Genetic elimination of known pheromones reveals the fundamental chemical bases of mating and isolation in *Drosophila*

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ABSTRACT Overexpression of the UAS-tra transgene in *Drosophila melanogaster* females led to the complete elimination of their cuticular pheromones. According to current models of *Drosophila* behavior, these flies should induce no courtship. In fact, they are still attractive to conspecific males. Three classes of stimuli are shown to induce courtship, with different effects on male behavior: (i) known pheromones produced by control females, (ii) stimuli produced by living control and transgenic flies, and (iii) as-yet-undetermined pheromones present on both control and transgenic flies. Only the latter class of pheromones are required for mating. They appear to represent a layer of ancestral attractive substances present in *D. melanogaster* and its sibling species; known cuticular pheromones modulate this attractiveness positively or negatively. The absence of inhibitory pheromones leads to high levels of interspecific mating, suggesting an important role for these cuticular hydrocarbons in isolation between species.

Sexual behavior in *Drosophila melanogaster* has been studied for over 80 years (1). Before mating, the male orients to the female, generally touching her before beginning to follow her while vibrating his wing; he then licks the female’s abdomen and attempts to copulate with her. This series of behaviors has been the subject of a wide range of genetic and neurobiological studies (2).

Over the last 2 decades, two female-specific cuticular hydrocarbons [7,11-heptacosadiene (7,11-HD) and 7,11-nonacosadiene] have been shown to induce one of the most obvious signs of male courtship, wing vibration (3–5). As yet, no decisive evidence exists for the involvement of cuticular hydrocarbons in the later stages of courtship, although there are strong indications that this is the case (6). The male-predominant cuticular hydrocarbon 7-tricosene (7-T) tends to inhibit the excitation of conspecific males (6, 7). Genes involved in the biosynthesis of these substances have been studied (8, 9), and a candidate has been sequenced (10). The view that cuticular hydrocarbons are critical elements in courtship has a high predictive power, especially with regard to patterns of interspecific courtship, but it cannot explain intra- or interspecific mating patterns (11, 12), which are thought to be a result of species-specific behaviors and auditory signals (8).

We have previously expressed a transgene (UAS-tra) containing the female-spliced form of the sex-determination gene transformer in male *D. melanogaster*. This procedure made it possible to feminize both the male’s brain and his cuticular profile and thus to investigate the perception and production of chemical messages in this species (13, 14). In this study, we overexpressed the UAS-tra transgene in female *D. melanogaster*, with the result that virtually all of their cuticular hydrocarbons, including all known pheromones, were completely eliminated. Surprisingly, these females were still attractive to both homo- and heterospecific males. Experiments showed that a hitherto-unstudied layer of substances acts as common copulation-inducing pheromones in *D. melanogaster* and its related species. These results lead us to revise our understanding of courtship and its evolution in *Drosophila*.

MATERIALS AND METHODS

*Drosophila* Stocks and Strains. *D. melanogaster*. Canton-S (Cs) males and C(1)DX females were used as control flies because of their clear behavioral responses (6, 13). *hs70-GAL4 × UAS-tra* females result from a cross between homozygous *UAS-tra* females, carrying the *UAS-tra* feminizing transgene (6, 13, 14), and homozygous p[F GAL4-Hsp70.PB] males (15). The *hs70-GAL4* transgene makes it possible to produce the GAL4 protein ubiquitously after heat shock, which in turn activates the *UAS-tra* transgene (14).

*Drosophila simulans*. The Seychelles strain has been maintained in our lab for more than 15 years; the c167.4 strain was provided by J. Roote (16). The *Drosophila sechellia*. The 228 strain is an isofemale strain that has been maintained for over 10 years in the laboratory (a gift of F. Lemeunier, Centre National de la Recherche Scientifique, Gif-sur-Yvette, France); the “Robertson” strain was provided by J. A. Coyne (University of Chicago).

*Drosophila mauritiana*. The 163.1 strain has been maintained for over 10 years in the laboratory (a gift of F. Lemeunier); the “synthetic” strain was provided by J. A. Coyne (17).

Overexpression of UAS-tra. *hs70-GAL4 × UAS-tra* females were collected under CO₂ anesthesia less than 1 hour after eclosion. Six-hour-old female flies were placed in groups of 10 in 100 × 12 mm polypropylene vials and transferred into a 37°C water bath for 1 hour. After heat shock (hs), these hs-tra flies were replaced in vials with medium until they were 4 days old. Non-heat-shocked *hs70-GAL4 × UAS-tra* flies were used as controls (non-hs-tra).

