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RESPONSES OF MALE AND FEMALE MOSQUITOES TO REPELLENTS IN THE WORLD HEALTH ORGANIZATION INSECTICIDE IRRITABILITY TEST SYSTEM[1]

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ABSTRACT. A study was conducted to compare responses of male and female *Aedes aegypti* (Linn.) and *Aedes taeniorhynchus* (Wiedemann) to 9 olfactory repellents in the World Health Organization insecticide irritability test system. An irritant insecticide (permethrin) and a control were included for comparison. *Aedes aegypti* exhibited significantly more takeoffs than *Ae. taeniorhynchus,* and female mosquitoes exhibited significantly more takeoffs than males. Permethrin induced significantly more takeoffs than the control, but olfactory repellents did not. Certain 2- and 3-factor interactions of test materials, species, and sexes were statistically significant. This study supports a previous conclusion that the World Health Organization test method does not measure contact repellency (irritancy) and olfactory repellency equally.

KEY WORDS *Aedes, Aedes taeniorhynchus, Aedes aegypti,* insecticides, irritability, repellents, permethrin, deet

INTRODUCTION

In 1960 the World Health Organization introduced a procedure for determining the irritability of adult mosquitoes to insecticides using a conical exposure chamber made of transparent plastic. In use, the exposure chamber is secured to an impregnated paper held vertically on a glass surface, and individual mosquitoes are introduced through a hole in its apex for observation. The time to first takeoff from the paper (step A) and the number of takeoffs in 15 min (step B) are recorded.

This basic procedure and modifications of it have been used in studies of the irritability of *Culex pipiens* Linn. to DDT, malathion, and permethrin (Gaaboub and Dawood 1974, Ree and Loong 1989); *Anopheles pulcherrimus* Theobald, *Anopheles albimanus* Wiedemann, *Anopheles darlingi* Root, and *Anopheles nuneztovari* Gabaldon to DDT (Bondareva et al. 1986, Quinones and Suarez 1989); *Anopheles gambiae* Giles to DDT, bendiocarb, and lamda-cyhalothrin (Evans 1993); *Anopheles arabiensis* Patton to lamda-cyhalothrin (Le Sueur et al. 1993); and *Anopheles farauti* Laveran and *Anopheles maculatus* Theobald to permethrin (Ree and Loong 1989).

Irritant insecticides have been called contact repellents, because they act in the solid or liquid phase on chemosensory organs of the tarsi, whereas conventional repellents act in the vapor phase on chemosensory organs of the antennae. The present study was conducted to determine the responses of male and female mosquitoes to conventional repellents in the World Health Organization irritability test system. An irritant insecticide (permethrin) and a control were included for comparison. The study was conducted from 1986 to 1989 at the former Letterman Army Institute of Research, Presidio of San Francisco, CA.

MATERIALS AND METHODS

Test species: The mosquitoes used in the study were *Aedes aegypti* (Linn.), University of California at San Francisco strain, and *Aedes taeniorhynchus* (Wiedemann), USDA Center for Medical, Agricultural and Veterinary Entomology strain. The colonies were maintained as described by Rutledge et al. (1978). Both sexes were tested, using males and nulliparous females 5–15 days old.

Test materials: Materials tested were MGK Repellent 326® (MGK Co., Minneapolis, MN 55427) (di-*n*-propyl 2,5-pyridinedicarboxylate); dimethyl phthalate, butopyronoxyl (butyl 3,4-dihydro-2,2-dimethyl-4-oxo-2*H*-pyran-6-carboxylate), Citronyl® (3-acetyl-2-(2,6-dimethyl-5-heptenyl)-oxazolidine), butoxy polypropylene glycol (butoxypropanediol polymer), deet (*N,N*-diethyl-3-methylbenzamide), ethyl hexanediol (2-ethyl-1,3-hexanediol), dibutyl phthalate (di-*n*-butyl phthalate), MGK Repellent 11® (1,5a,6,9,9a,9b-hexahydro-4a(4H)-dibenzofurancarboxaldehyde), and permethrin ((3-phenoxyphenyl) methyl 3-(2,2-dichloroethenyl)-2,2-dimethylcyclopropanecarboxylate ester).

Test materials were technical grade, obtained commercially. All are or have been employed in commercial formulations. All are chemically unrelated except dimethyl phthalate and dibutyl phthalate.

