A GENETIC SEXING SYSTEM IN *ANOPHELES GAMBIAE* SPECIES A

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ABSTRACT. To produce a sex separation system for use in genetic control of *Anopheles gambiae* species A, translocations were selected which linked the gene for resistance to dieldrin to the Y chromosome. Thus it could be arranged that females could be killed with dieldrin in the first instar, but males survived. In most of the stocks there were 10–15% of recombinant surviving females but in one stock (R70) the recombinant fraction was only about 0.25%. Maintenance of R70 by inbreeding the stock was compared with the more laborious, but also more reliable, method of outcrossing to susceptible homozygote females. Radiation sterilized R70 males were found to be fully competitive for mating in a large laboratory cage.

INTRODUCTION. A field experiment by Lofgren et al. (1974) has shown that the sterile male technique has considerable promise for control of anophelines. The major problem encountered was incomplete separation of males for release from females on the basis of pupal size, since in anophelines the sexes do not differ so markedly in this respect as do culicines. In making sterile male mosquito releases it is important to release few or no females as, even if these are sterilized, they could act as vectors and/or cause biting nuisance. Systems for sex separation at the adult stage are possible but these may be laborious and/or involve delay in the release of the sterile males, with adverse effects on their competitiveness. Sexing systems based on the sex linkage of a readily recognizable gene have a long history in poultry (see e.g. Crewe 1964). Sex linkage of suitable genes in insects can be artificially contrived (Serebrovskii 1971; Whitten 1969; Strunnikov 1975). The sex linkage of conditional lethal genes or their non-lethal alleles allows "automatic" sex separation (McDonald 1971; Sakai and Baker 1974). If the gene operates early in development this system would have the additional advantage that the females could be selectively destroyed when young and space need not be wasted in rearing them.

In *An. gambiae* resistance to the insecticide dieldrin is controlled by a single semi-dominant autosomal gene (Davidson 1956). In anophelines there is a differentiated Y chromosome (Mason 1967) and a translocation of the type illustrated in Fig. 1 should provide the desired male separation system. The use of such a system was discussed by Whitten and Foster (1975) and the system has been realized in *Lucilia cuprina* (M.J. Whitten, personal communication). In contrast to *Lucilia* there is frequent crossing over in male meiosis in anophelines (Mason 1967), and the translocation break point would have to be positioned so as to avoid an unacceptable proportion of recombinant, dieldrin-resistant, females being produced. Such a stock could be maintained by outcrossing dieldrin-treated males to females of a susceptible homozygote stock. If there was virtually no crossing over between the dieldrin locus and the translocation the stock could be less laboriously maintained by rearing the males and females without dieldrin treatment and allowing them to mate together. (Fig. 1)

MATERIALS AND METHODS. The stocks of *An. gambiae* sp. A used were as follows:

Pala RR—originated from Pala, Upper Volta; homozygous for dieldrin resistance.

16cSS—originated from Lagos, Nigeria; homozygous for dieldrin susceptibility and for the autosomal marker *collarless* (Mason 1967).

