ANOPHELES CULICIFACIES: OBSERVATIONS ON POPULATION ECOLOGY AND REPRODUCTIVE BEHAVIOR[1]

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ABSTRACT. During June 1979, 9323 ♀ and 7548 ♂ laboratory-reared progeny of wild-caught female *Anopheles culicifacies* were released as pupae in a self-marking device positioned at a breeding site near a rural village in Punjab Province, Pakistan. The numbers of marked adults collected resting indoors the first afternoon following release were disproportionately lower than the numbers taken in subsequent recapture attempts. In addition, the proportion of virgin and nulliparous unmarked females resting indoors was unexpectedly low, indicating that about half of the newly-emerged population may have rested outdoors. Relatively few pairings were observed at male swarms which formed at dusk in cattle-buffalo feed-lots, indicating most mating occurred elsewhere or at different times of the night.

INTRODUCTION

During a series of mark-release-recapture experiments aimed at estimating the mating competitiveness of genetically-altered *Anopheles culicifacies* Giles males, few unmarked indigenous females were inseminated by released males, even though these males mated with expected proportions of marked virgin female progeny from the same population (Baker et al. 1980). Concurrent dissections of unmarked females indicated that the proportions of virgin and nulliparous females was low, considering the estimated population addition and horizontal survivorship rates. This disparity was not anticipated, since congregations of resting female *An. culicifacies* have only been found indoors (e.g., Reisen 1978) and large crepuscular swarms of males characteristically form near diurnal resting sites (Reisen et al. 1977). The present study describes these mark-release-recapture observations in greater detail and describes the reproductive and resting behavior of adults emerging through a self-marking device positioned at a breeding site.

MATERIALS AND METHODS

PRODUCTION AND RELEASE. The rearing, marking and release procedures for the April and May mating competitiveness experiments were summarized previously in Baker et al. (1980). For the June release, the F_1 progeny of females collected resting at Kot Baghicha were reared under natural photoperiod (ca. 14L:10D) on a diet of liver powder. Pupal-larval separation was done daily using an ice water method (Weathersby 1963) and the pupae were counted by xerography (Baker et al. 1979). Pupae were allowed to emerge through a self-marking device (Singh and Yasuno 1971) which was positioned at a breeding site near a buffalo-cattle feed-lot 3 km northeast of the vil-

[1] This research was funded by Grant Nos. AI-10049 and AI-16289 from the U.S. Institutes of Allergy and Infectious Diseases, NIH, and PL-480 Grant No. 391-0455 from the U.S. Agency for International Development in Pakistan.

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lage of Kot Baghicha Singh Walla, Punjab Province, Pakistan (Baker et al. 1980). Each of the 3 cohorts was marked with a date-specific fluorescent dust. Dead pupae and adults which failed to leave the marking device were subtracted from the number produced to estimate the total number released. The sex ratio of each cohort was estimated from samples of 514, 632 and 218 pupae, respectively.

RECAPTURE. Starting on the afternoon following the first release, marked adults were recaptured daily for 7 days, using the following methods:

1) Adult mosquitoes resting indoors were sampled by mouth aspirator for 20 min and a battery-powered sweeper (Davis and Gould 1973) for 10-min in each structure at a cattle-shed compound (Shed C in Fig. 1 of Baker et al. 1980). Collections were standardized to the numbers of adults collected per man hour (MH) of aspirator or sweeper collection effort per day, summed and expressed as numbers collected/ 2 MH.

2) Attempts were made to collect outdoor resting adults from habitats productive for other mosquito species (Reisen 1978) using the same battery-powered sweeper.

3) Starting about 15 min. before sunset, 2 collectors sampled male swarms which formed at 4 to 6 locations near indoor resting sites using an aerial net. Additional habitats near the feed-lot compound were searched concurrently for swarming activity.

