Genetics of refractoriness to *Plasmodium falciparum* in the mosquito *Anopheles stephensi*

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Abstract. We previously selected a line of the malaria vector mosquito *Anopheles stephensi* refractory (resistant) to the human malaria parasite *Plasmodium falciparum*, using *in vitro* infections with *P. falciparum* gametocytes. This report presents data on the genetic background of refractoriness. The results of F_1-crosses and backcrosses show that refractoriness to *P. falciparum* in our *A. stephensi* line is autosomal and semi-dominant to susceptibility. The expression of refractoriness is apparently affected by a cytoplasmic factor. Interpretation of data from the crosses by quantitative trait locus analysis shows that one gene or two unlinked interacting autosomal genes, or groups of closely linked genes, are involved.

Key words. *Anopheles stephensi*, *Plasmodium falciparum*, vector parasite relationship, selection, refractoriness, genetic crosses, segregation, Quantitative Trait Locus (QTL) Analysis, Pakistan.

Introduction

Current methods of malaria control are unsatisfactory, so new approaches are being sought. These include manipulation of the vector *Anopheles* mosquito (Diptera: Culicidae) population in the field, aimed not at eradication but at decreasing its vector competence for malaria parasites (Collins & Paskewitz, 1995). Improving our understanding of the *Plasmodium falciparum*–vector relationship should facilitate the development of novel methods for malaria control, such as the new generation of transmission-blocking vaccines (Targett, 1990; Billingsley, 1994; Coleman *et al.*, 1994) or genetic control of malaria vector *Anopheles* mosquitoes. The possibility of reducing the vectorial capacity in the field through the dilution or complete replacement of a *P. falciparum*-transmitting vector population by a refractory field-adapted strain of the same anopheline species has attracted much attention (Jones, 1957; Graves & Curtis, 1982; Curtis & Graves, 1988; Curtis, 1994; Crampton, 1994).

Feldmann & Ponnudurai (1989) reported the selection of a line of *Anopheles stephensi* Liston, which greatly reduced susceptibility to *P. falciparum* when fed on gametocytes cultured *in vitro*. This paper presents the genetic analysis of

susceptibility to *P. falciparum* in that line of *An. stephensi*, an important vector of all four species of human malaria in the Indian subcontinent.

Materials and Methods

Anopheles stephensi *strains and lines*

The Kasur strain of *An. stephensi* was field-collected in 1981 from a cattle shed at Kasur, in the Punjab province of Pakistan, and has been maintained for over a decade at IPO-DLO. The Punjab strain (Pb) was obtained from the former Pakistan Medical Research Center through Dr R. Sakai. This Pb strain is highly susceptible to *P. falciparum* (Feldmann & Ponnudurai, 1989) and originates from a mixture of various stocks collected in the field in the Punjab province of Pakistan. The refractory line Pb3–9a (R) was genetically selected by Feldmann & Ponnudurai (1989) for reduced susceptibility to *P. falciparum*. The susceptible line Sda-500 (S) was genetically selected for increased susceptibility from the 'Sind' strain, a mixture of stocks from the Sind Province of Pakistan (Feldmann & Ponnudurai, 1989; Feldmann *et al.*, 1990a). Both the S- and R-lines have maintained good genetic stability and vigour in the IPO-DLO insectary in the Netherlands over 13 years.

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Rearing of mosquito larvae, maintenance of adults and Plasmodium falciparum *infections*

Mosquito colonies were maintained by supplying the adults with a 5% fructose solution in a bottle containing a paper wick; the females were occasionally blood-fed on mice anaesthetized with Nembutal® (Narcovet®; 0.4–0.7 mg/10 g bodyweight). Eggs were collected 3 days after blood-feeding, from a bowl of water lined with filter paper and placed in the cage on the previous evening. For eclosion, 400 eggs were placed within a floating paper rim in a tray (36–24 cm) containing 4.5 l of pre-warmed and de-gassed tap water + 250 ml aquarium water. The water and air temperature were 26 and 28°C, respectively, and a LD 16:8 h regime, with 45′ dawn-dusk simulation and ambient relative humidity of 70–80%. The first larval stage was fed by a few drops of Liquifry® No.1 (Interpet Ltd, Dorking, Surrey, U.K.) larval-fish food, added to the rearing tray on the first day. The later larval stages were fed twice daily with Micromin® (Tetra Werke, Melle, Germany) in quantities related to food consumption by developing larvae (Feldmann *et al.*, 1990b).

Mosquito infections with *Plasmodium falciparum* used the contents of one 'tipper' vessel (Ponnudurai *et al.*, 1982), containing about 0.25 ml of packed cells. *Plasmodium falciparum* parasitized cell suspension was washed on a cushion of pre-warmed red cells by centrifugation at 560 **g** for two min at 37°C and subsequently diluted with 6.5 ml of fresh pre-warmed bloodgroup A cells in compatible human serum at a haematocrit of 50%. The mixture, containing 2000–4000 mature gametocytes per ml, was distributed over six membrane feeders (blood chamber diameter 38 mm, depth of 1 mm), covered with stretched parafilm. Immediately after preparation of the feeders, 5-day-old adult female mosquitoes, seventy-six per cage, were allowed to engorge on the temperature-conditioned feeders for 15 min. For some experiments, the first group of females to obtain an infective feed was followed by a second group of females fed from the same membrane and culture. Thus, for experiment 11 the females were the first to engorge from the same feeder and parasite/blood mixture as used for experiment 7. Likewise, the females for experiment 9 followed those of experiment 14 on the same feeder 10 min later. Partially-fed or unfed females were discarded.

The infected females of *An. stephensi* were kept at 26°C and 70–80% r.h., supplied with 5% fructose diet in a containment facility. Dissection for oöcyst counting was undertaken 7 days after infection.