Detection of Hydrocarbons. GC analysis was performed on 4-day-old flies either singly or in groups of 30 flies according to established procedures (3, 9). Single flies were washed in a tube containing 50 µl of hexane. The absolute quantities and the relative levels (as percentages of the total amount) of all detectable hydrocarbons were noted for each fly. Identification of peaks was carried out by using MS on MD800 (Thermoquest) mass spectrometer coupled with a 8060 Fisons GC equipped with a 25-meter column. The MS was equipped with the publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: 7-T, 7-tricosene; 7,11-HD, 7,11-heptacosadiene; hs, heat shock; CI, courtship index.

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MASSLAB 1.2.7 software to scan compounds with a molar mass ranging from 40 to 650 g/mol.

Transfer of Hydrocarbons Between Dead Flies. Pools of 10 females of each genotype were washed for 1 min in 200 μl of hexane. This pooled extract was air-dried. Each female to be tested was flash-killed in liquid nitrogen, washed in hexane, and covered with 5 μl of the pooled extract, which had been redissolved in 50 μl of hexane. This is the equivalent to the extract of a single female (8).

Transfer of Hydrocarbons Between Living Flies. Twenty intact virgin hs-tra females were confined in a 4-ml space in a tube with food together with 100 virgin “donor” females [D. simulans Seychelles or D. melanogaster C(1)DX] for 24 hours. After crowding, 60–100% of the donor females’ cuticular hydrocarbon profile was transferred. No qualitative differences were observed between the donor profile and that of the receiver flies following crowding. Unlike previous interspecific experiments (12), transfers onto hs-tra females produced a “pure” hydrocarbon profile rather than a mixture of the hydrocarbons of two species. The results from these experiments can thus be interpreted unambiguously as being due to the presence of the donor’s hydrocarbon profile. Typical transferred amounts of known pheromones were 800-1000 ng of 7-T (from D. simulans donors) and 300–400 ng of 7,11-HD (from D. melanogaster donors).

Measures of Courtship and Mating. Courtship tests were performed on 4- to 5-day old flies at 25 ± 0.5°C. After eclosion, subject males were isolated in a food vial, while object females were kept in groups of 10. Subject males were individually aspirated into an observation chamber consisting of a watch glass (3.5 cm²) on a glass plate. After 10 minutes, the object female was aspirated into the chamber. A courtship index (CI) was established for each pair. The CI is the proportion of the observation period spent actively courting (i.e., engaged in vibration, licking, and attempting copulation). In intraspecific studies of D. melanogaster, all females were decapitated immediately before study to prevent locomotor activity affecting the data (6, 13). Each pair of flies was observed for 10 minutes. For interspecific studies in which mating was to be observed, females were left intact and were observed in single pairs for 60 minutes.

RESULTS

Genetic Elimination of Known Pheromones. Overexpression of UAS-tra after a heat shock in early imaginal life reduced the amount of cuticular hydrocarbons by ~93% and rendered both known and suspected sex pheromones (6) undetectable in female flies (Fig. 1). Only five substances could be identified on the cuticle of hs-tra females, none of which has previously been suggested as having a pheromonal role.

GC/MS studies of pools of 30 flies confirmed that no known pheromones can be detected on the cuticle of hs-tra females (data not shown). These substances may be present in very small quantities below the detection threshold of our GC (~2 ng). The maximum amount of each known pheromone that could go undetected would thus be <0.07 ng per fly. D. melanogaster male response thresholds for 7,11-HD have been estimated at 200 ng for a fractionated substance (5) and at 20 ng when present in a natural blend containing a number of other substances, including those found on hs-tra females (6). These thresholds are thus >2–3 orders of magnitude.

![Heat-shock vs. No heat-shock](image-url)