Test method: The test kit used in the study included exposure chambers, impregnated papers, tape, and an aspirator (World Health Organization 1960). A later kit containing preexposure and ex-

[1] Opinions and assertions herein should not be construed as official or as reflecting the views of the Department of the Army or the Department of Defense. Use of trade names does not imply official endorsement or approval of the products named.

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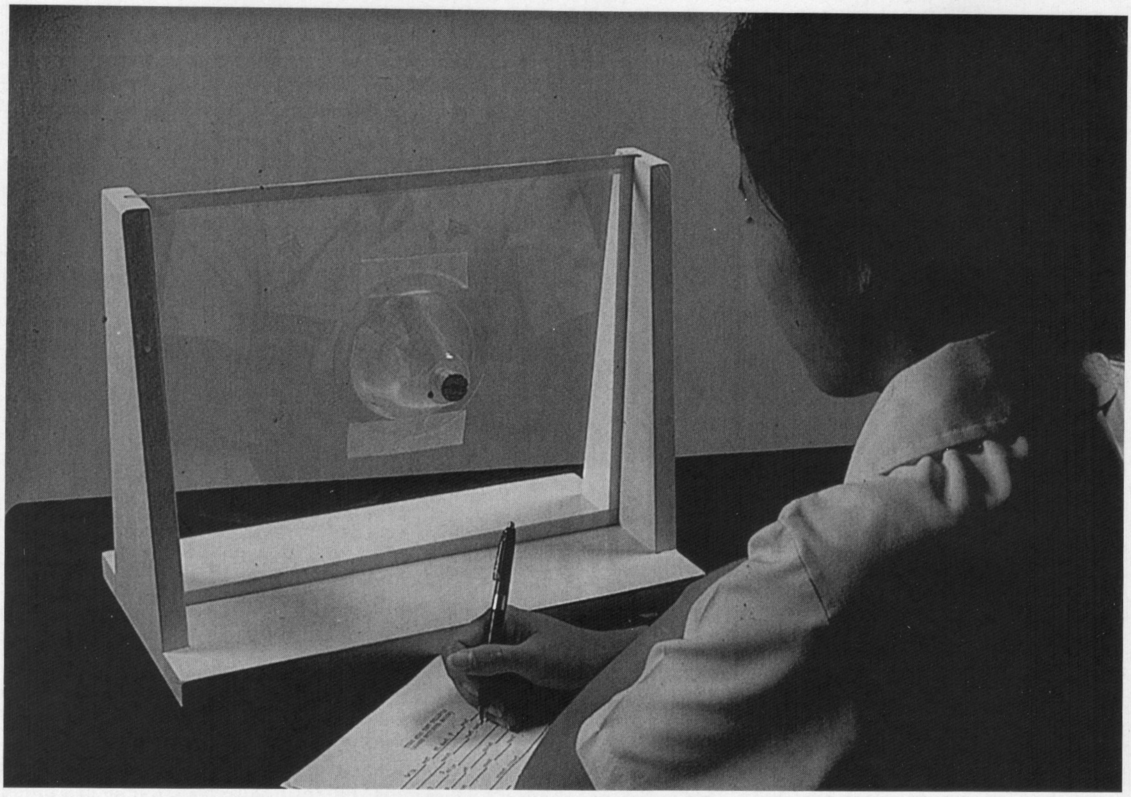

Fig. 1. View of test in progress showing glass pane in stand, test paper, and test chamber secured to glass with tape, and observer (N.M.E.) with test data sheet.

posure boxes (World Health Organization 1963, 1970) was not available for the study.

Procedures prescribed by the World Health Organization (1960) were followed, except that new papers were prepared to replace the oil- and DDT-impregnated papers furnished with the kit. Papers used in the study were 9-cm-diam filter papers treated with the test material in ethanol at 0.00001 mg of active ingredient (AI) per cm^2 of paper. Control papers were treated with ethanol only.

The test paper and exposure chamber were secured with adhesive tape to a pane of glass supported vertically in a wooden frame (Fig. 1). A single mosquito was introduced into the exposure chamber with an aspirator and allowed to settle for 3 min. The time to first takeoff from the paper was determined with stop watch (step A), and the number of takeoffs in 15 min (step B) was recorded. Each test material and control was tested against 20 mosquitoes of each species and sex, except dibutyl phthalate against male *Ae. aegypti* (18 only).

