Conditioned responses in courtship behavior of normal and mutant *Drosophila*

(reproductive behavior/learning and memory/behavior genetics)

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ABSTRACT   Male courtship behavior in *Drosophila melanogaster* is modified by prior sexual experience. Whereas naive males nearly always court virgin females persistently, males previously paired with unreceptive fertilized females subsequently court virgin females in an abbreviated manner, if at all. The probability of diminished male courtship is directly related to the duration of the prior "conditioning" period with a fertilized female. Naive males court fertilized females less vigorously than they court virgins; this depression of male behavior occurs even if the male is blind or if the fertilized female cannot actively reject his courtship. These results suggest that fertilized females are a source of both courtship-provoking and courtship-inhibiting olfactory cues and that the central association of these cues in males is sufficient to bring about the retention of modified courtship behavior. Mutant "amnesiac" males, selected as memory-deficient in a learning test unrelated to courtship [Quinn, W. G., Sziber, P. P. & Booker, R. (1979) *Nature* (*London*) 277, 212–214], are trainable by exposure to fertilized females, but the experience-dependent behavior—diminished courtship performance—wanes abnormally rapidly—i.e., less than 1 hour, compared to 2–3 hr for wild-type flies.

The well-known genetics of *Drosophila* has allowed the beginning of specific genetic analyses of learning and memory. Using olfactory discrimination tests, Quinn *et al.* (1) have demonstrated associative conditioning in *Drosophila*. These workers and their collegaues have subsequently isolated single-gene learning mutants; one, the "dunce" mutant, is defective in learning (2), whereas a second mutant, "amnesiac," learns well but forgets abnormally rapidly (3). It would be desirable to augment these studies of normal and mutant flies with new investigations using behavioral situations that do not involve the use of electric shocks (1, 4). One promising possibility is courtship behavior, which has been throughly described in *Drosophila* (5) and is amenable to manipulation (6). Naive individual males reliably court virgin females (5). If conditioning were found to alter components of this courtship behavior in wild-type flies, then experiments on learning and memory-defective mutants could be carried out. Previous studies have dealt with conditioning as a population phenomenon (1, 4). Learning revealed in individual flies in a courtship situation would make feasible experiments on single flies carrying learning mutations. Conditioned courtship might be of further interest in that it could suggest that the capacity to learn has adaptive significance connected to behaviors that occur naturally.

Courtship in *Drosophila* is a fixed action pattern, including orientation of the male toward the female, then extension and vibration of his wing to produce a courtship song, and finally a curling of the abdomen culminating in copulation (5). Because these activities seem to be performed normally by male flies

with no previous mating experience, their execution would appear to be independent of conditioning and specified only by the genotype. We show here that, perhaps unexpectedly, some components of reproductive behavior are modifiable by previous courtship experience. The discovery of this conditioning has permitted further exploration of the role of mutations in learning and memory.

MATERIAL AND METHODS

Flies (*Drosophila melanogaster*) were maintained on a cornmeal, agar, and molasses medium supplemented with yeast. Except for three temperature-sensitive stocks (see below), flies were reared at 25°C in rooms kept on a cycle of 12 hr of light and 12 hr of dark. The Canton-S strain provided wild-type males. Mutant amnesiac males came from a stock provided by W. Quinn, and the autosomes from this strain were made isogenic with our Canton-S stock before behavioral comparisons between the two strains were made. Blind males were derived from four different no-receptor-potential mutant stocks (*norpA*, ref. 7) or a glass-eye mutant (8). Sample males from blind stocks had their electroretinograms measured prior to courtship experiments; none had light-induced electrical activity in their eyes. Females, used either as virgins or as mated females, were usually from an attached-X, yellow forked stock. Three different shibire-temperature-sensitive stocks (shi^{ts-1}; shi^{ts-3}; shi^{ts-6}) provided females that could be immobilized at 27°C (9) and used in tests at that temperature. The higher temperature did not have major effects on the behavior of wild-type males tested with wild-type females (although the "courtship index," see below, was *ca.* 20% higher than that found at 25°C). At the permissive temperature for shi^{ts} females (20°C) behavior of wild-type males was virtually identical to that of wild-type males with normal females at this temperature. Males were collected under ether 0–10 hr after eclosion and kept singly in food-containing vials for 5 days before use. Females were collected in the same way and stored in groups of 5–15 in vials. Virgins were used 5 days after eclosion. Other virgins were put with males on the fourth day, observed to copulate then, and were used as mated females the next day.