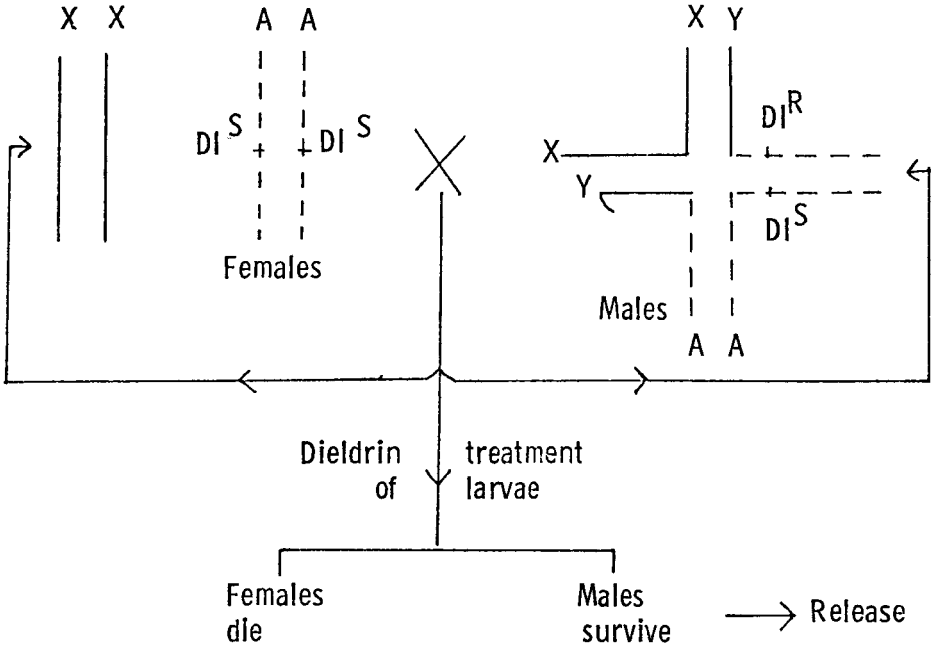

Fig. 1. Diagram of a translocation which links dieldrin resistance to the Y chromosome.

S3H and S3U—male linked translocation complexes with 65–75% sterility and induced by re-irradiation (Akiyama 1973, and unpublished data) of a previously radiation induced male linked translocation (Krafsur 1972).

In addition a test was carried out with the 5BTY male linked translocation in *An. gambiae* species B induced by Krafsur (1972).

Adult Pala RR males were irradiated with 4 k rad of X or gamma rays. Eggs were collected from females after their second and subsequent blood feed. These females were either tubed individually for egg laying or eggs were collected by placing bowls containing filter paper and water into cages containing gravid females overnight. Eggs were allowed to hatch in bowls and larvae were decanted leaving eggs adhering to the filter paper. Dieldrin treatment with 0.02 ppm for 24 hours was carried out over either the 2nd or 3rd night after the night of egg collection. Treatment over the 3rd night is now considered preferable because egg hatching has virtually finished by then, and there is almost no risk of accidental inclusion of unhatched eggs with the larvae for treatment. If this did happen late hatching larvae might "escape" dieldrin treatment at the larval stage. At the end of the treatment period the solution was diluted tenfold, larval food was added, and the surviving larvae were reared to adulthood and the sex ratio of the adults recorded at emergence.

RESULTS. Hitherto insecticide resistance tests in mosquitoes have been conducted with adults or 4th instar larvae. However, it was found that a dose of 0.02 ppm dieldrin to 1st instar larvae killed 100% of susceptible homozygotes and no heterozygotes. Treatment of the larvae produced by backcrossing heterozygotes to 16cSS gave the expected 50% kill.

The 3 pre-existing translocation stocks,

5BTY, S3H and S3U all had a high frequency of dieldrin resistance. Pure homozygous resistant stocks were produced by selection of adults on 4% dieldrin papers for at least 2 hr. The adult males were mated to 16cSS, the male progeny were backcrossed to 16cSS and their progeny treated with the dose required to discriminate heterozygotes from susceptible homozygotes. The results are shown in Table 1 and indicate no linkage of the dieldrin resistance locus to the Y chromosome in 5BTY and partial linkage with 11–15% crossing over between the dieldrin locus and the translocation in S3H and S3U.

It was concluded that the already existing translocations in *An. gambiae* would not serve the purpose of a sexing system and that new ones would have to be made and tested. Pala RR males were therefore irradiated, mated to 16cSS females, the male progeny back-crossed *en masse* to the maternal stock, 285 of the females were isolated for egg laying, and the egg hatchability was counted. Eighty-four of the egg batches were selected as they showed less than 75% hatch. This was taken as a probable indication of an induced translocation, though it was recognized that there was at this stage no certainty of the presence of a translocation in every case as about 9% of matings among unirradiated stock gave less than 75% hatch. From 4 of the 84 batches of larvae only male progeny were obtained following dieldrin treatment. The other 80 families yielded one or more females and were discarded, as there was no intention of accumulating translocations other than those potentially useful as a sexing system. From the 4 selected families the males were backcrossed to 16cSS females, larvae were treated with dieldrin, any female survivors were destroyed after emergence, and the males were backcrossed to 16cSS females. This process has been repeated for 4–5 generations. At each generation in each family characteristic levels of partial sterility of the eggs were found, and when the mated females were tubed individually for egg laying, all laid partially sterile egg batches. This indicates that translocations involving the Y chromosome were present in all these families and the translocation in the R70 family is of a type causing much less sterility than in the other families (Table 1).

Though no females survived dieldrin among the small numbers of individuals of the 4 families available at the 1st generation, some female survivors were found among the larger numbers reared in later generations. Tests of these females at the adult stage showed that about 80% of them survived exposure to 0.4% dieldrin papers for 1 hr, and their progeny showed a 1:1 segregation of resistance and susceptibility. It is concluded that at least the great majority of the female survivors were not susceptible homozygotes which

Table 1. Data on fertility and proportions of females among survivors of dieldrin treatment when males of the translocation stocks were backcrossed in 16cSS females.