PROCESSING. Mosquitoes were placed in a cold room at 5°C overnight and then examined under ultra-violet light for the presence of fluorescent dust, identified to species and counted. Samples of both marked and unmarked females collected resting indoors were dissected. Insemination was determined by examination of the spermatheca. Trophic status was scored as unfed, partially or fully blood-fed or gravid. Follicular development was classified by the degree of yolk deposition (Mer 1936). The number of previous

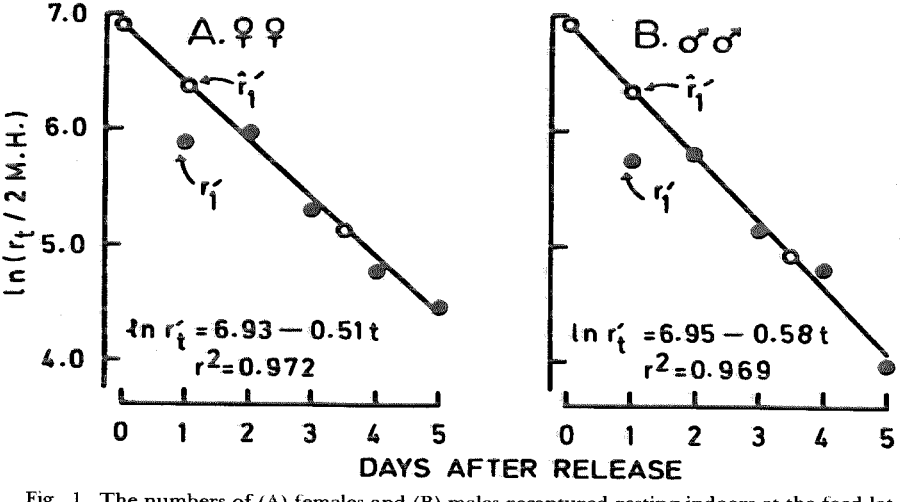

Fig. 1. The numbers of (A) females and (B) males recaptured resting indoors at the feed-lot compound per 2 MH of collection effort (r'_t) transformed to $\log_e$ and plotted as a function of days after release (t); open points are the intercept, estimated $\hat{r}'_1$ and mean r'_1 connected by the fitted regression function.

ovipositions was determined by the dilatation method of Polovodova (in Detinova 1962). Nulliparity in unfed females was confirmed by examining the degree of coiling of the ovarian tracheoles in one ovary (Detinova 1962).

CALCULATIONS. Daily survivorship was estimated horizontally over time by fitting a curvilinear regression function of the form $\ln r'_t = \ln a - t \ln s_h$, where r'_t = number of recaptured adults, r_t, collected on days t = 1 to 5 after release, corrected by MH of collection effort, a = the intercept, and s_h, the antilogarithm$_e$ of the regression coefficient, = horizontal survivorship rate (Reisen et al. 1980). Horizontal survivorship estimates included losses due to mortality, emigration and removal sampling. For pupal releases in June, s_h was calculated for days t = 2 to 4, with r'_1 estimated from the regression function. The goodness of fit of the regression function was expressed by the coefficient of determination, r^2, and $\ln s_h$ was tested for significant departure from 0 using an analysis of variance (ANOVA) (Sokal and Rohlf 1969). Significant regression slopes were compared by analysis of covariance (ANCOVA) (Sokal and Rohlf 1969). The loss rate, $L = 1 - s_h$.

Survivorship was also estimated vertically through the unmarked female population from the dissection age-grading data. Inherent in this approach are the assumptions that 1) all age groups are sampled proportionately, 2) the duration of the gonotrophic cycles are known and are relatively constant for the period of estimation and 3) the population age structure is relatively stationary; i.e., immigration $\approx$ emigration, emergence $\approx$ mortality. Vertical survivorship was estimated by 2 calculation procedures:

1) $s_v(1) = p^{1/g}$, with p = the proportion of females at ovarian stages III to V that were parous and g, the length of the gonotrophic cycle, = 2 days (Davidson 1954).

2) $s_v(2)$ was estimated from the regression of the number of females (y) in each dilatation class (i) on female age in days at the midpoint of each dilatation class (t_i), where $\ln (y_i+1) = \ln a - t_i \ln s_v(2)$ (Reisen et al. 1980). For the present study, g_1 (emergence to 1st oviposition) = 4 days with subsequent g_i = 2 days (Mahmood and Reisen 1981). Again, the slope, $\ln s_v(2)$, was tested for significant departure from 0 by ANOVA and compared by ANCOVA. Since the vertical s_v estimates presumably measured only mortality losses and the horizontal s_h estimates included mortality, emigration and removal sampling, the emigration rate, $er = s_v(1) - s_h$, considering removal sampling losses to be relatively minimal.