As the distribution of the number of oöcysts in a group of midguts follows the negative binomial distribution (Eyles, 1951), the median was taken as a standard for the susceptibility of the mosquitoes to *P. falciparum* infection.

Design of the genetic crosses

The following notation, without any presuppositions about the basis of the difference in susceptibility, is used throughout the text: R = Pb3–9a; S = Sda-500; RS = F_1 females from crossing R females with S males; SR = F_1 females from the reciprocal cross; RS.S and RS.R = backcross of RS-F_1 females to males of the S and R lines, respectively. Notation for the other backcrosses follows the same conventions.

Virgin females and males were obtained by sex separation through microscopic examination during the pupal stage. For crosses, 300 pupae of each sex were placed together in a small transparent plastic bowl containing 200 ml tapwater (de-gassed and temperature conditioned), which was placed into a 25 cm cubic cage. On the next day, bowls with the un-emerged pupae were removed from the cages. Mass mating started during the dusk periods from the second day onwards. Crosses were synchronized such that females of the same age, representing progenies of the different genetic crosses as well as the relevant control strains, could be infected on the same infective bloodmeal. Thus, F_1 females from reciprocal crosses between the R and S lines were fed on part of the same infective bloodmeal as females from the parental lines. Females derived from reciprocal backcrosses (RS or SR males or females mated to mates of the parental R and S lines) were fed on the same infective bloodmeal as control females. Exceptions to this general experimental set-up are indicated in Results.

Quantitative genetic analysis

Various simple hypotheses about the number of genes determining refractoriness can be tested in classical segregation analysis using 'mixture models' for segregating populations. Commonly, data from the different segregating populations are analysed separately. However, a joint analysis of all parental and offspring data can be much more powerful and recently a statistical procedure has been developed for fitting complex (normal) mixture models by the method of maximum likelihood (Jansen, 1993; Jansen & Stam, 1994).

We considered several simple genetic models with one or two unlinked loci only, and with or without a putative cytoplasmic factor interacting with these loci. For each locus there are two homozygous genotypes and one heterozygous genotype; the cytoplasmic factor can be present or absent. Therefore, in total, we have $3 \times 3 \times 2 = 18$ possible genotypes and twelve possible types of population: the parents (R and S), the two reciprocal F_1s (RS and SR), the four possible backcrosses to R (RS × R, R × RS, SR × R and R × SR) and the four possible backcrosses to S (RS × S, S × RS, SR × S, S × SR). The parental lines were highly inbred and repeatedly checked for the absence of individuals with aberrant phenotypes (Feldmann & Ponnudurai, 1989). Thus, only the backcrosses give genetically segregating populations and for each backcross we can specify the probabilities of each of the eighteen genotypes according to the model assumed.

In our joint analysis of all parental and offspring data we modelled eighteen expected genotypic means for susceptibility in terms of additive, dominance and epistatic gene effects. The populations were tested ('blocked') in a series of experiments and main block effects were part of the model. Parameters were estimated by the method of maximum likelihood. The likelihood values of the models were used to compare the relative goodness-of-fit of the different models.

Table 1. *Anopheles stephensi* susceptibility expressed as median (negative binomial distribution) number of *P. falciparum* oöcysts per mosquito (7 days post-infection), of parental susceptible (S) and refractory (R) lines and F_1 progeny from reciprocal crosses between them. Also is included the unselected Punjab (Pb) strain (see Materials and Methods for further details).

	Parents				F_1-progeny			
Experiment	Strain	No. females dissected	Median oöcysts	P^1	Females × males	No F_1-females dissected	Median oöcysts	P^2
1	R	9	9.3	0.001	R × Pb	70	77.6	< 0.01
	Pb	20	108.5					
2	R	15	5.3	< 0.001	Pb × R	45	23.0	
	Pb	19	52.1					
3	R	0	–		R × S	30	49.4	0.030
	S	0	–		S × R	30	32.5	
4	R	0	–		R × S	19	147.4	0.038
	S	0	–		S × R	17	104.9	
5	R	10	2.2	< 0.001	R × S	35	33.5	< 0.001
	S	20	84.2		S × R	59	13.0	
6	R	30	9.1	< 0.001	R × S	38	92.1	0.898
	S	12	165.6		S × R	49	97.2	

[1] comparing the R and the Pb or S strains. [2] comparing the F_1 hybrids from the reciprocal crosses.

Differences between the various likelihoods of the models were measured relative to the model having the highest likelihood. Twice this difference, the so-called 'deviance' is approximately χ^2 distributed, where the difference in parameters involved determines the number of degrees of freedom (d.f.), for 'nested' models.

Results

Crossing the susceptible and refractory lines

The susceptibility of F_1 females from reciprocal crosses between the R line and the unselected Punjab strain was determined in experiments 1 and 2 (Table 1). In experiments 3, 5 and 6, susceptibility of the F_1 females was measured from reciprocal crosses between the selected susceptible (S) and refractory (R) lines. It was observed that the susceptibility of F_1 females was intermediate between that of the parental lines, but (except in experiment 6) the susceptibility of F_1 females was significantly dependant on the direction of the cross, tending to resemble the male parent.

The oöcyst distributions of the R and S lines rarely overlapped (5% overlap in experiment 5 and none in experiment 6). In experiment 5, where relatively low levels of infection were observed, 40% of SR and 17% of RS F_1 females were found within the parental R-distribution. However, in experiment 6, with higher infection levels, the oöcyst distributions of F_1 females from both crosses were significantly ($P < 0.01$) different from that of the parental R line, but not significantly different from that of the parental S line.

Backcrossing to the susceptible and refractory line

The results of SR backcrosses (experiments 7, 8, 11 and 12/13) are presented as distributions of the number of oöcysts per female in Fig. 1 and those of RS (experiments 9, 10 and 14) are given in Fig. 2.