Stock	No. of eggs counted	Percent hatch	No. of adult progeny scored after larval dieldrin treatment	No. females	% females (with 95% confidence limits *)
5BTY	....	....	157	75	47.7 (39.9–55.5)
S3H	1779	32.2	649	76	11.7 (9.2–14.2)
S3U	1063	29.4	95	14	14.7 (8.2–23.9)
R39	3131	26.5	554	66	11.9 (9.2–14.6)
R70	8169	59.1	2459	6	0.24 (0.08–0.53)
R86	4760	25.4	583	72	12.3 (9.6–15.0)
R96	2221	24.0	231	24	10.4 (6.5–14.3)

* Based on binomial distribution using method of Stevens (1943).

had "escaped" killing at the larval stage, but were resistant heterozygotes which had arisen as a result of cross-overs between the resistance gene and the translocation break point. In the families R39, R86 and R96 10–12% of females were obtained but in R70 the proportion was only about 0.25%. Thus R70 showed promise as a practical sex separation system whereas the R39, R86 and R96 stocks did not. The properties of the latter 3 stocks were so similar that it seems possible that they all carry the same translocation and that they arose from 3 matings made by a single translocated male at the stage of mass mating of the F_1 progeny of the irradiated males to 16cSS females.

When larvae of R39, R70, R86 or R96 were reared without dieldrin treatment, approximately 1:1 sex ratios were obtained, which confirmed the supposition that the biased sex ratios following dieldrin treatment were due to selective killing of susceptible females and not to some entirely different phenomenon such as meiotic drive. Only in the R70 family was the rate of recombination between the dieldrin locus and sex low enough to justify an attempt to maintain the stock by allowing mating between the males and females reared without dieldrin treatment. One to 4 generations of such rearing followed finally by dieldrin treatment of larvae yielded the proportions of surviving females shown in Table 2. The proportions of surviving females following inbreeding were a little higher than that found from repeated backcrossing of R70 males to 16cSS females (Table 1), and a comparison of the pooled data for all generations of inbreeding with that from backcrossing gave $X^2_1=11.4$, $P<0.001$. This difference is presumably because recombinant females carrying dieldrin resistance genes had been included in the breeding stock in the inbred material. The adverse effects of such recombinant females could be reduced by the addition of females from the 16cSS stock and Table 2 indicates tests of the addition of 16cSS females in numbers equal to those of the females of the inbred population. When the progeny larvae were dieldrin-treated the observed proportion of surviving females was approximately as low as in the line maintained by continual outcrossing of R70 males to 16cSS females.

The quality of R70 males, which survived treatment with dieldrin at the 1st instar, was evaluated in the laboratory with the results shown in Table 3. Percentage survival through the aquatic stages of the survivors of dieldrin treatment was approximately the same as for untreated larvae of R70 or 16cSS. The adult survival of R70 males was similar to that of the wild type control. Mating competition tests were carried out with R70 males which had survived the larval dieldrin

Table 2. Proportions of females in R70 strain following rearing without dieldrin treatment.

	No. adult progeny counted	% females
Reared without dieldrin treatment	833	47.4
Treated with dieldrin following indicated nos. of generations inbreeding without dieldrin treatment and with or without supplementation of females by equal nos. of 16cSS females:		

	No supplementation		Supplementation	
	No. adult progeny scored	% females	No. adult progeny scored	% females
1 generation	1756	1.02	....	....
2 generations	649	0.62	1291	0.23
3 generations	282	0.71	....	....
4 generations	445	1.57	273	0.37

Table 3. Data on performance of R70 males in comparison with wild type.

(1) Survival through aquatic stages:		survival
R70 following dieldrin treatment of 1st instar		69%
R70 untreated		58%
16cSS untreated		77%
(2) Adult male survival:		
	Days for 50% mortality	Days for 100% mortality
R70 following dieldrin treatment of larvae	14	36
16cSS untreated	16	23
(3) Mating competitiveness test: Ratio of adults	Fertile egg batches	Sterile egg batches
1 S R70 M : 1 U Pala M : 1 U Pala F	39	48
2 U Pala M : 1 U Pala F	42	4

treatment and had been irradiated as young adults with 12 k rad of gamma rays which induces about 99.5% dominant lethality in sperm. Such males (symbolized SR 70 M) were caged in a 1 m^3 laboratory cage at a 1:1:1 ratio with untreated males and untreated virgin females both of the Pala RR strain (symbolized U Pala M and U pala F). After leaving for 5 days for mating, the females were blood fed and isolated in tubes for egg laying, and the egg batches were classified as fertile or sterile. As shown in Table 3, the results indicated a slight but non-significant excess of sterile egg batches. A control with untreated males and females gave a small proportion of sterile egg batches. Correcting the results of the competition experiment for this proportion of sterility found in the Pala RR stock led to the conclusion that the irradiated R70 males were virtually equal to Pala RR in competitiveness.

