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Identification of Stimuli that Mediate Experience-Dependent Modification of Homosexual Courtship in *Drosophila melanogaster*

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A naive, sexually mature *D. melanogaster* male tested with a young, sexually immature male will perform vigorous courtship, but the mature male will perform much less courtship if he is subsequently tested with a second young male. This phenomenon is called experience-dependent courtship modification (EDCM). We have shown that exposure to either or both of the two courtship-stimulating pheromones that immature males synthesize is sufficient to induce EDCM.

KEY WORDS: *Drosophila melanogaster;* sex pheromone; courtship; homosexuality; behavioral plasticity.

INTRODUCTION

Immature *Drosophila melanogaster* males elicit courtship from naive, sexually mature males that is quantitatively and qualitatively indistinguishable from the courtship that immature or sexually mature virgin females stimulate. Typically, a mature male will orient to a young male and follow the "sex object," tap the male's abdomen, extend and vibrate a wing to produce a courtship song, lick the male's genitalia, and attempt to copulate (reviewed by Tompkins, 1989). With regard to the evolutionary significance of this behavior, males that elicit courtship when they are young mate more quickly with females when they become sexually mature (McRobert and Tompkins, 1988), suggesting that elicitation of homosexual courtship benefits the sex object. However, performance of homosexual courtship does not confer any obvious reproductive advantage to mature males (McRobert and Tompkins, 1988). Thus, since males that perform homosexual courtship presumably waste time and energy and expose themselves to predators, it is not surprising that a mechanism has evolved to reduce its occurrence. This phenomenon, called experience-dependent courtship modification (EDCM) (Tompkins, 1989; see Gailey *et al.*, 1982), is most easily demonstrated by observing a mature male with two immature males, presented sequentially. In this situation, the mature male performs much less courtship in response to the second young male (Gailey *et al.*, 1982).

With regard to the stimuli to which mature males are exposed when they perform homosexual courtship in response to immature males, it is clear that young males provide a visual stimulus to courting males, since mature males tested with young males in a dim red light, in which the males cannot see (see Bixler *et al.*, 1992), perform less courtship than males tested in normal laboratory illumination (Tompkins, 1984). In addition, young males synthesize courtship-stimulating sex pheromones. Evidence for this is provided by the observation that hydrocarbons extracted from young (1- to 8-h-old) males, unlike extracts from mature males, stimulate mature males to court each other (e.g., Tompkins *et al.*, 1980). More recently, two of the cuticular hydrocarbons that immature males synthesize, (*Z*)-11-tritriacontene and (*Z*)-13-tritriacontene, have been identified as aphrodisiac pheromones (Schaner *et al.*, 1989). Finally, immature males probably begin

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to synthesize inhibitory pheromones when they are a few hours old, since the courtship that they elicit starts to decline when the young males are approximately 3 h old (Curcillo and Tompkins, 1987). With regard to the identity of these antiaphrodisiacs, sexually mature males synthesize two compounds, octadecenyl acetate (*cis*-vaccenyl acetate) and Z-7-tricosene, which have antiaphrodisiac activity in bioassays (Jallon *et al.,* 1981; Scott, 1986). The times at which males begin to synthesize these two compounds have not yet been determined, although it is known that only traces of octadecenyl acetate can be extracted from newly eclosed males (Butterworth, 1969).

Several lines of evidence suggested that exposure to the aphrodisiac pheromones associated with immature males might mediate experience-dependent courtship modification. A mature male that is confined with another mature male, then tested with a young male, will court the young male as vigorously as he would if he were sexually naive (Gailey *et al.,* 1982). Since mature males do not synthesize biologically relevant quantities of courtship-stimulating pheromones (Antony *et al.,* 1985; but see Tompkins and McRobert, 1989), this observation suggests that exposure of a mature male to the inhibitory pheromones associated with males is not sufficient to induce EDCM. Moreover, a mature male that has been confined in an observation chamber that was previously occupied by young males will perform less homosexual courtship than a mature male that has been isolated in a clean chamber (Gailey *et al.,* 1982), which implies that exposure to residual stimuli associated with immature males is sufficient for EDCM. On the basis of these observations, Gailey *et al.* (1982) hypothesized that experience-dependent modification of homosexual courtship occurs because mature males adapt or become habituated to the aphrodisiac pheromones that immature males synthesize. However, since the immature males used in Gailey and co-workers' (1982) experiments were 1 to 6 h old, it is likely that some or all of them had also begun to synthesize inhibitory pheromones. Moreover, in addition to the two aphrodisiac pheromones, immature males synthesize several cuticular hydrocarbons that mature flies do not make (Pechine *et al.,* 1988), any or all of which could mediate EDCM. Thus, Gailey and co-workers' (1982) experiments do not prove that exposure to the courtship-stimulating pheromones that immature males synthesize is sufficient for EDCM.