**FIG. 1.** Representative mirrored gas chromatograms of hexane extracts of two individual female flies resulting from the hsp70-GAL4 × UAS-tra cross. The Upper signal comes from a fly that was subjected to a 37°C heat shock for 60 minutes at 6 hours old, the Lower signal from a fly that was not heat shocked. The profile of the non-heat-shocked fly is qualitatively similar to those of most wild-type D. melanogaster females (30). The two chromatograms are to the same scale: they were aligned and calibrated by using an added standard of hexacosane on each chromatogram (removed for the sake of clarity, and indicated by two vertical lines). Peaks marked with * have been suggested to have a pheromonal function (3–7). Peaks are labeled with a number and were identified by comigration with the results of mass spectrum studies on pools of 10 flies. Peak 1, 7-T; peak 2, n-tricosane; peak 3, 9-pentacosene; peak 4, 7-pentacosene; peak 5, n-pentacosane; peak 6, 7,11-HD; peak 7, 2-methyl hexacosane; peak 8, 7-heptacosene; peak 9, n-heptacosane; peak 10, 7,11-nonacosadiene; peak 11, 2-methyl octacosane. The five substances found on both hs-tra and non-hs-tra females were present in far higher mean levels on non-heat-shocked flies: n-pentacosane, 14 ± 3 ng for hs-tra, (128 ± 5 ng for non-hs tra); 2-methyl hexacosane, 21 ± 3 (262 ± 12); 7-heptacosene, 14 ± 6 (91 ± 12); n-heptacosane, 16 ± 2 (68 ± 3); 2-methyl octacosane, 70 ± 6 (93 ± 3). In all other respects (morphology, locomotor activity), hs-tra flies were normal. Viability at 24 hours was normal, but at 4 days old, hs-tra females showed 40% mortality as opposed to 2% for non-hs-tra flies (data not shown). This result may indicate the importance of cuticular hydrocarbons in protecting flies from dessication (31).
magnitude greater than the maximum amount of known pheromone that could go undetected in our system.

**Intraspecific Male Responses to hs-tra Females.** According to the current model of courtship in *D. melanogaster*, these “pheromone-free” hs-tra females should induce no courtship from conspecific males. Surprisingly, 53 of 55 control males courted these females, showing the full range of courtship—for example, attempted copulation (the highest level of courtship) was observed in half of the courtships with these decapitated hs-tra females. We conclude that known female cuticular pheromones are not necessary for the higher stages of courtship in *D. melanogaster* because a male can perform the full range of courtship behavior in the absence of these substances. However, male courtship of these hs-tra females as measured by the CI was significantly weaker [less than 2/3 of that for control females (Fig. 2; $t = 4.34$, df = 88, $P < 0.0001$)], indicating that known pheromones do play a behavioral role.

**Dissecting Intraspecific Male Responses to “Pheromone-Free” Females.** A series of experiments was carried out to reveal the nature of the courtship-inducing stimuli remaining on hs-tra females. Killing females with liquid nitrogen did not change cuticular hydrocarbon profiles but did reduce both the CI and the frequency of attempted copulation by over half in both control and experimental flies (Fig. 2). This shows that an important part of courtship is induced by unknown signals produced by the immobile living fly. As expected (3), washing dead flies in hexane removed all cuticular hydrocarbons and also abolished courtship (Fig. 2). To demonstrate the role of hexane-soluble substances, dead washed flies were covered with their own hexane extract (8). Washed and covered hs-tra females induced no courtship (CI = 1.9 ± 0.4), whereas covering washed control females in their own extract induced wing vibration (CI = 11.2 ± 1.6; Fig. 2), but no attempted copulation. Reciprocal covering with extracts from hs-tra and control females (i.e., covering washed hs-tra females with control extract and vice versa) was carried out (data not shown). CI levels were only affected by the origin of the extract ($F_{1,104} = 79.26$, $P < 0.0001$), with no significant effect of the receiver fly and no interaction.

A striking result is that the difference in CI values between the two types of flies is virtually the same under all three conditions in which courtship was observed. This was confirmed by an ANOVA on the data in Fig. 2. As well as a significant effect of treatment (alive, killed, covered) ($F_{0,217} = 110.36$, $P < 0.0001$) in which all three levels of treatment were significantly different from each other, the overall difference between heat-shock and non-heat-shock flies was highly significant ($F_{1,217} = 30.12$, $P < 0.0001$) and was constant over treatments (interaction $F_{2,217} = 1.33$, not significant). This constant difference, which corresponds to 10–20 index points, can be imputed to the action of known pheromones, which are absent in hs-tra females.

We conclude that hs-tra females are attractive because, like control flies, they carry unidentified cuticular pheromones that induce all stages of courtship. Whereas the stimulatory action of known contact pheromones on male wing vibration can be restored by covering control females in their own extract, the action of the unidentified pheromones that induce attempted copulation is no longer present following washing and covering with extracts of hs-tra female. This result will be discussed further.

**Interspecific Male Responses to hs-tra Females.** Four of the five hydrocarbons detected on the cuticle of hs-tra females are also found on males and females of all four species in the *D. melanogaster* complex, i.e., the two island species *D. melanogaster* and *D. simulans* and the two cosmopolitan species *D. sechellia* and *D. mauritiana* (18).

