STUDIES OF FORCED MATING TECHNIQUES ON ANOPHELINE MOSQUITOES

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ABSTRACT. The effect of age on the success of the forced mating technique was studied in *Anopheles farauti* No. 1. Success of this technique appeared to be independent of age within the range 1½ to 4½ days old. Overall only 56.4% of 1099 females which had copulated with males during this experiment were inseminated. In *An. gambiae, An. arabiensis* and *An. farauti* No. 1 it was found that when males were cooled for 24 hrs prior to being used for the forced mating technique, the number of inseminations per 100 males was increased compared with the number of inseminations per 100 males of the same species which were kept under normal laboratory conditions. In all 3 species there were low rates of inseminations per 100 copulations using both the cooled and control males (45.8% to 67.3%).

INTRODUCTION

Before 1957, laboratory studies on mosquitoes were limited because many of the so-called eurygamous mosquitoes would not mate under laboratory conditions. The development of a forced mating technique by McDaniel and Horsfall (1957) overcame this limitation. Modifications have since been made by Baker et al. (1962) and it is now possible to carry out single pair matings for genetic and cytogenetic studies.

At the Ross Institute of Tropical Hygiene, the forced mating technique has been in use for many years, particularly for identification of the members of the *Anopheles gambiae* Giles complex (Davidson et al. 1967, Davidson and Hunt 1973) and the *An. punctulatus* Dönitz complex (Bryan 1973a and 1973b), for studies on the relationships between the members of these complexes and for work on insecticide resistance. Material for such studies is usually received as eggs sent by post and often only a small percentage of the eggs hatch on arrival.

In many cases failure to identify material or to test for insecticide resistance occurs as insufficient females obtained from the material lay viable eggs. This is sometimes due to the males not copulating and sometimes to the failure of the males to inseminate their mates in spite of copulating with them.

Thus a lot of potentially valuable material, often collected with considerable difficulty in the field, is lost. Some species could not be maintained for long periods in the insectary, e.g. *An punctulatus* Dönitz and *An. koliensis* Owen, and failure to inseminate the females contributed to the extinction of their colonies. In view of these losses it was considered important to see if the efficiency of the forced mating technique could be improved so that maximum use could be made of field material. Also if the technique could be improved a lot of time would be saved as the technique is rather time-consuming.

It has already been shown in *Aedes aegypti* (Linnaeus) that the age of the female influenced the success of the forced mating technique (Gwadz and Craig 1968). It was therefore decided to study the effect of age on the success of mating. Studies were also made to see if cooling males prior to mating would increase the number of inseminations as this had already been shown to improve the success of the forced mating technique in some species of *Anopheles* (Baker et al. 1962).

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MATERIALS AND METHODS

COLONIES USED AND INSECTARY

METHODS. *An. farauti* Laveran No. 1 (Bryan 1973a, 1973b, 1973c, 1974) were obtained from a colony established in the Ross Institute laboratories in 1965 from eggs received from Rabaul, Papua, New Guinea. *An. gambiae* Giles, formerly known as species A of the *An. gambiae* complex (White 1975), came from a colony which had been established for 3–4 years and originated from eggs obtained at McCarthy Island, The Gambia. The colony of *An. arabiensis* Patton was established in 1956 from material collected in Kano, Nigeria, and was formerly known as species B of the *An. gambiae* complex (White 1975, Mattingly 1977).

The larvae were reared and the adults maintained as described by Bryan (1973a). Newly emerged adults were collected from the rearing bowls each morning and the males and females separated and caged independently. No matings were attempted within 24 hrs of emergence so as to allow the male terminalia to rotate.

For the first series of experiments on the effect of age on mating, after separation, the males and females were held for periods of 1, 2, 3, and 4 days in an insectary at 24°C and 80% relative humidity. As the adults begin to emerge in the late evening, some individuals would have been at least 12 hrs old before separation, so that after the holding periods they would have been 1½, 2½, 3½ and 4½ days old. Within each age group the mosquitoes would have varied in age but no attempt was made to determine the exact age of the mosquitoes.