Accordingly, to test the hypothesis rigorously, we attempted to induce EDCM by exposing mature wild-type and genetically blind males to male flies of various ages and immature females, which are similar to immature males in that they synthesize long-chain cuticular hydrocarbons that mature flies do not make (Pechine *et al.,* 1988). In addition, we observed wild-type males with residual stimuli associated with males. Since the results of these experiments suggested that exposure to hydrocarbons associated with very young males, which do not synthesize inhibitory pheromones, was sufficient for EDCM, we then observed mature males that had been confined with the courtship-stimulating pheromones. Although preliminary results suggested that these hydrocarbons had no effect on mature males' courtship (Tompkins, 1989), the experiments described in this report, in which we tested a wide range of hydrocarbon concentrations, indicate that exposure to either or both of the aphrodisiac pheromones that immature males synthesize is necessary and sufficient for experience-dependent modification of homosexual courtship.

MATERIALS AND METHODS

Stocks: The wild-type Canton-S (CS) stock used in these experiments was derived from a single male–female pair in 1976 and has since been maintained in mass culture. A *glass*^{3} stock was obtained from the Mid-America Drosophila Stock Center in Bowling Green, Ohio. Flies that express the *glass*^{3} mutation lack functional photoreceptors and are thus totally blind (Meyerowitz and Kankel, 1978).

Both stocks were maintained at 25°C in a 12:12 light:dark cycle, with lights on at 8 AM. Stocks were maintained on a standard cornmeal–corn syrup medium.

Courtship-Stimulating Pheromones. (*Z*)-11-tritriacontene and (*Z*)-13-tritriacontene, synthesized as described by Schaner *et al.* (1989), were provided by Dr. Larry Jackson (Montana State University). A 160 μg/ml stock solution of (*Z*)-11-tritriacontene and a 240 μg/ml stock solution of (*Z*)-13-tritriacontene were prepared by dissolving each of the hydrocarbons in analytical-grade hexane. Each stock solution was serially diluted 1:1 with hexane to yield a nine-member dilution series. Since 16.0 ng of (Z)-11-tritriacontene and 24.0 ng of (Z)-13-tritriacontene can be recovered from the cuticle of an immature male (Schaner *et al.,* 1989), the stock solutions of each hydrocarbon were 10 fly-equiva-

lents/μl, while the members of the dilution series were 5, 2.5, 1.25, 0.63, 0.32, 0.16, 0.08, 0.04, and 0.02 fly-equivalents/μl.

Behavioral Assays. All flies were collected as virgins under CO_2 anesthesia. CS and *glass*[3] males that were to be tested when they were sexually mature were collected within 2 h of eclosion, then stored individually in vials. These flies were tested when they were 4 days old. Three- to five-hour-old CS males and females ("young males" and "young females") were collected within 1 h of eclosion, then aged for 3–4 h in vials. CS males that were less than 2 h old ("very young males") were collected within 30 min of eclosion, then aged for 30–60 min. All experiments were conducted at 22 ± 1°C during the flies' subjective day.

In the first series of experiments, a mature CS or *glass*[3] "test male" was aspirated without anesthesia to a small cylindrical observation chamber (volume, ca. 0.2 cm^3) with either no fly, a CS male, or a CS female. After 30 min, the test male was aspirated into a clean chamber with a young CS male and observed for 10 min under 7× magnification. A courtship index (CI), the percentage of time that the test male spent performing any of the courtship behaviors, was calculated for the 10-min observation period.

In the second series of experiments, three CS males were aspirated without anesthesia into a larger cylindrical observation chamber (volume, ca. 0.4 cm^3) whose bottom and sides were lined with a glass microfiber filter disk (Whatman GF/C) that had been moistened with distilled water. After 30 min, the males were removed, and a mature CS test male was aspirated into the chamber. Ten minutes later, the test male was transferred to a small chamber with a young CS male, and the test male's CI was determined as described above.

In the third series of experiments, 2 μl of one of the *(Z)*-11-tritriacontene solutions and 2 μl of one of the *(Z)*-13-tritriacontene solutions were applied to a glass microfiber filter disk. The disk was air-dried for 1–2 min to allow the solvent to evaporate, then pressed into the bottom and sides of a 0.4-cm^3 observation chamber. A mature CS test male was then aspirated into the chamber. Ten minutes later, the test male was transferred to a 0.2-cm^3 observation chamber with a young CS male, and the mature male's CI was determined as described above.