A plastic mating wheel (10) with 10 observation chambers, each about 0.4 cm³, was used to train males and to observe courtship performance. All training and courtship tests were conducted at 25°C ± 1°C and during the "daytime" hours of the light–dark cycle. Male courtship behavior was quantified in the following way. Each experimental or control male was gently aspirated to a clean chamber of the mating wheel that contained an active or an immobilized female (either a lightly etherized attached-X female or a shi^{ts} female at 27°C). The courtship index (CI) for a given male is defined as the fraction

Abbreviation: CI, courtship index, the fraction of an observation period that a male spends courting a female.

of the observation period during which any courtship behavior occurs. In tests with active or with *shi*[ts] temperature-paralyzed females, pairs were observed for periods of 10 min. Observations involving etherized females were approximately 10 min, depending on how long the females remained immobile. The CIs reported are unweighted means ± SEM; weighted means, taking into account variations in observation periods, were virtually identical. Immobile virgins were usually used to derive the CI because, although active virgins are generally courted more persistently (11, 12), the variation among trials is greater.

RESULTS

Experience-associated modification of male courtship behavior

Naive male *Drosophila* courted active virgin females in 97% of cases observed and copulated in 38% of them ($n = 420$, observed for 10 min each; CI = 0.52 ± 0.03). Such males courted active mated females with a slightly lower probability (78%, $n = 121$), with less persistence (CI = 0.13 ± 0.04, $n = 40$), and copulation was very rare (<1%). The experience of a male with a mated female differs from that with a virgin in that courtship triggers apparent rejection behaviors (13), the most conspicuous of which is extrusion of the female ovipositor without egg laying (0.30 extrusions per min in the above tests with mated females vs. 0.02 extrusions per min by virgins). The extrusion rate for premated females was positively correlated with the CI (Spearman's $r_s = 0.55$, $P < 0.01$); in fact the extrusion rate for previously mated females that were courted was 3-fold higher than that for fertilized females that were ignored by males. Can the experiences of a male with a mated female eventually bring about differences in his courtship behavior and is the conspicuous extrusion the key feature of this experience? Wild-type males were paired with females in the chambers of a mating wheel and observed for 60 min. The duration of all male courtship behavior was recorded for each of the six 10-min intervals comprising the total observation time. Fig. 1 shows

that males expressed less courtship toward mated than virgin females. There was a small and progressive decrease in the amount of time a male spent courting a mated female during the course of the total observation period; no comparable decrement was found among males paired with virgin females.

To test the significance of visual cues for male behavioral modifications, naive blind males were presented with active virgins; all of the males ($n = 87$) courted, 14% copulated, and the CI was 0.29 ± 0.03 for the 10-min observation period (somewhat less than the CI for normal males). When these males were presented with active mated females, the percentage of males courting was 79%, none copulated, and the CI was substantially reduced—i.e., to 0.08 ± 0.01 ($n = 98$). These data are pooled from tests of males derived from various blind stocks; interstock behavioral differences were negligible. Although the mated females actively extruded their ovipositors in these trials (0.35 per min), the males apparently did not require visual input from this female activity to be inhibited.

Other observations show that extrusion of the ovipositor is not essential for inhibition of male courtship (cf. ref. 14). Mated females that were immobilized by ether or heat treatment of the temperature-sensitive mutant never extruded, yet they stimulated from wild-type males much less courtship than similarly immobilized virgins. CIs for these four situations were etherized–mated: 0.02 ± 0.01, $n = 40$; immobilized *shi*[ts]–mated: 0.06 ± 0.02, $n = 43$,; etherized normal virgins: 0.25 ± 0.03, $n = 24$; and immobilized *shi*[ts] virgins: 0.35 ± 0.03, $n = 133$. It is worth noting that blind males can be stimulated to court immobilized *shi*[ts] virgins (CI = 0.23 ± 0.05, $n = 27$).