When the chronological age at which any biological event occurs is determined, the proportion of the population expected to be in that class may be calculated from the constant vertical survivorship rate. Thus, the expected proportion of females nulliparous, $NP = 1 - s_v(1)g_1$ and the expected proportion not inseminated, $NI = 1 - s_v(1)^m$ with m = female age at mating. $s_v(1)$ was used since it measures survivorship during the nulliparous period.

Daily population size (P_t) was estimated using Bailey's (1952) modification of the Lincoln Index where $P_t = R_t U_t / r_t$, with R_t, the number of marked specimens remaining in the study area, $= s_h(M_{t-1} + R_{t-1})$, M_t = number of marked specimens released, and U_t = the numbers of unmarked specimens collected. The number of daily additions, emergence + immigrants, (A_t) was calculated using the method of Manly and Parr (1968) with $A_t = P_t - (P_{t-1}s_h)$. The addition rate, $ar = \overline{A_t}/\overline{P_t}$ and the overall rate of population change, RC = ar − L.

RESULTS

ONSET OF REPRODUCTIVE ACTIVITY. In June, marked males did not swarm on their night of emergence, but were collected swarming at Shed C compound on the following evening (Table 1). The proportion of each cohort in aerial net samples, standardized by the total numbers of males collected swarming, consis-

Table 1. Numbers of marked and unmarked *An. culicifacies* males collected swarming at the feed-lot compound from 5 to 11 June 1979.

Date (June)	Number of swarms	Marked[1] Release Date (June) 4	5	6	unmarked
5	7	0 (.00)	0 (.00)	—	92
6	8	4 (.04)	9 (.09)	0 (.00)	88
7	9	2 (.01)	39 (.20)	51 (.26)	101
8	8	3 (.05)	9 (.16)	21 (.37)	24
9	5	3 (.02)	20 (.13)	51 (.34)	78
10	6	1 (.01)	17 (.12)	35 (.25)	89
11	4	1 (.01)	10 (.14)	14 (.20)	46
Total	47	14 (.02)	104 (.13)	172 (.21)	518

[1] Proportion of recaptured males among all males presented within parentheses.

tently increased on the 2nd dusk following the night of emergence, and then declined. Swarms were not observed away from cattle sheds despite repeated searches along water-course breeding sites, agricultural fields, and brush.

The 281 marked females collected resting indoors the afternoon following their night of emergence were all uninseminated (Table 2). As indicated by the proportion of inseminated females sequentially recaptured resting indoors, approximately 66% (256/390) mated on the second night and almost all of the remainder mated on the third night after emergence.

Of 281 females recaptured resting indoors on the afternoon following their night of emergence, 55 (20%) were blood-fed (Table 2). Oögenesis had progressed to stage IIa in 38% of the females. By the following afternoon, 265 of 390 females (68%) had follicles matured beyond stage IIa. In June when afternoon

Table 2. Reproductive status of marked *An. culicifacies* females recaptured resting indoors from 5 to 11 June 1979.

Attributes	Chronological Age in Days <1	2	3	4	5	6	7
No. dissected	281	390	200	127	85	35	4
No. uninseminated	281	134	2	0	0	0	0
Trophic status: unfed	226	63	1	0	0	0	0
Bloodfed: partial	49	128	2	0	0	0	0
replete	6	199	107	61	46	18	4
gravid	0	0	90	66	39	17	0
Ovarian stage: Ia	30	0	0	0	0	0	0
Ib	135	22	0	0	0	0	0
IIa	106	103	0	0	0	0	0
IIb	1	77	1	0	0	0	0
III	0	166	64	16	14	9	1
IV	0	22	46	44	26	3	2
V	0	0	87	67	45	23	1
No. dilatations: 0	281	390	200	63	12	2	0
1	0	0	0	49	48	19	3
2	0	0	0	0	0	1	1
Parity not determined	0	0	0	15	25	13	0

indoor temperatures averaged 37°C, 90 of 200 females (45%) were gravid by day 3, and 49 of 112 females (44%) collected on day 4 were parous with follicles at stage II or IV. These results indicated that the time from emergence to first oviposition (g_1) was slightly less than 4 days, and that females refed the same night that they oviposited. Oögenesis from stage IIa to stage V took about 2 days. Thus, $g_1 = 4$ days, with subsequent $g_i = 2$ days was an appropriate chronology to be used in vertical survivorship estimates.

SURVIVORSHIP AND POPULATION SIZE ESTIMATES. During the pre-monsoon season (April to June), horizontal survivorship was highest in May (Table 3) when the

Table 3. Reproductive status and selected population attributes of unmarked *An. culicifacies* females collected resting indoors.