The fourth series of experiments was identical to the third except that 4 μl of *(Z)*-11-tritriacontene (2.5 fly-equivalents/μl) or 4 μl of *(Z)*-13-tritriacontene (2.5 fly-equivalents/μl) was applied to a glass microfiber filter.

Statistical Analysis. In all experiments, the significance of differences between means was determined by calculating t (two-tailed) for arcsin-transformed courtship indices, using Statgraphics statistical software for most of the calculations.

RESULTS

Effects of Confinement with Male and Female Flies. The results of the first series of experiments are presented in Table I. In experiment 1, mature CS males were isolated in observation chambers for 30 min, then transferred to chambers with young males. The mature males' courtship indices were high, confirming previous observations that mature, sexually naive males court young males vigorously (e.g., Tompkins *et al.,* 1980). In experiment 2, mature CS males were confined with young CS males for 30 min, then tested with young CS males. The test males performed significantly less courtship than isolated males (cf. experiments 1 and 2), indicating that experience-dependent courtship

Table I. Homosexual Courtship Performed by Mature Males that Have Been Confined with Young Females and with Males of Various Ages[a]

Expt	Test male	Stimulus	*CI*
1	CS	None	88 ± 3
2	CS	Young CS male	40 ± 6*
3	CS	Mature CS male	77 ± 5**
4	CS	Very young CS male	54 ± 7*
5	CS	Young CS female	34 ± 5*
6	*glass*	None	41 ± 5
7	*glass*	Young CS male	26 ± 3***
8	*glass*	Very young CS male	29 ± 6***

[a] Mature (4-day-old) CS or *glass*[3] males were isolated in observation chambers or confined with mature (4-day-old), young (3- to 5-h-old) or very young (<2-h-old) CS males or females, then tested with young (3- to 5-h-old) CS males as described in the text. CI, courtship index (±SE). $N = 20$ for all experiments.

* Significantly different from CS controls (experiment 1); $p < 0.01$.

** Not significantly different from CS controls (experiment 1); $p > 0.05$.

*** Significantly different from *glass* controls (experiment 6); $p < 0.01$.

modification occurs under our experimental conditions.

In experiment 3, two mature CS males were confined in a chamber for 30 min, after which time one of the mature males was tested with a young CS male. The test males' CIs were not significantly different from those of isolated males (cf. experiments 1 and 3). In experiment 4, mature CS males were confined with very young (<2-h-old) CS males for 30 min, then tested with young CS males. The test males' courtship indices were significantly different from those of naive males (cf. experiments 1 and 4). These results confirm Gailey and coworkers' (1982) observation that contact with mature males does not induce EDCM. In addition, they demonstrate that confinement with very young males is sufficient for courtship modification.

In experiment 5, mature CS males were confined with young CS females for 30 min, after which time the males were tested with young CS males. The test males performed significantly less courtship than isolated males (cf. experiments 1 and 5). Since immature males and females synthesize the same cuticular hydrocarbons (Pechine *et al.,* 1988), this observation suggests that exposure to chemical stimuli associated with young flies, rather than perception of sex-specific visual stimuli associated with immature males, mediates EDCM.

In experiments 6 through 8, mature *glass*[3] males were isolated in observation chambers, confined with young CS males, or confined with very young CS males for 30 min, then tested with young CS males. Confinement with young or very young males significantly reduced the blind test males' courtship indices (cf. experiments 6 and 7 and experiments 6 and 8). These results confirm that perception of the attractive visual stimuli associated with immature males is not necessary for EDCM.

Effects of Confinement in Chambers that Had Been Previously Occupied by Male Flies. The results of the second series of experiments are presented in Table II. In experiment 1, CS test males were isolated in large chambers, then tested with young CS males. In experiments 2 and 3, the test males were isolated in large chambers that had previously been occupied by three young or very young CS males, respectively. Test males that were isolated in chambers that had previously been occupied by young or very young males performed significantly less courtship than test males that had been isolated in clean chambers (cf. experiments 1 and 2 and experiments 1 and 3). These results confirm Gailey and co-workers' (1982) observation that EDCM can be induced by residual stimuli, presumably chemicals, associated with young males. In addition, they demonstrate that exposure to residual stimuli associated with very young males is sufficient for EDCM.