Data analysis: In some tests, no takeoffs occurred during the 15-min test period and the time to first takeoff was recorded as ">15 min." Data of this kind are said to be censored and are not amenable to conventional methods of analysis. Accordingly, analysis of the data obtained in the study was limited to that obtained in step B, the number takeoffs occurring in a 15-min test period.

The data were analyzed by 2 × 2 × 11 (2 species × 2 sexes × 11 treatments) analysis of variance (ANOVA) with unequal replication, using multivariate methods (Kachigan 1991). The software employed in the analysis was the Statistical Analysis System for Microcomputers, version II.0, 1984 (Statistical Consultants, Inc., Lexington, KY). Main effects and 2- and 3-factor interactions were tested for significance of differences at the 1 and 5% levels. The significance of differences between means was tested by Fisher's (protected) least significant difference (Steel and Torrie 1980).

RESULTS AND DISCUSSION

Forty-two female *Ae. taeniorhynchus* died in tests of various repellents during the period May–October 1986. No mortality occurred in tests of permethrin or in tests against male *Ae. taeniorhynchus* or either sex of *Ae. aegypti.* In view of this pattern of occurrence, the observed mortality was interpreted as a quality control failure in the *Ae. taeniorhynchus* rearing program, not related to the materials and methods of testing. Data obtained in

Table 1. Mean numbers of takeoffs of male and female *Aedes aegypti* and *Aedes taeniorhynchus* from treated and untreated test papers in 15 min.

Test material[1]	*Ae. aegypti*[2]		*Ae. taeniorhynchus*[3]		
	Male	Female	Male	Female	Mean
Control	0.6	4.2	2.4	6.0	3.3
MGK R-326	0.0	2.8	3.1	3.6	2.4
DMP	0.2	3.5	1.0	4.8	2.4
Butopyronoxyl	0.0	2.6	2.0	6.1	2.7
Citronyl	0.4	5.8	3.4	2.3	3.0
BPG	0.4	4.2	1.6	7.6	3.4
deet	1.4	4.4	1.1	7.5	3.6
EHD	1.3	8.4	1.7	4.0	3.8
DBP	0.1	6.4	1.0	8.8	4.1
MGK R-11	0.3	4.9	3.6	9.8	4.6
Permethrin	0.9	13.7	10.4	22.8	12.0
Mean	0.5	5.5	2.8	7.6	4.1

[1] MGK R-326, MGK Repellent 326; DMP, dimethyl phthalate; BPG, butoxy polypropylene glycol; EHD, ethyl hexanediol; DBP, dibutyl phthalate; MGK R-11, MGK Repellent 11.

[2] Number of observations was 20 except males tested with DBP (18).

[3] Number of observations was 20 except females tested with DMP (9), butopyronoxyl (18), deet (8), EHD (5), DBP (19), and MGK R-11 (19).

tests against the affected mosquitoes were excluded from the analysis.

Of 836 mosquitoes tested, 293 (35%) did not take off in the 15-min test period. These mosquitoes were primarily males, including 161 male *Ae. aegypti* and 96 male *Ae. taeniorhynchus.* No male *Ae. aegypti* took off in any test of MGK Repellent 326 or butopyronoxyl. The largest numbers of takeoffs recorded were 33, 35, 37, 37, 41, 45, and 49. These 7 records were obtained in tests of dibutyl phthalate (1), MGK Repellent 11 (1), and permethrin (5) against female *Ae. taeniorhynchus.*

Mean numbers of takeoffs by test material, species, and sex are given in Table 1. The fewest takeoffs were observed in tests of MGK Repellent 326 and dimethyl phthalate (mean = 2.4), and the most were observed in tests of permethrin (12.0). *Aedes aegypti* took off less frequently than *Ae. taeniorhynchus,* regardless of sex, and males took off less frequently than females, regardless of species.

The ANOVA of the data obtained in the study is shown in Table 2. All main effects and interactions were significant at the 1 or 5% level except the species × sexes interaction. Fisher's (protected) least significant difference for the 5% level was 2.83 for comparisons of cell means of 20 observations each, 1.42 for comparisons of row means of 80 observations each, and 0.85 for comparisons of column means of 220 observations each (Table 1). Values for comparisons of means involving fewer observations were computed individually (Steel and Torrie 1980).

Permethrin induced significantly more takeoffs than all other test materials. This result confirms the irritancy of permethrin as reported previously by Ree and Loong (1989) and Chareonviriyaphap et al. (1997).