SR.S and S.SR females were equally susceptible and were not significantly less susceptible than S females (experiments 7 and 8), but both types of backcross females were significantly more susceptible than SR females (experiment 7).

SR.R females were significantly less susceptible than R.SR females (experiment 12/13). In experiment 11, SR.R females again had a median less than that of R.SR, but the difference was not significant ($P = 0.115$). Both types of backcross females were significantly less susceptible than S females and SR females (experiment 11), but more susceptible than the parental R females.

RS.S and S.RS females were equally susceptible, and were significantly less susceptible than S females (experiments 9 and 10, Fig. 2). Both types of backcross females were not significantly more susceptible than RS females (experiment 9).

In experiment 14, R.RS females were almost significantly ($P = 0.08$) less susceptible than RS.R females. R.RS and RS.R females were significantly less susceptible than S females (experiment 10) and significantly more susceptible than the parental R females, but both types of these backcross females were not less susceptible than RS females (experiments 9 and 14).

One can make direct comparisons of the susceptibility of a range of genotypes, by comparing results of experiment 11 vs. experiment 7 (Fig. 1) and experiment 14 vs. experiment 9 (Fig. 2), because of the use of identical parasite cultures in these pairs of experiments, for mosquito infection (see Materials and Methods). SR.R and R.SR females (experiment 11) had median oöcyst numbers about one third of those of the SR.S and S.SR females (experiment 7). Also, according to expectation RS.R and R.RS females (experiment 14) were less susceptible, though not significantly, than RS.S and S.RS females (experiment 9).
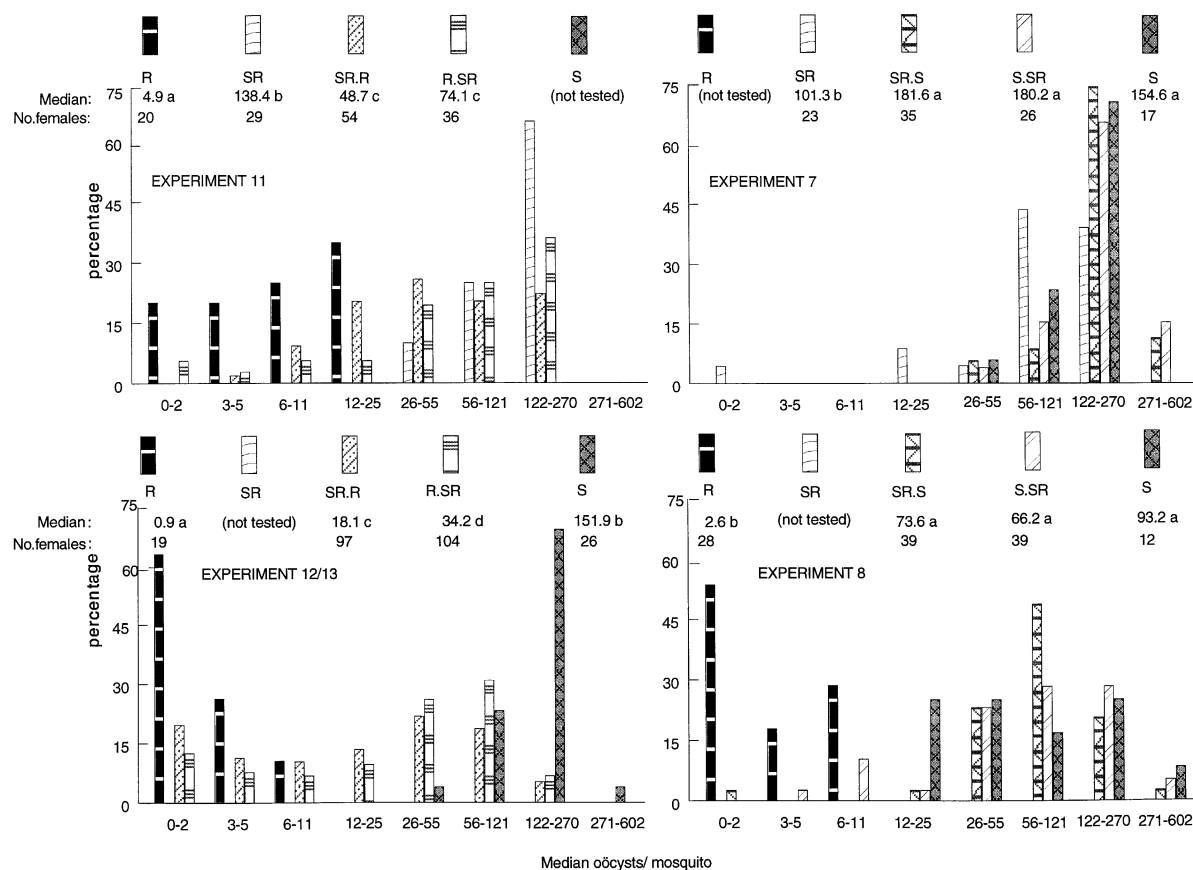
Figs 1 and 2 (overleaf). The observed frequency distributions of malaria oöcyst numbers in *An. stephensi* for parental females, reciprocal hybrid F_1 females and backcrosses females. The refractory line Pb3–9a was genetically selected by for reduced susceptibility to *P. falciparum*. The susceptible line Sda-500 was genetically selected for increased susceptibility (Feldmann & Ponnudurai, 1989). R = Pb3–9a; S = Sda-500; RS = F_1 females from crossing R females with S males; SR = F_1 females from the reciprocal cross; RS.S and RS.R = backcross of RS-F_1 females to males of the S and R lines, respectively. Notation for the other backcrosses follows the same conventions. Within each experiment, the medians (negative binomial distribution) of the number of oöcysts per female do not differ significantly from each other, if followed by the same letter. In experiments 12 and 13, mosquitoes were fed on an infective bloodmeal derived from the same parasite culture, but on two successive feeds, each taking 10 min.