In summary, male courtship behavior is depressed in the presence of a mated female, and this modified behavior is not dependent upon the activity of the female or upon visual cues received by the male.

Duration of pretest experience is directly related to the probability that male behavior will be modified

The next experiments were designed to ask if the courtship response of a male to a virgin female is modified by previous experience with a mated female. Wild-type males were paired with mated females in the mating wheel for predetermined periods, then transferred at once to fresh chambers of the mating wheel for tests of courtship behavior with immobile virgin females. The results shown in Table 1 indicate that the courtship index with the virgin is related to the duration of the pretest experience. There appears to be an inverse relationship between the amount of time a male is paired with a mated female and the probability that he will court vigorously in the subsequent presence of a virgin. The maximum effect is ap-
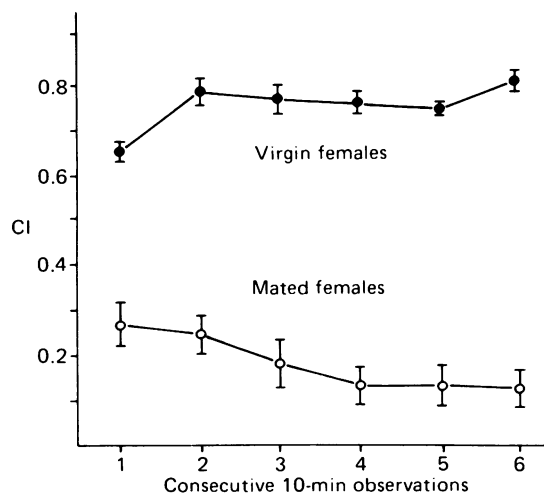

FIG. 1.   Courtship of wild-type males paired with active mated females or active virgin females. Males were observed individually in mating chambers, each containing a fertilized female (24 tests) or a virgin female (24 tests). Each chamber was observed for 1 hr, and the mean CIs (±SEM) are expressed separately for six consecutive 10-min intervals. There were no copulations involving the mated females (O); however, 14 males copulated with virgin females (●) in the first 10-min period; four additional males copulated over the next 50 min. After these males began copulation, recording of their courtship behavior was discontinued.

Table 1.   Effect of the duration of the "training" period on the subsequent CI for wild-type males

Period with fertilized female, min	CI, mean ± SEM	No. of males per arbitrary CI value			
		0.0	>0.0–0.1	>0.1–0.3	>0.3
5	0.35 ± 0.05	0	3	9	12
15	0.28 ± 0.05	2	7	3	12
22	0.13 ± 0.04	6	9	5	4
30	0.08 ± 0.03	10	9	2	3
60	0.03 ± 0.02	14	7	3	0
120	0.07 ± 0.03	11	8	3	2

In each part of the experiment, 24 males were individually paired with fertilized females for the period indicated; then the CI for each male was determined with an etherized virgin.

Table 2. Frequency and duration of courtship activities for naive males (control) and males previously paired with fertilized females (experimental)

		Mean ± SEM, per male, of three courtship activities					
		Orientation		Wing extension		Attempted copulation	
Males	CI, mean ± SEM	Number	Duration, min	Number	Duration, min	Number	Duration, min
Control	0.32 ± 0.05	16.0 ± 1.8	0.06 ± 0.01	13.0 ± 2.3	0.07 ± 0.01	8.3 ± 2.0	0.24 ± 0.07
Experimental	0.03 ± 0.02	3.5 ± 0.7	0.05 ± 0.01	5.5 ± 1.7	0.07 ± 0.02	1.8 ± 0.6	0.10 ± 0.02