None of the 9 olfactory repellents induced significantly more takeoffs than the control. This result can be interpreted in terms of the differing modes of action of conventional repellents, which are volatile and act through olfaction, and permethrin, which is persistent and acts through contact. In tests of olfactory repellents, the atmosphere within the test chamber would be quickly saturated with repellent vapor, leading to rapid habituation of the olfactory organs. Directional cues such as gradients and currents would be absent. These effects would not affect the irritancy of permethrin by contact.

Aedes aegypti and *Ae. taeniorhynchus* differed significantly in numbers of takeoffs (Tables 1 and 2). The mean number of takeoffs for *Ae. aegypti* (sexes combined) was 3.0, compared with 5.2 for *Ae. taeniorhynchus.* Because these are composite values computed from data obtained in tests of controls, olfactory repellents, and permethrin, they must be interpreted in terms of the significant test materials × test species interaction.

Mean numbers of takeoffs by test material and test species (sexes combined) are shown in Table 3. Fisher's (protected) least significant difference for the 5% level was 2.00 for comparisons of spe-

Table 2. Analysis of variance of the numbers of takeoffs of male and female *Aedes aegypti* and *Aedes taeniorhynchus* from treated and untreated test papers in 15 min.

Source of variation	Degrees of freedom	Sum of squares	Mean square	F
Treatments (A)	10	5,928.63	592.86	28.41[1]
Mosquito species (B)	1	860.68	860.68	41.24[1]
Sex (C)	1	5,276.29	5,276.29	252.82[1]
AB interaction	10	1,416.93	141.69	6.79[1]
AC interaction	10	1,767.34	176.73	8.47[1]
BC interaction	1	2.60	2.60	0.12
ABC interaction	10	401.46	40.15	1.92[2]
Error	792	16,527.06	20.87	
Total	835	32,180.99		

[1] Significant at the 1% level.

[2] Significant at the 5% level.

Table 3. Mean numbers of takeoffs of *Aedes aegypti* and *Aedes taeniorhynchus* (sexes combined) from treated and untreated test papers in 15 min.

Test material[1]	*Ae. aegypti*[2]	*Ae. taeniorhynchus*[3]	Difference
Control	2.4	4.2	1.8
MGK R-326	1.4	3.4	2.0
DMP	1.9	2.1	0.2
Butopyronoxyl	1.3	4.0	2.7[4]
Citronyl	3.1	2.8	−0.3
BPG	2.2	4.6	2.4[5]
deet	2.9	2.9	0.0
EHD	4.8	2.2	−2.6[5]
DBP	3.4	4.8	1.4
MGK R-11	2.6	6.6	4.0[4]
Permethrin	7.3	16.6	9.3[4]

[1] MGK R-326, MGK Repellent 326; DMP, dimethyl phthalate; BPG, butoxy polypropylene glycol; EHD, ethyl hexanediol; DBP, dibutyl phthalate; MGK R-11, MGK Repellent 11.

[2] Number of observations was 40 except tests with DBP (38).

[3] Number of observations was 40 except tests with DMP (29), butopyronoxyl (38), deet (28), EHD (25), DBP (39), and MGK R-11 (39).

[4] Significant at the 1% level.

[5] Significant at the 5% level.

Table 4. Mean numbers of takeoffs of male and female mosquitoes (species combined) from treated and untreated test papers in 15 min.

Test material[1]	Males[2]	Females[3]	Difference
Control	1.5	5.0	3.5[4]
MGK R-326	1.6	3.2	1.6
DMP	0.6	3.9	3.3[4]
Butopyronoxyl	1.0	4.3	3.3[4]
Citronyl	1.9	4.1	2.2[5]
BPG	1.0	5.9	4.9[4]
deet	1.2	5.3	4.1[4]
EHD	1.5	7.5	6.0[4]
DBP	0.6	7.6	7.0[4]
MGK R-11	2.0	7.3	5.3[4]
Permethrin	5.6	18.2	12.6[4]

[1] MGK R-326, MGK Repellent 326; DMP, dimethyl phthalate; BPG, butoxy polypropylene glycol; EHD, ethyl hexanediol; DBP, dibutyl phthalate; MGK R-11, MGK Repellent 11.

[2] Number of observations was 40 except tests with DBP (38).