Collective analysis of experiments 3–14

Various genetic hypotheses were tested (see Table 2; Figs 3 and 4), using the observed data from experiments 3–14 and estimated the best fit by a likelihood method (Jansen, 1993).

Table 2 shows the results of fitting various hypothetical models for the genetic basis of refractoriness to the data of all the crossing experiments. Model XIII has the maximum likelihood of fit. Model XI is 'nested' within model XIII: it can be obtained by dropping the cytoplasmic suppressor factor (SUP +). The significance of the cytoplasmic effect can be tested by comparing the deviance (11.9) with χ^2 for 1 d.f. ($P < 0.005$). Similarly, model VIII is nested within model XIII, by dropping the interaction between the two putative autosomal genes, and the difference is highly significant ($\chi^2 = 56.8$, d.f. = 2, $P < 0.001$). Model V, however, is not nested within model XIII and it cannot be tested formally for which one fits the best. These two models have similar deviance and

both are therefore equally plausible in a statistical sense. Model II is nested within model V and can be obtained by dropping the cytoplasmic suppressor factor. The cytoplasmic effect is significant ($\chi^2 = 6.2$, d.f. = 1, $P < 0.05$). Models I and V are nested and the effect of segregation distortion on goodness of fit is highly significant ($\chi^2 = 67.2$, d.f. = 1).