	Date Sampled		
	23–28 April	20–25 May	7–11 June
Mean afternoon indoor:			
Temp. (°C)	32.5	29.7	37.0
Relative humidity (%)	58	60	51
Number examined	726	150	1263
Obs. uninseminated (%)	2.4	1.3	4.0
Exp. uninseminated (%)[1]	49.6	41.2	38.5
Trophic status:			
fully bloodfed	421	105	799
gravid	286	37	383
gravid/bloodfed	0.679	0.352	0.479
No. parity determinations	144	144	168
0 parous	94	83	90
1 parous	45	57	75
2 parous	5	4	1
5 parous	0	0	2
Expected no. nullipars ($\hat{y}_0$)[2]	152.9	148.4	160.8
Estimated % nullipar exophily[3]	38.5	44.1	44.0
Obs. nullipars (%)	65.3	57.6	53.6
Exp. nullipars (%)[4]	87.6	80.2	77.3
s_h[5] (♀ − ♂)	0.583−0.583	0.634−0.709	0.599−0.559
$s_v(1)$[6]	0.593	0.667	0.690
Emigration rate (s_v (1) − s_h)	0.010	0.033	0.091
s_v (2)[7]	0.486	0.516	0.517
Addition rate (♀ − ♂)	0.366−0.246	0.333−0.275	0.361−0.369
% virgin additions	6.6	3.9	11.1
Loss rate (♀ − ♂)	0.417−0.417	0.366−0.291	0.401−0.441
Rate of change (♀ − ♂)	−0.051−(−0.171)	−0.033−(−0.016)	−0.040−(−0.072)

[1] Expected uninseminated = $1 - s_v(1)^{1.31}$.

[2] $\hat{Y}_1$ Expected number of nullipars that are 1 day old, calculated from $\ln(Y_1 + 1) = \ln a - [\ln s_v(2)]\, t$ for $t = 1$.

[3] % Exophily = $(\hat{Y}_o - Y_0)/\hat{Y}_0 \times 100$.

[4] Expected nullipars = $1 - s_v(1)^4$.

[5] Horizontal survivorship estimated by regression.

[6] $s_v(1)$ vertical survivorship estimated by Davidson's method for females with Stage III to V ovaries.

[7] $s_v(2)$ vertical survivorship estimated by the dilatation regression method.

weather was unseasonably cool (i.e., the mean maximum temperature of 29.7°C was 7°C less than the 40 year normal). In April and May, survivorship was estimated for days t = 1 to 5 using cohorts of 1-day old F_1 adults released at the Shed C compound, while in June survivorship was estimated for days t = 2 to 5 using adults emerging from pupae. Daily population size estimates were relatively similar within months and were highest during May (Table 4). Except for males in April, the rate of population size change was less than 0.1, indicating that the population was relatively stationary within each estimation period. Vertical survivorship estimated for females at follicular stages III to V using Davidson's (1954) formula ($s_v(1)$) approximated the horizontal estimates (s_h) and indicated that losses due to emigration were less than 0.1 (Table 3).

EVIDENCE FOR EXOPHILY. The proportion of unmarked, uninseminated females resting indoors was less than 0.04 throughout; much lower than the estimated daily addition rate which was always greater than 0.33 (Table 3). Fur-

Table 4. Releases and recaptures of ♀♀ − ♂♂ *A. culicifacies* at the feed-lot compound during April, May and June, 1979[1].

Date	U_t ♂−♂	r_1 ♀−♂	r_2 ♀−♂	r_3 ♀−♂	p_t ♀−♂
22 April		m_o=3453–2947			
23 "	980–1201	192–275			10268–7506
24 "	935–1244	215–215			5097–5800
25 "	926–839	87–90			7268–5449
26 "	818–591	47–61			6924–3303
27 "	1055–600	43–39			5686–3059
28 "	611–494	13–19			6346–3015
				$\overline{P}_t$ =	6932–4689
Total	5325–4969	597–699		±95%CI =	1907–1943
20 May		m_o=3428–2262			
21 "	901–1059	179–113			10933–15032
22 "	530–636	81–72			9004–10047
23 "	812–935	57–77			12421–9794
24 "	675–1119	40–67			9323–9552
25 "	685–788	26–21			9222–15217
				$\overline{P}_t$ =	10181–11928
Total	3603–4537	383–350		±95%CI =	1688–3362
4 June		m_o=1269–1392			
5 "	268–423	17–18	m_o=2913–2937		
6 "	250–346	35–36	135–137	m_o=5141–3219	
7 "	352–224	23–12	183–129	267–191	3273–1997
8 "	353–221	11–8	99–61	196–150	3039–1670
9 "	247–209	4–6	38–41	86–86	3045–1454
10 "	355–197	6–5	37–19	63–58	3166–1242
11 "	343–142	2–1	29–3	43–23	2624–1520
				$\overline{P}_t$ =	3029–1577
Total	2168–1762	98–86	521–390	655–508	
				±95%CI =	306–349