[3] Number of observations was 40 except tests with DMP (29), butopyronoxyl (38), deet (28), EHD (25), DBP (39), and MGK R-11 (39).

[4] Significant at the 1% level.

[5] Significant at the 5% level.

cies means of 40 observations each. Values for comparisons of means involving fewer observations were computed individually (Steel and Torrie 1980). *Aedes taeniorhynchus* exhibited significantly more takeoffs than *Ae. aegypti* in tests of butopyronoxyl, butoxy polypropylene glycol, MGK Repellent 11, and permethrin, but *Ae. aegypti* exhibited significantly more takeoffs than *Ae. taeniorhynchus* in tests of ethyl hexanediol. *Aedes taeniorhynchus* exhibited an average of 9.3 more takeoffs than *Ae. aegypti* in tests of permethrin.

Male and female mosquitoes differed significantly in numbers of takeoffs (Tables 1 and 2). The mean number of takeoffs for males (species combined) was 1.6, compared with 6.6 for females. Because these are composite values computed from data obtained in tests of controls, olfactory repellents, and permethrin, they must be interpreted in terms of the significant test materials × sexes interaction.

Mean numbers of takeoffs by test material and sex (species combined) are shown in Table 4. Fisher's (protected) least significant difference for the 5% level was 2.00 for comparisons of sex means of 40 observations each. Values for comparisons of means involving fewer observations were computed individually (Steel and Torrie 1980). Females exhibited significantly more takeoffs than males in the controls and in tests of all materials except MGK Repellent 326. Females exhibited an average of 12.6 more takeoffs than males in tests of permethrin.

The species × sexes interaction was not statistically significant (Table 2). This result can be interpreted to mean that the differences between *Ae. aegypti* and *Ae. taeniorhynchus* in numbers of takeoffs did not differ by sex or, alternatively, that the differences between males and females in numbers of takeoffs did not differ by species.

The significant 3-factor interaction (Table 2) can be regarded as the interaction of the materials × species interaction (Table 3) with sex, or as the interaction of the materials × sexes interaction (Table 4) with species, or as the interaction of the species × sexes interaction (not shown) with materials (Steel and Torrie 1980). The numerical results are the same in any case.

To evaluate the 3-factor interaction, we computed the separate contributions of the individual test materials to the 3-factor interaction sums of squares. This analysis revealed that 75% of the 3-factor interaction sum of squares was accounted for by deet, ethyl hexanediol, and Citronyl alone. To evaluate this result, we computed the 3-factor interactions of each test material and the control (factor A) with test species (factor B) and sexes (factor C).

The values obtained for deet, ethyl hexanediol, and Citronyl were 3.2, 4.9, and 6.6 takeoffs per mosquito per 15 min (sign ignored), compared with 0.48 (dimethyl phthalate) to 2.35 (MGK Repellent 326) for other test materials. Thus, the significant 3-factor interaction reflects multiple differences between the species and sexes in their responses to the test materials. These differences were greatest in tests of deet, ethyl hexanediol, and Citronyl.

Although olfactory repellents did not induce significantly more takeoffs than the control, several 2- and 3-factor interactions of these materials with species and sex were statistically significant (Table 2). These interactions indicate that certain repellents did, in fact, induce significantly more takeoffs in one species than the other and/or significantly more takeoffs in one sex than the other (Tables 3 and 4). Because repellents have toxic as well as repellent properties (Rutledge et al. 1981, Mehr et al. 1990), this activity could reflect low-level irritant and/or fumigant effects.

In conclusion, this study supports the finding of Roberts et al. (1997) that the World Health Organization test system measures primarily responses to physical contact with the test material. Only permethrin, a known irritant, induced significantly more takeoffs than the control. Because insecticides can exhibit significant vapor repellent activity (Chareonviriyaphap et al. 1997), this should be recognized as a shortcoming of the World Health Organization test method.

In addition, we recommend that step A, time to first takeoff, be deleted from the instructions for the procedure, because many mosquitoes do not take off during the 15-min test period. Finally, we believe that the requirement for exposure of 20 or more mosquitoes to the treated papers in the procedure should be reduced. In the present study, exposure of 20 mosquitoes to the treated papers resulted in 792 degrees of freedom for error (Table 2), which is far beyond the range of standard tables of F. There are definite practical limits on increasing accuracy by the repetition of measurements (Wilson 1990), and this is an important consideration when each test takes 15 min plus preparation and cleanup time to complete.

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