Each group consisted of 12 wild-type males tested with ether-immobilized virgin females. Prior to testing, control males were confined alone in a chamber of the mating wheel for 30 min, whereas experimental males were paired for 30 min with fertilized females. The mean number of events per male is shown for each activity. For each male, the total time for a given activity was divided by the number of times that activity occurred and the means of these values appear under the headings "Duration."

parently achieved in 30–60 min; replicate trials of the 30- and 60-min pretreatments (using a new set of 48 males and females) yielded CI values statistically indistinguishable from those shown in Table 1. Other behaviors such as locomotion, preening, phototaxis, and geotaxis did not appear to be affected among males previously paired with fertilized females.

Controls were performed in order to determine whether the decrement in courtship activity is due solely to the pretreatment with mated females. The relationship between the time spent in the mating wheel and the subsequent reduction in the CI cannot be due merely to handling or to the duration of confinement, for males placed alone in a chamber for 30 min and then tested were found to have a normal CI of 0.28 ± 0.03, n = 24 (cf. previous section). Second, the results in Table 1 cannot be related simply to the presence of a yellow-bodied fly, because males paired for 30 min with yellow males gave a subsequent CI of 0.23 ± 0.03 (n = 24); and other males confined with active yellow forked virgin females for 5–30 min had a CI of 0.31 ± 0.03 (n = 24) upon subsequent testing with etherized virgins. (In the latter trial, 20 of the 24 males copulated with the virgin females during the 30-min pretreatment.) It might be supposed that males that actively court mated females without success for 30 min or longer become either physically or sexually exhausted and hence the CI value is low. This possibility is hardly likely in view of the results with virgin females shown in Fig. 1. Moreover, the CI (on subsequent tests) for 24 selected males that courted active virgin females for 30 min without achieving copulation was 0.24 ± 0.03. It is worth noting that, in contrast to the results in Table 1, only one among 120 individual control males failed to court the test virgin and only 19 had a CI of 0.1 or lower.

The aftereffect of experience with mated females can be

Table 3. Effect of postconditioning rest period on courtship of amnesiac and wild-type males

Period with fertilized female, min	Time between training and CI tests, min	CI, mean ± SEM	
		Wild-type	Amnesiac
0	0	0.36 ± 0.05 (24)	0.26 ± 0.06 (24)
15	0	—	0.25 ± 0.06 (12)
30	0	—	0.14 ± 0.06 (12)
60	0	0.04 ± 0.02 (31)	0.11 ± 0.02 (64)
60	15	0.12 ± 0.05 (25)	0.18 ± 0.04 (28)
60	30	0.07 ± 0.04 (34)	0.29 ± 0.08 (10)
60	60	0.09 ± 0.03 (10)	0.19 ± 0.04 (34)
60	120	0.11 ± 0.06 (10)	0.31 ± 0.10 (10)
60	180	0.32 ± 0.04 (36)	0.24 ± 0.04 (24)

CI was determined, after the training and rest periods, by observing males with etherized virgins. The numbers of males in each test are in parentheses.

revealed by using test virgins other than those immobilized with ether. Thus, males that had been trained with mated females and then tested with immobilized *shi*^ts virgins at 27°C gave essentially the same results as those in Table 1. On the other hand, when trained males were subsequently tested with *active* virgin females, only pretreatments of 30 min or longer led to noticeable decreases in the CI and the variations from one trial to the next were relatively high.

The quality of male courtship towards a virgin is not modified in an obvious way as a result of prior courtship with a fertilized female. Although males that had previously courted fertilized females showed about a 3-fold reduction in the incidence of various courtship activities in comparison with controls (Table 2) the sequence of these activities—orientation, then wing extension and vibration, and finally abdominal curling and attempted copulation—and their execution was identical for every control and experimental male.