Discussion

Genetic crosses

If there was no dominance between refractoriness and susceptibility in both reciprocal crosses (experiments 1–6; Table 1), it might be expected that the susceptibility of F_1 females would be equal to the geometric mean of the medians of the parental refractory and susceptible lines. This was approximately observed in crosses where the parental male
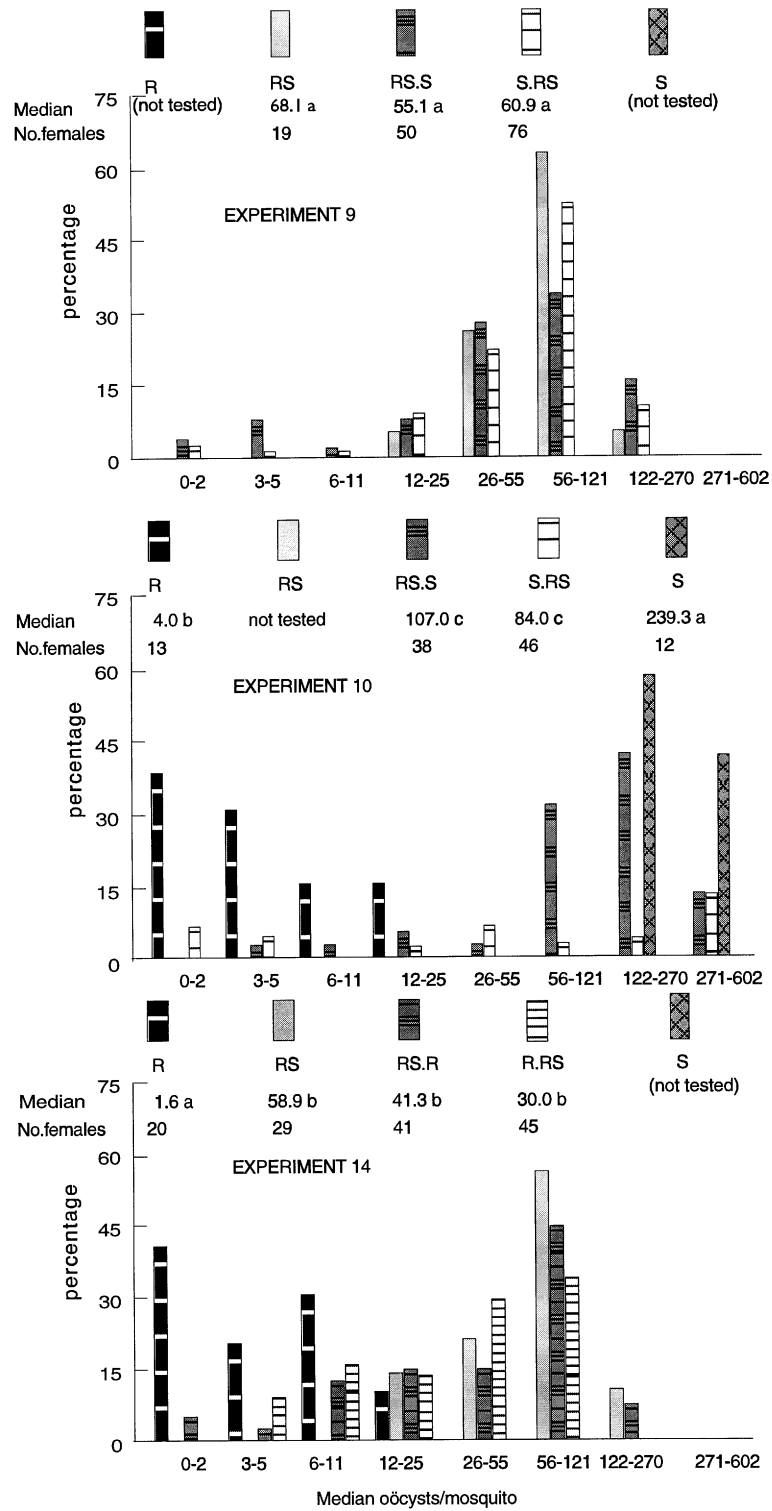
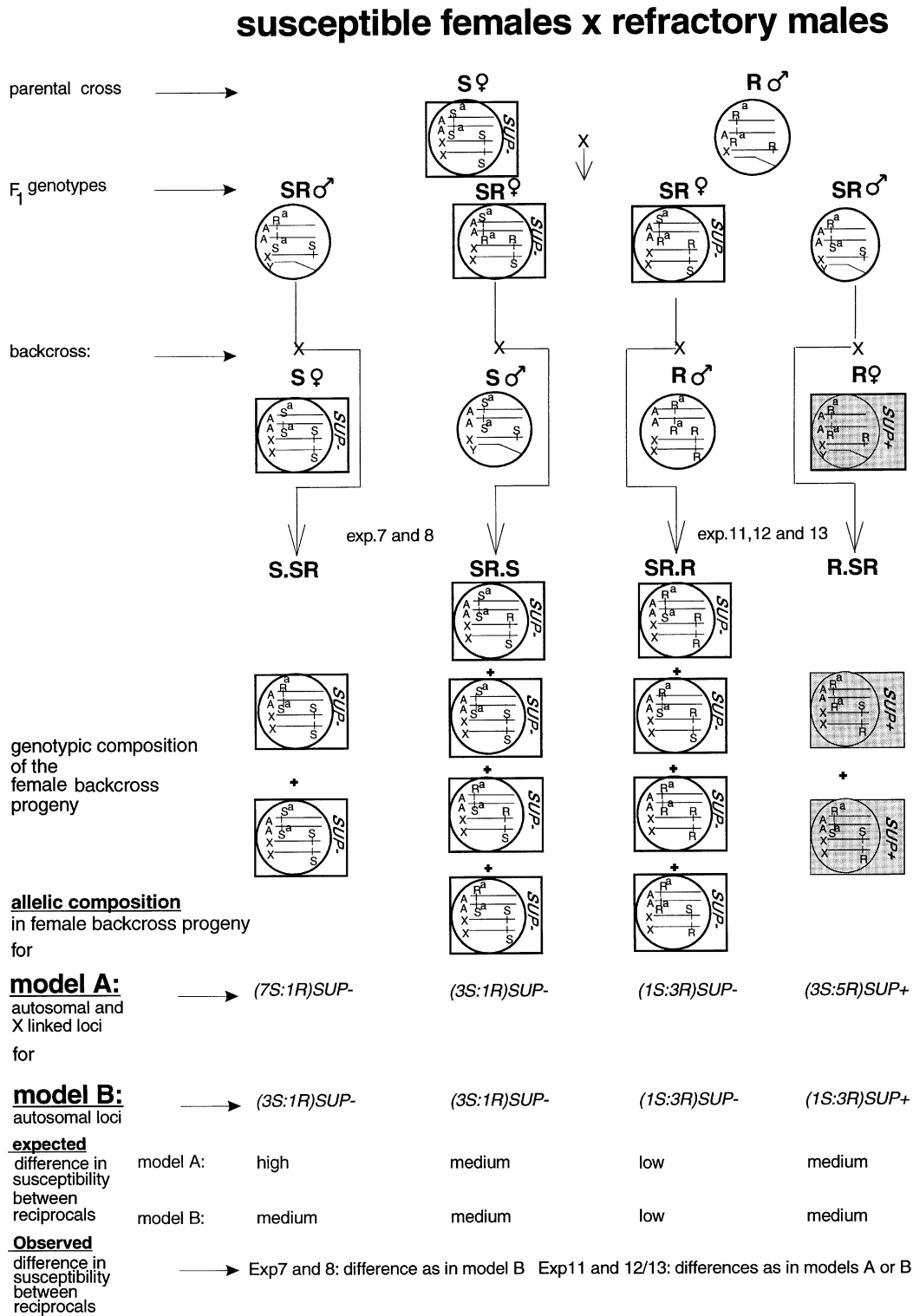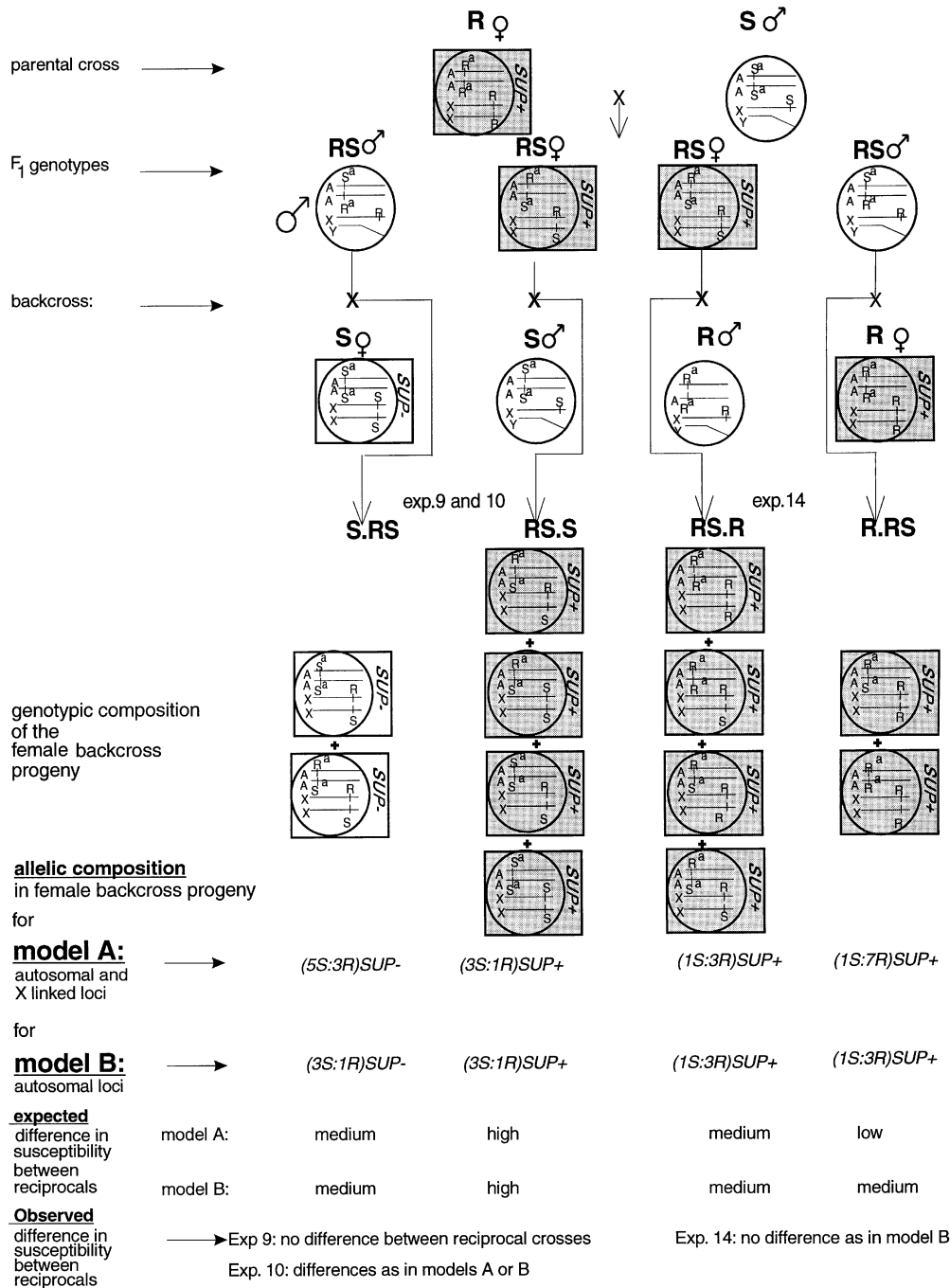**Fig. 2.** See previous page for legend.