Table II. Homosexual Courtship Performed by Mature Males that Have Been Confined in Chambers Previously Occupied by Males[a]

Expt	Test male	Previous occupants	*CI*
1	CS	None	81 ± 2*,**
2	CS	Young males	55 ± 5*
3	CS	Very young males	44 ± 5**

[a] Three young (3- to 5-h-old) or very young (<2-h-old) CS males were confined in observation chambers for 30 min, then removed. Mature (4-day-old) CS males were isolated in the same chambers, then tested in clean chambers with young (3- to 5-h-old) CS males as described in the text. CI, courtship index (±SE). $N = 20$ for all experiments.
* Significantly different ($p < 0.001$).
** Significantly different ($p < 0.001$).

Effects of Exposure to Aphrodisiac Pheromones. The results of the third and fourth series of experiments are presented in Fig. 1 and in the text. Control males (CS) were confined for 10 min in chambers lined with glass microfiber filter disks to which hexane had been applied, then tested with young CS males. Experimental males (CS) were confined in chambers lined with disks to which various concentrations of *(Z)*-11-tritriacontene, *(Z)*-13-tritriacontene, or both hydrocarbons had been applied, then tested with young CS males. Although most concentrations of hydrocarbons had no significant effect on the mature males' courtship, CS males exposed to 2.5 fly-equivalents of (Z)-tritriacontene and 2.5 fly-equivalents of (Z)-13-tritriacontene or 5 fly-equivalents of both hydrocarbons (concentrations 7 and 8 in Fig. 1) performed significantly less courtship than controls. CS males exposed to 10 fly-equivalents of *(Z)*-11-tritriacontene also performed significantly less courtship than controls (CI for experimental males = 33 ± 10, $N = 6$; $p < 0.01$), as did experimental males exposed to 10 fly-equivalents of *(Z)*-13-tritriacontene (CI = 36 ± 2, $N = 6$; $p < 0.01$). These observations confirmed that exposure to either or both of the cuticular hydrocarbons synthesized by immature males that have been identified as aphrodisiac pheromones is sufficient to induce EDCM.

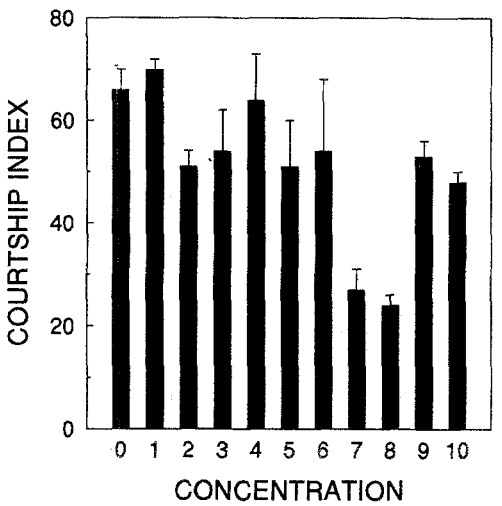

Fig. 1. Homosexual courtship performed by mature males that have been exposed to the courtship-stimulating hydro-carbons that immature males synthesize. Mature (4-day-old) CS males were confined in observation chambers lined with filter disks that had been impregnated with 4 μl of hexane (concentration 0) or 2 μl of (*Z*)-11-tritriacontene plus 2 μl of (*Z*)-13-triacontene. Fly-equivalents of hydrocarbon in each solution were 0.04 (concentration 1), 0.08 (concentration 2), 0.16 (concentration 3), 0.32 (concentration 4), 0.63 (concentration 5), 1.25 (concentration 6), 2.5 (concentration 7), 5 (concentration 8), 10 (concentration 9), and 20 (concentration 10). After 10 min, the males were tested with young (3- to 5-h-old) CS males as described in the text. CI, courtship index (± SE). $N = 6$ for each concentration. Courtship indices of males exposed to concentrations 7 and 8 were significantly different from those of males exposed to concentration 0 ($p < 0.02$ for both comparisons). Courtship indices of mles exposed to the other concentrations were not significantly different from those of males exposed to concentration 0 ($p > 0.05$ for all comparisons).

DISCUSSION

In the experiments described in this report, we addressed the question of whether exposure to the courtship-stimulating pheromones synthesized by immature males was necessary and sufficient for experience-dependent courtship modification of homosexual courtship. We observed that wild-type males that court immature females subsequently exhibit EDCM, as do genetically blind males that court immature males, suggesting that perception of visual stimuli associated with immature males is not necessary for courtship modification. Wild-type males confined in chambers that have previously been occupied by immature males perform less homosexual courtship than controls, implying that contact with chemical stimuli associated with immature males is sufficient for EDCM.