Courtship behavior of amnesiac, a memory-defective mutant

Amnesiac flies carry an X-linked mutation (3) that causes rapid decay of memory after training in the original system of olfactory learning (1). Wild-type flies learn to avoid specific odors when they are associated with an electric shock, more than half of their learned behavior is still present 1 hr later (1, 15), and some memory is detectable 6 hr after training (15). An amnesiac fly learns well in these experiments but learned performance decays to half its initial value in 15 min and is undetectable 60 min after training (3). As is the case with wild-type males (see above), *amn* males direct far less courtship toward immobile fertilized females (CI = 0.02 ± 0.01, n = 14) than toward immobile virgins (CI = 0.25 ± 0.06, n = 14), demonstrating that the mutant is not sensory deficient with respect to differences between these two kinds of females. The results in Table 3 show the amount of time after conditioning required for wild-type males or amnesiac males to again show a normal, vigorous, response to a virgin female. Results in the top four rows of the table demonstrate that courtship behavior in both strains can be negatively conditioned. To obtain the results shown in the last three rows of the table, males were paired with mated females for 60 min and then aspirated into individual "rest" vials with food, for periods of 15–180 min. Then the courtship index was determined in the standard way. When amnesiac males were given only a 15-min rest period after training with a mated female, their courtship activity was similar to that of naive (untrained) control amnesiac males (Fig. 2); in other words, no statistically significant effect of the prior experience with mated females remained (Student's t test, two-tailed, P >0.05). After longer rest periods the mutant males again showed no evidence of the prior experience. In contrast to these results, the CIs of wild-type males remained significantly depressed (P < 0.01) after rest periods of 15–120 min. (Table 3, Fig. 2). These tests

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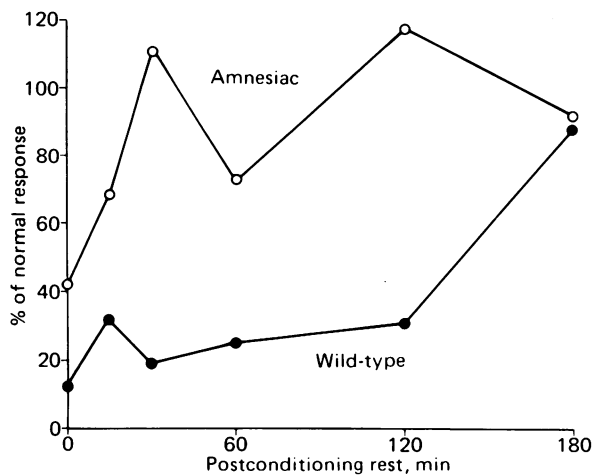Proc. Natl. Acad. Sci. USA 76 (1979)   3433

FIG. 2.   Effect of postconditioning rest period on male behavior with virgin females. Males were placed individually with mated females for 60 min. The males were then tested for courtship with immobilized virgin females either immediately or after the indicated periods of postconditioning rest. Males were either wild-type (●) or mutant amnesiac (○). For each genotype, the CIs for each point are expressed as a percentage of the courtship performance for males courting virgins without having had previous exposure to mated females (see Table 3).

involving postconditioning rests of amnesiac and wild-type males were done in two completely separate experiments, both of which gave basically the same results. Thus the initial and confirming data are pooled in Table 3 and Fig. 2.

DISCUSSION

As a consequence of a specific previous experience, the quantity of male courtship behavior is reduced in a predictable way. The reduced courtship response requires association with fertilized females as the previous experience. Moreover, the probability that a given male will entirely avoid the test female is related in a direct way to the duration of that prior exposure (Table 1).

What factors are involved in the male–female interactions in *Drosophila*, and how are they possibly related to the changes in male behavior that can be induced by a previous courtship experience? Females elicit males to courtship by a variety of cues. Specific visual cues from female actions are necessary for complete mating behavior in some *Drosophila* species such as *D. subobscura* (16). In *D. melanogaster* visual cues are not required, because blind males court well, mating readily occurs in the dark (reviewed in ref. 17), and immobile females provoke male courtship. On the other hand, chemical cues may be important for stimulation of *D. melanogaster* males. Olfactometric experiments (18, 19) suggest the involvement of volatile compounds in the courtship of this species, and female-specific volatile compounds have been detected by gas chromatography (20, ‡). Moreover, female-specific substances extracted from virgins stimulate male courtship while volatile compounds from males do not.‡ The putative chemical cues may be augmented by visual cues, because active females stimulate more male courtship than inactive females. Mated females elicit quantitatively less courtship than virgins, and copulation is very rare. Because blind males court mated females less well than they court virgins, the decrement in courtship associated with the fertilized females does not require that a male see overt rejection responses (e.g., ovipositor extrusion). In fact, no active repelling