When testing for the effect of cooling the males, all adults were kept in the insectary for 1 day after separation. The males were then divided into 2 groups, half being kept in the insectary and the remainder being transferred to a cooling incubator for 24 hrs at 15°C to 20°C. After this time they were used for mating with females of the same age, i.e. 2½ days. The males were transferred to the laboratory just prior to mating.

MATING TECHNIQUE. The legs, wings and heads of the males were removed. Each male was then glued by the thorax to the edge of a petri dish, ventral side up, with the terminalia facing away from the center of the dish. Up to 20 males were prepared at one time. An anaesthetized female held by suction to a micropipette was presented to a male dorsal side uppermost. The male was stimulated by gentle stroking of the genital region with the female genitalia to induce copulation. Each male was used for only one copulation so that depletion of spermatozoa would not influence the results. If a male failed to respond, the female was presented to another male.

In the experiment on the effect of age, males of each age group were mated with at least 50 females from each age group. Most males could be induced to copulate and no record was made of the number of males that did not copulate.

When testing for the effect of cooling the males, the number of males tested and the number of copulations which resulted was recorded.

The success of the forced mating technique was assessed by scoring the spermathecae of the females for the presence or absence of spermatozoa.

RESULTS AND DISCUSSION

EFFECTS OF AGE ON THE FORCED MATING TECHNIQUE IN *An. farauti* NO. 1. The results of these experiments are set out in Table 1. Careful examination of this table reveals no obvious trend in success when the two independent variables, age of males and age of females are inspected separately. It is therefore appropriate to test the data by means of a two-way analysis of variance. Although the extreme limits of the percentages of successful inseminations were only 43.7% (4½ day-old females, 4½ day-old males) and 67.7% (2½ day-old females, 3½ day-old males), analysis of variance was carried out after an angular or arcsine transformation of the percentages to stabilize variance; as the various percentages were calculated from groups of roughly equal size, no weighting was applied (Reimer

Table 1. Numbers of females of *An. farauti* No. 1 mated and inseminated with percentages of success in insemination, classified by age of males and age of females in days at the time of mating by the forced mating technique.

		Age of males in days									
		1½		2½		3½		4½		Total	
		+ ve*	– ve*	+ ve	– ve	+ ve	– ve	+ ve	– ve	+ ve	– ve
Age	1½	34	26	37	27	37	25	40	30	148	108
		56.7%		57.8%		59.7%		57.1%		57.8%	
of	2½	34	26	34	26	42	20	36	25	146	97
		56.7%		56.7%		67.7%		59.0%		60.1%	
females	3½	63	36	32	28	38	24	38	30	171	118
		63.6%		53.3%		61.3%		55.9%		59.2%	
in	4½	27	32	29	31	50	30	49	63	155	156
		45.8%		48.3%		62.5%		43.7%		49.8%	
days	Total	158	120	132	112	167	99	163	148	620	479
		56.8%		54.1%		62.8%		52.4%		56.4%	

* + ve = mated and inseminated.
* – ve = mated but not inseminated.

1959). The variance-ratio was not significant at the 5% level, and it can be concluded that the observed deviations of the individual percentages of successful inseminations shown in Table 1, could have occurred by chance.

Subjection of the results in Table 1 to dredging procedures (Armitage 1971) reveals one pairing—2½ day-old female × 3½ day-old male—which is statistically significant in successful inseminations in contrast to the pairing 4½ day-old male × 4½ day-old female, 67.7% against 43.7%, but this contrast would certainly not have been expected on *a priori* grounds, and has almost certainly arisen by chance.

It is however, reasonable, in view of the very small element of doubt introduced by this last observation to test the data in an alternative way, involving a non-parametric hypothesis tested by a distribution-free method.

This has been done by testing the marginal total percentages for rows and columns in Table 1 by calculating the coefficient of rank correlation; this yields a coefficient of 0.04. The significance of this figure for r is tested by the statistic z, which in this case equals 0.423; the standard deviation of z is 1.0, and thus the coefficient of rank correlation is clearly insignificant. (Details of statistical analyses may be obtained from the 2nd author.)