With regard to the nature of the chemical stimuli that effect EDCM, we have confirmed Gailey and co-workers' (1982) observation that exposure to mature males does not induce EDCM. We have also shown that contact with very young males or their residual compounds induces EDCM in wild-type and genetically blind males. As previously noted, mature males do not synthesize courtship-stimulating pheromones, but they do synthesize hydrocarbons that inhibit courtship, while males that are less than 3 h old synthesize courtship-stimulating pheromones but do not synthesize hydrocarbons that inhibit courtship. Accordingly, these observations suggest that exposure to the courtship-stimulating pheromones and/or other hydrocarbons that very young males synthesize is necessary and sufficient for experience-dependent modification of homosexual courtship, while exposure to the inhibitory pheromones that males that are more than 3 h old synthesize is neither necessary nor sufficient for EDCM.

Finally, the results of the experiments in which mature males were confined with (*Z*)-11-tritriacontene, (*Z*)-13-tritriacontene, or both hydrocarbons indicate that exposure to either or both of the courtship-stimulating pheromones that immature males synthesize is sufficient to induce experience-dependent courtship modification. However, in the experiments in which males were exposed to both hydrocarbons, only those males that were exposed to 2.5 or 5 fly-equivalents of each compound (concentrations 7 and 8 in Fig. 1) exhibited EDCM. Since males that court one immature male perform less homosexual courtship afterward (e.g., Gailey *et al.*, 1982; this report), the question arises as to why males that were exposed to 1.25 fly-equivalents of each pheromone (concentration 6 in Fig. 1) did not exhibit EDCM. Although (*Z*)-11-tritriacontene and (*Z*)-13-tritriacontene are the only hydrocarbons synthesized by immature males that stimulate mature males to court each other (Schaner *et al.*, 1989), it is possible that other hydrocarbons associated with immature males' cuticles act in conjunction with the two aphrodisiac pheromones to induce EDCM. If this were the case, 1.25 fly-equivalents of each of the two aphrodisiac pheromones, in the absence of the other cuticular hydrocarbons that immature males synthesize, might not be sufficient to induce EDCM. Alternatively, (*Z*)-11-tritriacontene and (*Z*)-13-tritriacontene may be the only cuticular hydrocarbons that induce EDCM, but these compounds, when present on the cuticles

of immature males, may be more effective stimuli than equivalent quantities of the same pheromones on filter disks. Evidence in support of the latter alternative is provided by observations of mature males confined with immature males, which courted the sex objects vigorously. While courting, the mature males repeatedly tapped the immature males' abdomens with the tarsal segments of their forelegs; thus, the tarsal taste receptors that would be expected to respond physiologically to long-chain hydrocarbons were often in contact with the sex objects' cuticles. In contrast, mature males that were isolated in chambers with filter disks spent much of their time on the top surfaces of the chambers, where their tarsal taste receptors were not in contact with the hydrocarbon-impregnated filter disks that lined the bottom and sides of the chambers.

Another question raised by the experiments in which males were exposed to pheromones on filter disks concerns the inability of high concentrations of the hydrocarbons to induce experience-dependent courtship modification. Gailey *et al.* (1982) suggested that EDCM occurs because mature males habituate or adapt to the courtship-stimulating pheromones that immature males synthesize. If this were the only long-term effect of exposure to the pheromones, males that were exposed to 10 and 20 fly-equivalents of each hydrocarbon (concentrations 9 and 10, respectively, in Fig. 1) would exhibit EDCM; however, they do not. It is possible that high concentrations of either or both of the cuticular hydrocarbons are repellent or irritating. If this were the case, males that touched a filter disk that was impregnated with the hydrocarbon soon after the confinement period began might spend most of the remaining time on the top of the chamber, thus reducing their contact with the pheromone to the point where adaptation or habituation did not occur. Alternatively, exposure to very high concentrations of either or both of the courtship-stimulating pheromones might induce sensitization, which would counteract the behavioral effects of adaptation or habituation that repeated contact with these relatively high concentrations of these compounds induces. Although sensitization to sex pheromones has not previously been demonstrated in *Drosophila* (see Tompkins *et al.*, 1980), high concentrations of sucrose induce a central excitatory state in *D. melanogaster* (Duerr and Quinn, 1982; Vargo and Hirsch, 1982), providing evidence for the occurrence of sensitization in response to taste stimuli in this species.

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