[1] U_t = number unmarked; r_t = number recaptured each day, m_o = number released, $\overline{P}_t$ = mean of daily population size estimates, P_t, ±95%CI = ±95% confidence interval.

thermore, the expected proportion of uninseminated females which ranged from 38.5 to 49.6% far exceeded the observed proportion of uninseminated females indoors which ranged from 1.3 to 4.0% (Table 3). If the female immigration rate approximated the low emigration rate, the majority of female population additions emerged within the study area, but mated before resting indoors. A similar disparity was observed between the observed proportion of nullipars resting indoors and the calculated expected proportion in the whole population (Table 3). In agreement with this, the observed number of nullipars resting indoors was always less than the number expected from the regression function (Fig. 1). The regression coefficients, ln s_v (2), were significantly greater than 0 when tested by ANOVA and did not differ among months when tested by ANCOVA ($\alpha = 0.05$). The regression estimates suggested that between 38 and 44% of the newly-emerged nullipars may have rested outdoors.

When regression functions were fitted to the numbers of males or females recaptured per 2 MH per day (r'_1) for each of the 3 cohorts released in June, the regression coefficients significantly differed from 0 when tested by ANOVA, but did not significantly differ among cohorts when tested by ANCOVA ($\alpha = 0.05$). The data were subsequently pooled over the three cohorts and regression expressions fitted for females and males collected on days t = 2 to 5 (Fig. 2). The expected number of adults resting indoors was calculated from the regression functions to be 636 ♀♀ and 645 ♂♂. The observed r'_t were 373 ♀♀ and 329 ♂♂ and suggested that 58.7 and 51.1% of the released females and males, respectively, were not available to be sampled on the day following their evening of release. In agreement, the recapture rate on day 2 exceeded the recapture rate on day 1 (Fig. 2). In June only one marked female and one marked male were recaptured in over 2.5 MH of outdoor sweeper collections in habitats which appeared favorable. Both adults had just emerged and were collected within 10 m of the release site. One unmarked female and 3 unmarked males were collected resting in a tree hole and 1 unmarked male was collected from a monitor lizard burrow. The

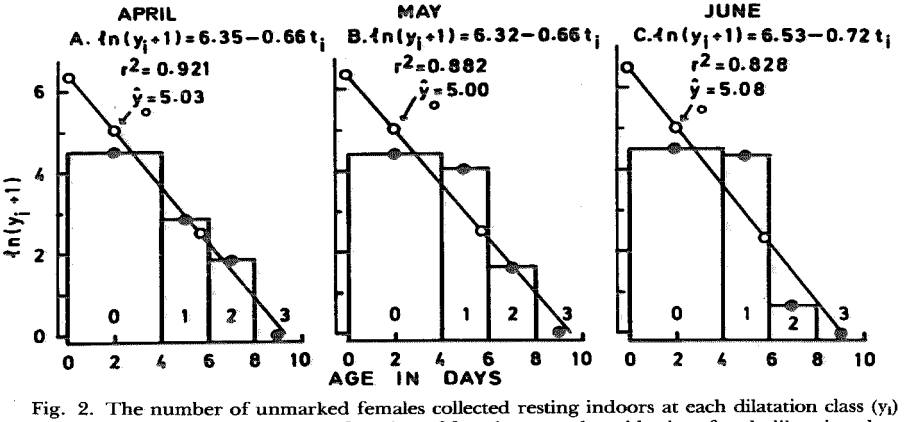

Fig. 2. The number of unmarked females collected resting indoors at each dilatation class (y_i) transformed to $\log_e$ and plotted as a function of female age at the midpoint of each dilatation class (t_i); open points are the intercept, estimated $\hat{y}_o$ and the mean of y connected by the fitted regression function.

female was uninseminated, unfed, at ovarian stage Ib and nulliparous, while all the males had their terminalia fully rotated.