Gwadz and Craig (1968) noted that in *Ae. aegypti*, refractory and receptive females behaved differently when presented to males; refractory females (young or previously inseminated ones) retracted their 8th abdominal segments thereby preventing union of the terminalia and insemination; receptive females responded to the males by extending their 8th abdominal segments and cerci thus facilitating union and the transfer of spermatozoa. The actions of the receptive and refractory females were sufficiently different so that the 2 types of females could be separated by their behavior with over 70% accuracy. During the present study such behavioral differences in the females of *An. farauti* No. 1 were looked for but not seen.

The low overall percentage of inseminations (56.4) was surprising as in every case firm union was established between the partners. There was no obvious difference in the positioning of the male and female terminalia between successful and unsuccessful couplings.

EFFECT OF COOLING THE MALES ON THE FORCED MATING TECHNIQUE. Three comparisons were made between cooled and control males:

a) Percentage of males copulating;

b) percentage of males producing insemination;

c) percentage of copulations producing insemination.

For each species, standardized normal deviates were calculated from standard errors of the differences between percentages of the 3 factors which were compared.

The results are set out in Tables 2 and 3.

Table 2. Numbers of males used, numbers of copulations achieved and numbers of inseminations achieved.

	Cooled Males			Control Males		
Species	No. of males	No. of copulations	No. of inseminations	No. of males	No. of copulations	No. of inseminations
An.f. 1*	278	260	173	302	267	165
An.g.	215	94	60	221	96	44
An.a.	222	158	105	222	150	101

* *An.f.* 1 = *An. farauti* No. 1.
An.g. = *An. gambiae.*
An.a. = *An. arabiensis.*

In *An. farauti* No. 1 both the percentage of copulations per 100 males was significantly higher (at the 5% level) when the males were cooled. The percentage of inseminations per 100 copulations was also higher but not significantly so at the 5% level.

In *An. gambiae* the percentage of "warm" and "cool" males copulating was similar but the cool males were insignificantly more efficient at inseminating their mates.

Warm and cool males performed similarly in *An. arabiensis* but in contrast to the other 2 species the percentage of inseminations per 100 copulations was slightly higher using the warm males, although the percentage of inseminations per 100 males was slightly higher using cool males.

From the results it is clear that there are marked differences between the species, with *An. farauti* No. 1 producing the highest insemination rate per male and *An. gambiae* the lowest rate, but these differences are less marked when the numbers of inseminations per 100 copulations are considered.

These results apply only to mosquitoes of the particular ages studied, and very different results might have been obtained if older mosquitoes had been used. However, the main objective of our work was to improve the recovery of material received from the field, and it was not possible to risk the total loss of small samples of eggs by holding unmated emerged adults for longer than 5 days, in view of the mortality associated with such a procedure.

Table 3. Copulations and inseminations per 100 males, and inseminations per 100 copulations.

Species	Cooled Males	Control Males	P value
	Copulations per 100 males		
An.f. 1*	93.5	88.4	0.015
An.g.	43.7	43.4	0.8
An.a.	71.2	67.6	0.20
	Inseminations per 100 males		
An.f. 1	62.6	54.6	0.03
An.g.	27.9	19.9	0.02
An.a.	47.3	45.5	0.34
	Inseminations per 100 copulations		
An.f. 1	66.5	61.8	0.08
An.g.	63.8	45.8	0.005
An.a.	66.5	67.3	0.42

* *An.f.* 1 = *An. farauti* No. 1.
An.g. = *An. gambiae.*
An.a. = *An. arabiensis.*

CONCLUSIONS

No optimum age was found for the forced mating technique for *An. farauti* No. 1 within the age range 1½ to 4½ days.

In all 3 species there were low rates of insemination per 100 copulations (45.8% to 67.3%), and this should be taken into account when using the forced mating technique for experimental work. The number of copulations per 100 males could be increased by using some of the males more than once. In practice this is often possible but was not done in these experiments to simplify the analysis of results.

Even using each male only once, the number of inseminations per 100 males was improved in every case by cooling the males, and where practicable, it is suggested that male mosquitoes be cooled before using the forced mating technique.

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