susceptible females x refractory males

parental cross ——————▶

F₁ genotypes ——————▶

backcross: ——————▶

exp.7 and 8   exp.11,12 and 13

S.SR   **SR.S**   **SR.R**   **R.SR**

genotypic composition
of the
female backcross
progeny

allelic composition
in female backcross progeny
for

model A: autosomal and X linked loci	——▶ (7S:1R)SUP-	(3S:1R)SUP-	(1S:3R)SUP-	(3S:5R)SUP+

for

model B: autosomal loci	——▶ (3S:1R)SUP-	(3S:1R)SUP-	(1S:3R)SUP-	(1S:3R)SUP+

expected
difference in
susceptibility
between
reciprocals

model A:	high	medium	low	medium
model B:	medium	medium	low	medium

Observed
difference in
susceptibility
between
reciprocals

——————▶ Exp7 and 8: difference as in model B   Exp11 and 12/13: differences as in models A or B

Fig. 3. See next page for legend.

refractory females x susceptible males

Figs 3 (see previous page) **and 4.** Diagrammatic presentation of the segregation of genotypes following reciprocal crossing and backcrossing of the *P. falciparum* refractory and susceptible *An. stephensi* lines. Codes used for the genotypes and crosses are explained in Methods. The refractory line Pb3–9a was genetically selected by for reduced susceptibility to *P. falciparum*. The susceptible line Sda-500 was genetically selected for increased susceptibility (Feldmann & Ponnudurai, 1989). R = Pb3–9a; S = Sda-500; RS = F_1 females from crossing R females with S males; SR = F_1 females from the reciprocal cross; RS.S and RS.R = backcross of RS-F_1 females to males of the S and R lines, respectively. Notation for the other backcrosses follows the same conventions. It is assumed that a maximum of two unlinked genes determines refractoriness. Model A assumes both an autosomal and X-linked refractory gene. Model B assumes that there is only one autosomal gene. $R^x\backslash\backslash S^x$ and $R^a\backslash\backslash S^a$ are allelic, semi-dominant and additive in expression. A cytoplasmic factor (SUP +) partially suppresses the effect of the refractoriness genes. SUP + occurs in the cytoplasm of the refractory line (R) and is absent, or inactive (SUP–) in the susceptible line (S). Differences of the median number of oöcysts in certain of the reciprocal backcross progenies are expected from both models and are compared with the observed results shown in Figs 1 and 2.

Table 2. Comparison of various hypothetical models for the genetic basis of refractoriness fitted to the results of reciprocal crosses and backcrosses of *An. stephensi* lines selected for refractoriness and susceptibility to *P. falciparum*.

Model number	Number of autosomal genes	Number of genes on X-chromosome	Cytoplasmatic suppressor (SUP) effect[1]	Segregation distortion[2]	Deviance[3]	Number of parameters[4]
I	1	0	SUP–	No	82.4	15
II	1	0	SUP–	Yes	13.1	15
III	1	0	SUP +	No	80.1	15
IV	1	1	SUP–	No	68.9	15
V	1	0	SUP +	Yes	2.9	16
VI	2	0	SUP–	No	72.1	16
VII	1	1	SUP +	No	63.8	16
VIII	2	0	SUP +	No	62.2	17
IX	2	0	SUP–	Yes*	12.5	17
X	2	0	SUP–	Yes**	12.7	18
XI	2($\times$)	0	SUP–	No	11.9	18
XII	1($\times$)	1	SUP–	No	65.8	18
XIII	2($\times$)	0	SUP +	No	0	19
XIV	2($\times$)	0	SUP–	Yes*	11.6	19
XV	2($\times$)	0	SUP–	Yes**	11.8	20

($\times$) = interaction of genes; yes* = segregation distortion (SD) acting on one gene; yes** = SD acting on two genes.

[1]cytoplasmic suppressor effect (SUP +) partly suppresses the expression of the refractoriness genes. SUP + is only present in the cytoplasm of the refractory line (see Fig. 4); SUP– indicates absence of suppression.