DISCUSSION. Maintenance of the R70 stock by inbreeding would be considerably less laborious than by outcrossing of males to virgin females of a homozygous susceptible strain, because the outcrossing system would require the separate maintenance of the susceptible strain, the collection of virgin females from it and the elimination of recombinant resistant females from among the dieldrin-treated R70 males prior to mating. However, the production of material for a release programme extending over several months by the inbreeding system would run the risk of serious decline in the "quality" of the sexing process in the later stages as a result of the steady accumulation of recombinants in the breeding stock. Such a steady accumulation is not seen in Table 2, probably only because of "sampling error" in this small scale experiment. The "compromise" system in which an inbred R70 stock is supplemened with virgin females from a relatively small homozygous susceptible colony might well prove to be optimal but the breeding system to be used would have to be decided taking into account the circumstances of the particular release program concerned.

Another possible means of improving the quality of sex separation would be to use a pupal grid separator (Sharma et al. 1972) in addition to the genetic system to try to remove the recombinant resistant females. Alternatively an improved genetic system could be constructed by incorporation of a natural or artificial crossover suppressing inversion around the dieldrin resistance locus prior to the induction of a translocation with a break point inside the inverted region. Information on the location of the required inversion based on a cytogenetic study of the existing translocation stocks will be published separately.

Provided that the proportion of females in release material can be kept down to

about 0.5%, we consider this acceptable since it would allow the use of a 20:1 ratio of released:wild males with a temporary increase in the female population of only 10%. Such a small and temporary increase would be of no epidemiological significance nor would it interfere appreciably with the population control process even if a sterility system which only affects the male were utilized.

As indicated in Table 1, the R70 system causes less than 50% sterility. Males separated by use of the R70 system may be radiation sterilized and, as far as can be determined in the laboratory, such males are fit and competitive (Table 3). A field check on their performance is, however, required. The use of a highly sterile translocation complex might enable one to avoid the cost of a radiation source at every installation from which mosquitoes were to be prepared for release. A program to add an additional translocation to R70 is in progress. The objective is to produce a complex which is not subject to disintegration by crossing over and which maintains male linked sterility of about 75%. A stock with such a sterility level is considered to be capable of being reared economically but sufficiently sterile to have a reasonable chance of overcoming the recovery capacity of wild populations and bringing about effective control.

The R70 translocation is also being backcrossed into stocks selected for refractoriness to *Plasmodium* (Al Mashhadani 1974). Sex separation prior to release of such a stock would probably be required since, even where the females presented no danger as vectors of *Plasmodium*, they might be able to transmit filaria and/or to create a serious biting nuisance.

We do not consider that the mass release of R70 males carrying a gene for dieldrin resistance could reduce the effectiveness of future insecticide programs in the same area because: (1) dieldrin resistance genes are already widespread in wild populations of *An. gambiae* (Coz et al. 1968); (2) the use of dieldrin for mosquito control has now been abandoned and the use of HCH (resistance to which is controlled by the same gene as that to dieldrin) is uncommon; (3) the males released would be sterilized and/or the gene would be linked to a partially sterilizing male linked translocation, and the establishment of the gene would therefore tend to be prevented by natural selection.

The R70 translocation involves the Y chromosome and therefore cannot enter females so that any possible effects of the translocation on vectorial capacity need not be considered. Before release of R70 males into any wild population, they should be backcrossed to dieldrin susceptible females from the same wild population to ensure adaptation of the males to the local conditions and to remove any gene which, if inherited by females of the wild population, might conceivably make them more dangerous than the indigenous population. Any "foreign" genes, which were so tightly linked to the R70 translocation as not to be removed by repeated backcrossing, could not be dangerous because such tight linkage would also prevent their transmission to females of the wild population.

It is concluded that the R70 system is a safe and effective one for separation of males for use in the genetic control of *An. gambiae* sp. A.

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