lower levels of infection than did the colony as a whole. The least

Table 3. Relative susceptibility of different selected pupal color phenotypes of *Anopheles freeborni* to the Santa Lucia (El Salvador) strain of *Plasmodium falciparum*.

Comparison	No. of comparisons	Mosquitoes examined	Mean percent infection	Geo. mean Gut infection index
Base : Brown striped	19	516 : 409	66.4 : 63.1	394 : 295
Base : Brown nonstriped	23	632 : 472	64.7 : 63.9	380 : 437
Base : Green nonstriped	23	632 : 409	64.7 : 65.6	380 : 398
Brown striped : Brown nonstriped	19	409 : 389	63.1 : 66.1	295 : 525**
Brown striped : Green nonstriped	19	409 : 336	63.1 : 68.3	295 : 461*
Brown nonstriped : Green nonstriped	23	472 : 409	63.9 : 65.6	437 : 398

* Significant difference at the 5 percent level.
** Significant difference at the 1 percent level.

Table 4. Comparative susceptibility of different selected pupal phenotypes of *Anopheles freeborni* to the Salvador II strain of *Plasmodium vivax*.

Comparison	No. of comparisons	Mosquitoes examined	Mean percent infection	Geo. mean Gut infection index
Base : Brown striped	29	779 : 698	63.1 : 74.0**	316 : 402
Base : Brown nonstriped	38	1,020 : 857	66.7 : 70.3	349 : 335
Base : Green nonstriped	36	967 : 830	68.7 : 78.4**	383 : 526**
Brown striped : Brown nonstriped	29	675 : 733	74.0 : 66.2*	402 : 302**
Brown striped : Green nonstriped	28	698 : 702	76.3 : 75.7	450 : 508
Brown nonstriped : Green nonstriped	35	819 : 819	71.8 : 78.0*	373 : 536**

* Significant difference at the 5 percent level.
** Significant difference at the 1 percent level.

susceptible appeared to be those with the brown striped character. Selection of lines containing 3 different pupal phenotypes resulted in a marked increase in relative susceptibility to infection in all lines. The striped character continued to be associated with the least susceptible mosquitoes. These differences were extended to another strain of *P. falciparum* from Africa. It would appear that the selective inbreeding of a small segment of the F-1 colony of *An. freeborni* for any of these pupal characters resulted in a marked increase in susceptibility to *P. falciparum*. This increase in susceptibility through selection was also apparent for *P. vivax*, although to a somewhat lesser degree. The greatest change with *P. vivax* appeared to be an increased advantage of the green nonstriped forms over both the base and the brown nonstriped forms.

The selection for pupal phenotype in *An. freeborni* compared with that of *An. albimanus* (Table 6) indicated that the relationship between green pupal color and susceptibility to *P. vivax* was directly reversed (brown nonstriped versus green nonstriped comparisons). With *An. freeborni*, the naturally occurring brown nonstriped forms were more susceptible than the green nonstriped; with the derived lines, the green nonstriped were more susceptible than the brown nonstriped. With *An. albimanus* and *P. vivax*, the naturally occurring green nonstriped forms were more susceptible than the brown nonstriped; with the derived lines, the brown nonstriped were more susceptible than the green nonstriped forms. Changes in the level of susceptibility to *P. falciparum* were more pronounced in *An. freeborni* than in *An. albimanus* (5 changes in the relationships in *An. freeborni* following selection, versus

Table 5. Relative susceptibility of different selected pupal color phenotypes of *Anopheles freeborni* to the West African I strain of *Plasmodium falciparum*.

Comparison	No. of comparisons	Mosquitoes examined	Mean percent infection	Geo. mean Gut infection index
Base : Brown striped	13	367 : 240	56.9 : 75.5*	245 : 324*
Base : Brown nonstriped	14	395 : 315	55.6 : 80.3**	240 : 631**
Base : Green nonstriped	13	366 : 174	55.6 : 71.6**	247 : 468*
Brown striped : Brown nonstriped	13	240 : 301	75.5 : 78.8	324 : 613*
Brown striped : Green nonstriped	12	231 : 167	76.2 : 71.6	330 : 474
Brown nonstriped : Green nonstriped	13	295 : 174	79.6 : 71.6	608 : 468

* Significant difference at the 5 percent level.
** Significant difference at the 1 percent level.

Table 6. Changes in patterns of susceptibility between naturally occurring and selected pupal color phenotypes of *Anopheles freeborni* and *An. albimanus* to the Salvador II strain of *Plasmodium vivax* and the Santa Lucia strain of P. falciparum.

Comparison##	*P. vivax*#				*P. falciparum*			
	An. freeborni		*An. albimanus**		*An. freeborni*		*An. albimanus*	
	Natural	Derived	Natural	Derived	Natural	Derived	Natural	Derived
Base : Brown nonstriped	=	=	<	<	>	=	=	=
Base : Brown striped	=	<	<	<	>	=	=	=
Base : Green nonstriped	>	<	<	=	>	=	<	=
Brown striped : Brown nonstriped	=	>	=	>	=	<	=	=
Brown striped : Green nonstriped	=	=	<	>	=	<	=	=
Brown nonstriped : Green nonstriped	>	<	<	>	=	=	=	=

* From Warren et al., 1977, 1979.
\# Based on statistically significant differences in the Gut Infection Index.
\## Sign (=, <, >) indicates relationship of first pair member to second.

only one change with *An. albimanus*). It is obvious that there were distinct differences in the susceptibility relationships of both the *An. freeborni* and *An. albimanus* for the 2 species of *Plasmodium*. This is again readily demonstrated in the brown nonstriped versus green nonstriped comparisons. Whereas there were dramatic differences in both the *An. freeborni* and the *An. albimanus* between the naturally occurring and the derived lines exposed to *P. vivax*, there were no differences between occurring and derived phenotypes with either species of mosquitoes when exposed to infection with *P. falciparum*.

These results support the thesis that although there are direct relationships between pupal phenotype and susceptibility to *Plasmodium* infection, there is no consistency either between species of mosquito or species of malaria parasite. What qualitative or quantitative factors associated with the pupal color are responsible for increased or decreased susceptibility to infection are at present unknown, although the results of these studies would indicate that it is a complex relationship involving both the mosquito host and the parasite.

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