‡ L. Tompkins, J. C. Hall, and L. M. Hall, unpublished data.

behavior at all is required for the decrement: mated females that cannot move elicit less courtship than immobilized virgins. What, then, is the function of extrusion of the mated female's ovipositor? The fact that the frequency of extrusion by mated females is positively correlated with the quantity of male courtship suggests that this behavior does not cause reduced levels of male courtship but instead serves a copulation-blocking function.

We suggest then that mated females are a source of both courtship-provoking and courtship-inhibiting chemical cues: there is a substantial amount of courtship elicited by such females, but it is significantly subnormal. Spieth's (5) observations of courtship led him to postulate that males receive "noxious" chemical information from mated females, and Tompkins *et al.*‡ have recently discovered a volatile substance unique to mated females. We infer that chemical cues that are sufficient to inhibit male courtship are associated with mated females, and that these cues play a significant role in the modification of male courtship behavior.

The experiment that most convincingly demonstrates the effects of experience on subsequent behavior is summarized in Table 1. If courtship-inhibiting chemical cues from the mated female are indeed responsible for diminished courtship with the subsequent virgin female, then a similar depression in male courtship might be brought about by prior experience with an immobilized mated female. This point can be tested by utilizing *shi*ts mated females immobilized by exposure to 27°C. (Males exposed for 20–30 min to males or females immobilized by ether show a general decrement in all behavior, probably due to the lingering presence of the anesthetic; it should be stressed that all CIs—e.g., those in Table 1—were determined by placing males in the presence of etherized virgins for only about 10 min, which leads to no noticeable defects in overall behavior.)

The effects on male behavior of prior experience with a mated female are most clearly revealed by subsequent tests of the male with an immobilized (virgin) female. This deserves comment. Because naive males court active females more vigorously than immobilized females, then males that had been previously paired with mated females may receive sufficient olfactory and optomotor stimuli from active females to push them over thresholds necessary for normal courtship. Males tested with immobile females may be less stimulated, and for this reason the effects of the previous experience can be brought to light.

Modification of male courtship behavior occurs as a specific consequence of an earlier experience with a mated female. The points discussed above suggest that the experience includes simultaneous courtship-provoking and courtship-inhibiting stimuli. Thus it may be that males associate the two stimuli and hence tend to avoid courtship upon presentation with a test virgin female. Alternatively, this result could be a manifestation of a more general debilitation caused by association with mated females, such that males merely have a diminished capacity to respond to females or court in a normal way; if so, the phenomenon would have no connection with central conditioning. Data on the courtship behavior of mutant amnesiac males are important in this regard. Amnesiac was isolated as a memory-deficient mutant in an associative learning task having nothing to do with courtship behavior. In addition, the capacity of amnesiac to distinguish odors appears to be normal (3). Because this single-gene mutation decreases the "refractory period" for courtship behavior, the notion that normal males are merely debilitated, habituated, or fatigued by exposure to mated females seems implausible; and the idea that appropriately conditioned males can learn and remember to avoid otherwise

attractive females gains support. It would be useful to study the effects of mated females on additional learning and memory mutant males (2, 3) and to analyze the involvement of these genes in other courtship situations in which the effects of a previous experience are suspected (21–23). Nevertheless, it already appears, from the experiments on the one mutant so far analyzed, that some components of courtship in *Drosophila* are modifiable by experience, the information for which may be processed centrally. Thus, it will be useful in the future to view reproductive behavior in this insect as not comprised of completely fixed action patterns.

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