[2] segregation distortion affects the random segregation of a gene following meiosis.

[3]deviance = twice the difference of the maximum likelihood value.

[4]The deviance is χ^2 distributed, the number of degrees of freedom (d.f.) corresponds with the difference in number of parameters specific to the models compared.

was originating from the refractory line (experiments 2 and 5). For example, in experiment 2 the expected median for F_1 females is antilog $\{[\log 52.1 + \log 5.3]/2\}) = 16.6$ and the observed median of was 23.0. However, when the parental male was from the susceptible line, F_1 females were found consistently with a median susceptibility, which was markedly higher than the median susceptibility of females from the reciprocal cross (Table 1). For example, the observed median susceptibility of the F_1 females in experiment 1 was 77.6, compared with an expectation under 'no dominance' of 31.8 (= antilog $\{[\log 108.5 + \log 9.3]/2\})$.

It is concluded that the susceptibility of F_1 females is generally dependent on the direction of the parental cross, being lower if the parental male is from the refractory line. However, experiment 6 showed no difference in the susceptibility of reciprocal F_1 females; this may have been due to experimental error in making the crosses, i.e. only R females may have been mated to S males, giving RS F_1 females in both crossings, with a 2.4-fold higher susceptibility than expected (38.8) if there were no dominance.

Autosomal and X-linked genes would not explain reciprocal differences in the F_1 female progeny, because these inherit their haploid chromosomes equally from each parent (Figs 3 and 4). On the hypothesis of an X-linked factor, RS-F_1 males backcrossed to R females (R.RS; experiment 14) would not be expected to transfer the susceptibility gene to their female progeny (Fig. 4). However, experiment 14 shows that R.RS females are more susceptible than the parental R females (Fig. 2). This shows that a proportion of R.RS females possesses

S genes, which excludes the possibility of exclusive linkage of refractoriness to the X-chromosome.

Exclusively cytoplasmic inheritance of refractoriness also does not explain the observed intermediate susceptibility of SR-F_1 females, which inherited their cytoplasm from the susceptible parental female (Table 1; Fig. 3). Cytoplasmic inheritance could, however, explain reciprocal differences between F_1 progeny; if refractoriness is determined by interaction of a chromosomal-linked genetic factor and a cytoplasmic factor. As, firstly, RS-F_1 females are more susceptible than SR-F_1 females and, secondly, the susceptibility of SR-F_1 females corresponds with the expected susceptibility calculated from values from the parental lines (see above), it is proposed that expression of the gene for refractoriness is partially suppressed in RS-F_1 females by a cytoplasmic factor (SUP +) inherited from the refractory line.

The hypothesis of a cytoplasmic factor interfering with the refractory gene(s) is consistent with the backcross results (Figs 1 and 2 and Figs 3 and 4: model B). According to this hypothesis, reciprocal differences in susceptibility of backcross progenies are expected in backcrossing SR individuals to the refractory line (Fig. 1; experiments 11 and 12 + 13; Fig. 3 model B). The chromosomal composition of both types of reciprocal backcross progeny is the same (1S:3R) but there is a difference in the presence (R.SR) or absence (SR.R) of the postulated cytoplasmic factor. Thus, the former progeny should be more susceptible than the latter, and this is consistent with what was found in experiments 11 and 12 + 13, although the difference was not statistically significant in experiment 11.

Reciprocal differences in susceptibility of backcross progeny are also expected on backcrossing RS individuals to the susceptible line (Fig. 2: experiments 9 and 10, Fig. 4: model B). The chromosomal composition will be the same for both reciprocal types of backcross female progeny, but susceptibility from the RS.S is expected to be higher because these females would inherit R cytoplasm. This expectation is consistent with the results of experiment 10, because S.RS females tended to be less susceptible than RS.S females, although they did not differ significantly (Fig. 2). Results of experiment 9 do not support the hypothesis, as the progeny with S cytoplasm had slightly but not significantly higher susceptibility.

Following our hypothesis further, no reciprocal differences are expected, and were not observed, between the reciprocals tested in experiments 7 and 8 (Fig. 1: S.SR and SR.S; Fig. 3: model B). Also, no difference in susceptibility between RS.R and R.RS (Fig. 2: experiment 14) reciprocals was observed. This is according to expectation, due to the presence of R-cytoplasm in both types (Fig. 4).

Some pairs of experiments were planned so that the susceptibility of different backcross progenies could be compared with each other, across experiments, by using the same infective feed for each member of the pairs. This applies to experiments 7 and 11 and to experiments 14 and 9. According to our hypothesis, we do not expect an affect of a cytoplasmic factor on the expression of refractoriness in experiments 7 (SR.S and S.SR) and 11 (SR.R) (Fig. 3: model B). However, it should be possible to observe the effect of autosomal composition, i.e. (3S:1R) vs. (1S:3R), in the absence of cytoplasmic complications. Indeed it was observed that SR.S and S.SR females (experiment 7) had a median 3.7-fold higher than that of SR.R females (experiment 11), which is consistent with the expected difference in autosomal composition. Equivalent difference in autosomal composition is expected between the backcross progenies of experiments 9 (3S:1R) and experiment 14 (1S: 3R), but the backcross females of experiment 14 would have the hypothetical cytoplasmic suppressor (SUP +) factor (Fig. 4: model B). The RS.R and R.RS females (experiment 14) tended to be less susceptible (1.6-fold) than S.RS females (experiment 9; Fig. 2), suggesting that the suppressing effect by the R-cytoplasmic suppressor factor compensates partly for the higher frequency of R alleles in R.RS than in S.RS.

Overall analysis

A meta-analysis of all our data can be obtained by analysing all the experiments together using quantitative genetic methods, not restricting analysis to pairs of experiments.