Other outdoor resting sites sampled that were negative included irrigation culverts, an abandoned "Persian wheel" irrigation system, snake and lizard holes near the release site, and maize and vegetable fields. These collections yielded adults of 7 mosquito species (2 ♀♀ – 2 ♂♂ *An. stephensi* Liston, 0–1 *Culex bitaeniorhynchus* Giles, 3–4 *Cx. quinquefasciatus* Say, 0–1 *Cx. fuscocephala* Theobald, 2–6 *Cx. pseudovishnui* Colless and 40–54 *Cx. tritaeniorhynchus* Giles), but no *An. culicifacies*. These species, with the exception of *An. stephensi,* were considered completely or partially exophilic (Reisen 1978).

DISCUSSION

At Kot Baghicha about half of the newly-emerged adult *An. culicifacies* population was not collected by indoor aspirator or battery-powered sweeper collections. Remarkably low numbers of unmarked, virgin and nulliparous females were collected resting indoors, even though the indigenous population exhibited a rapid turnover with high loss and addition rates. The recapture rate on day 2 of marked adults released as pupae was equal to or greater than the recapture rate on day 1. Although congregations of exophilic *An. culicifacies* were not found during the present or previous studies (Reisen 1978, Reisen et al. 1980), exophilic adults were sampled by Shalaby (1971) using a Muirhead-Tompson pit shelter in Gujarat State, India. In agreement with our results, Shalaby (1971) classified 55.8 and 3.9% of the 1585 exophilic resting females examined as unfed and gravid, respectively.

Presumably, the unsampled segment of the newly-emerged population at Kot Baghicha rested outdoors. After the first day of adult life, all age classes rested indoors and exhibited an equal probability of capture as indicated by the close fit of the regression function to the constant decrease in the recapture rate with time. Exophilic resting by newly-emerged adults would cause underestimation in monitoring relative abundance or estimating population size using indoor resting collection.

Apparently a considerable amount of mating in *An. culicifacies* occurs away from crepuscular male swarms, considering the population size and turnover rate of the Kot Baghicha population. Swarms seemed to be formed only in the immediate vicinity of the diurnal resting site and were not found near breeding habitats, agricultural fields or directly over bloodmeal hosts during the present or previous studies (Reisen et al. 1977). Virgin females resting indoors, egressing at dusk, and flying near swarms were pursued and mated, and presumably represented the pairings observed during the present and previous studies (Baker et al. 1980, Reisen and Aslamkhan 1976, Reisen et al. 1977). Swarms always dissipated after dusk, but the males remained outdoors for the remainder of the night, entering shelters at dawn (Reisen et al. 1976, Baker et al. 1980). Some males mated after swarming terminated, as Baker et al. (1980) found that females entrained on an unnatural insectary photoperiod egressed from indoor resting sites after swarming was completed but were mated by indigenous males. Possible situations when females may be inseminated thus included: 1) the crepuscular egress from indoor and/or outdoor resting sites, 2) traversing the distance from the resting site to the blood-meal host, and 3) flying from the host to the post blood-meal resting site and then to the diurnal indoor resting site at dawn. Dissections of unmarked females feeding throughout the night at buffalo baits in May indicated that, except for a few newly-emerged females feeding early in the evening, host-seeking females were inseminated (96.5%, n = 259). During May, unmarked females collected in ingress traps at dawn were mostly inseminated (94.6%, n = 56). Dawn swarms were not observed in any

month. Thus, the actual place of mating remains cryptic but seems to be associated with the initiation of female flight from indoor or outdoor resting sites. Since virgin females rest away from shelters, future genetic control studies should endeavor to release altered males as pupae near a breeding site so they may be in the proper place at the proper time to successfully compete for exophilic virgin females from the target population.

ACKNOWLEDGMENTS. We especially thank Messrs. J. Ahmad, S. H. Chisti, S. Amar, and T. J. Rathore for their assistance with the collection and identification of the mosquitoes and Messrs N. Hussain, M. Saghir, I. H. Zafar, T. Mahmood, L. Chowdhery, Z. Ahmad, R. Yasmin and M. R. Sindhoo for producing the cohorts for release. The administrative assistance of the School of Public Health, University of California, Berkeley is gratefully acknowledged.

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