Model V is based on a single gene affected by a cytoplasmic factor and subjected to segregation distortion (1R: 3S gives the best fit) and fits almost as well as model XIII (Table 2). As a working hypothesis, both Models V and XIII are considered as a plausible genetic basis for refractoriness. Segregation distortion is a phenomenon which has been observed to occur for various culicine mosquito chromosomes and genes (Hickey & Craig, 1966; McGivern & Rai, 1974; Dennhöfer, 1975;

Sweeny & Barr, 1978; Severson *et al.*, 1994). Model V has a deviance of only 2.9 relative to Model XIII (d.f. = 3; $0.5 > P > 0.1$ *cf* Table 2), and differs from model XIII by dropping one hypothetical autosomal gene and the assumption of meiotic drive of the S- against the R-allele in heterozygotes.

Model XIII, which has the best fit, is based on the assumption of two dose-dependent complementary and interacting autosomal refractory genes which are suppressed for 83% of full expression when combined with a cytoplasmic factor (SUP +). The predicted values, according to Models XIII and V, for median oöcyst distributions of the different genotypic combinations can be subdivided into three categories. These values are determined by chromosomal genotype and further differentiated if associated with the hypothetical cytoplasmic factor (SUP +). One category consists of the homozygous refractory genotype ($R_1//R_1-R_2//R_2$), representing interaction between two sets of heterologous refractory genes ($/R_1-/R_2$), with the predicted median value = 3.7; the second category comprises three types of heterozygotes, where interaction is only possible between one set of heterologous refractoriness genes, resulting in a marked increase in susceptibility with the predicted median ≈ 52; the third category also has three genotypes, where interaction between heterologous R genes is excluded, with the predicted median = 121.5.

Al-Mashhadani *et al.* (1980) observed differences like ours between reciprocal crosses of *Anopheles gambiae* Giles lines selected for refractoriness and susceptibility to *Plasmodium berghei* rodent malaria. They found that SR-F_1 females [F_1(PB × LD)] had intermediate susceptibility but RS-F_1 females [F_1(LD × PB)] were completely susceptible. A sex-linked effect on the expression of refractoriness was deduced from co-segregation of the X-linked *white-eye* locus, together with the refractoriness level. Al-Mashhadani *et al.* (1980) observed that RS.R backcross females were more susceptible than the reciprocal R.RS females, and that SR.R backcross females were less susceptible than R.SR females. These observations are in agreement with the expectations presented in Figs 3 and 4 (Model A).

The Pb-3–9a line went through several 'bottlenecks' during the selection procedure and the chance fixation of a cytoplasmic factor, interfering with refractoriness, might have taken place. However, it is unlikely that such a chance fixation would have occurred during both our selection experiments and those of Al-Mashhadani *et al.* (1980*)*. Alternatively, during the selection process the cytoplasm of the R line might have acquired genetic factors, which promote *Plasmodium* development. Due to the increasingly unfavourable conditions for the parasite in the mosquito host during the course of the selection for refractoriness, a genetic factor might have been transfected from the parasite to the host. Such a mechanism (although in the reverse direction) has been found in both plant and insect parasitic viruses, some of which have acquired in their genomes host gene sequences which, after expression, interfere with development of the host in such a way that survival of these parasites is favoured (Fraser *et al.*, 1983; O'Reilly, 1995). Cytoplasmic and/or sex-linked effects on the inheritance of refractoriness have not been noticed for other relationships between the insect vectors of *Plasmodium* species (Huff, 1934;

Ward, 1963; Kilama & Craig, 1969; Frizzi *et al.*, 1975; Collins *et al.*, 1986; Vernick *et al.*, 1989). The manipulation of vectors by plasmodial parasites in order to enhance the probability for transmission has been shown and discussed by Fialho & Schall (1995), although only as an adaptive manipulation of the host's behaviour by the parasite. The present authors believe that we have demonstrated for the first time a genetic modification of the vector by *P. falciparum* to increase its probability for transmission under conditions becoming gradually more inimical for the parasite in its natural vector.

The isolation and identification of genes involved in host–parasite relationships is one of the primary targets for the development of strategies to manage field populations of disease-transmitting insects (Jones, 1957; Curtis, 1968; Lorimer *et al.*, 1976; Gubler *et al.*, 1982; Curtis & Graves, 1988; Cockburn *et al.*, 1989; Crampton *et al.*, 1990; Crampton, 1994; Warren & Crampton, 1994; Collins & Paskewitz, 1995). Such specific resistance genes are also required for a test of the gene-for-gene hypothesis, as applied to host–vector relationships in the study of genetic variability of the parasite (Wakelin, 1978; Walliker, 1982, 1991). This information will contribute to the assessment of the feasibility for reduction of vector competence in wild mosquito populations through the release of refractory genotypes of the same species. Strong selection may be expected on the parasite population to evade the mechanism of refractoriness in the mosquito population. The likelihood of such a compensatory process can only be assessed properly in field trials following extensive tests on a wide array of *P. falciparum* clones in the laboratory.

The present authors intend to repeat the backcross experiment of SR-F_1 females with R males, followed by selection from the backcross progeny of females homozygous for the R alleles. The selected females will then have the R genotype with S cytoplasm and may be more, or completely, refractory to *P. falciparum*. This was attempted previously, but not achieved completely, by repeated reselection within the Pb3–9a line from females with no oöcysts (Feldmann & Ponnudurai, 1989). Also, the outcome of ongoing research using molecular markers and Quantitative Trait Locus Analysis (QTL-analysis; Jansen & Stam, 1994) is relevant for determining whether the assumptions of hypothesis V or XIII are the genetic basis for refractoriness. This approach has been successfully applied earlier to investigate susceptibility of the mosquito *Aedes aegypti* (L.) to the bird malaria parasite *P. gallinaceum*, and two unlinked loci affecting susceptibility for this malaria parasite have been mapped by Severson *et al.* (1995).

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