To see whether one or more of these substances play a common excitatory pheromonal role, males of all four species were tested with intact *D. melanogaster* hs-tra females. Unlike in the previous experiment with headless females (Fig. 2), *D. melanogaster* males showed no reduction of courtship with intact hs-tra females (Fig. 3). This is presumably because the other stimuli provided by these mobile flies were sufficiently exciting to make up for the absence of known pheromones. All three heterospecific males showed relatively high levels of courtship with hs-tra females (Fig. 3), suggesting that the copulation-inducing pheromones produced by hs-tra *D. melanogaster* females are also attractive to males of the other species. No significant intraspecific variation was observed when these tests were carried out with males from another *D. sechellia* strain (Robertson), from the *D. mauritiana* synthetic strain, and from the *D. simulans* c167.4 strain (data not shown). The attraction shown by heterospecific males toward hs-tra females in single pairs was so intense that mating took place in all three interspecific crosses (Table 1). *D. mauritiana* males showed unprecedentedly high levels of interspecific mating when crossed with hs-tra females; 28% of pairs mated within 60 minutes (Table 1). Interspecific insemination levels of 3% after 48 hours in mass mating conditions is a more typical figure (19).

**Dissecting Interspecific Male Responses.** The predominant female cuticular hydrocarbon in *D. melanogaster* and *D. sechellia* is 7,11-HD, whereas in *D. simulans* and *D. mauritiana*, it is 7-T (18, 20).
**DISCUSSION**

These data substantially enrich our model of *Drosophila* courtship and indicate that cuticular hydrocarbons may play a fundamental role in isolation between species.

**An Enriched Model of Courtship.** We suggest that the four species studied here (*D. melanogaster*, *D. sechellia, D. simulans*, and *D. mauritiana*) share a layer of ancestral cuticular substances that are able to induce all stages of courtship necessary for copulation. The action of these "ur-pheromones" can be modulated by the presence of known cuticular pheromones. This result implies that previous studies of chemical communication in *D. melanogaster* and its related species (3–7, 11, 12, 14, 20) have focused on modulatory pheromones rather than on the key stimulatory pheromones.

Our data suggest that in *D. melanogaster* males, 7-T acts either as a mask or as an inhibitor of these *ur*-pheromones and thus tends to inhibit intraspecific male–male courtship. In *D. melanogaster* females, the principal known cuticular pheromones (7,11-HD and 7,11-nonacosadiene merely reinforce the

which showed similar CIs with intact and decapitated females (data not shown). In *D. melanogaster* and *D. sechellia*, the main female cuticular hydrocarbon is 7,11-HD; in *D. simulans* and *D. mauritiana*, it is 7-T. GC analysis revealed the following levels of cuticular hydrocarbons: 7,11 HD = 30.6% ± 1.4 for *D. melanogaster* control and 25.4% ± 0.7 for *D. sechellia*, 7-T = 61.2% ± 3.1 for *D. simulans* and 58.3% ± 0.8 for *D. mauritiana*. These results are close to those previously reported (18).
conspecific wing vibration induced by the ur-pheromones described here.

The unknown stimuli produced by living, immobilized flies account for \( \approx 30\% \) of courtship induced by control and hs-tra flies and apparently reinforce the response induced by the copulation-inducing pheromones and other known pheromones. The signal(s) involved may be visual (e.g., the posture of the immobile fly), chemical (e.g., unknown volatile pheromones), or even auditory. Identifying their nature and function will be a major challenge.

A summary description of our revised conception of male courtship is as follows. The male orients to a fly on the basis of visual stimuli (8) and the presence of pheromones, both the previously known ones and the candidates postulated here. Wing vibration may begin at this point. The male detects known pheromones by tapping or by being in close contact with the fly; if the pheromones indicate that the courted fly is an appropriate target (i.e., it smells or tastes like a conspecific female), courtship continues. If the courted fly carries a substance that indicates that the target fly is not a conspecific female, courtship stops. Female movement and the presence of both known and unknown pheromones stimulate the male further, inducing following and licking and leading to copulation, for which only the fundamental pheromones revealed here are required. Two things should be noted about this description. First, it is essentially one-sided, focusing on female stimulation of the male; the interaction between the sexes remains poorly understood. Second, although different courtship-inducing factors can be distinguished (modulatory pheromones, unknown signals produced by the living fly, fundamental pheromones) and their behavioral effects separated, in a nonexperimental context all three kinds of factor contribute to courtship and its outcome.

**Detecting Pheromones.** Male fruit flies simultaneously detect both stimulatory and inhibitory chemical stimuli (6) with their foretarsi (21), which in *D. melanogaster* and *D. simulans* show a sexual dimorphism for the number of chemosensory hairs (22). The male normally “taps” a female before courting her, and interspecific mating increases substantially after removal of the male’s foretarsi (19, 21, 23), suggesting that (i) the foretarsi are used to detect modulatory pheromones and (ii) the ancestral stimulatory pheromones that enable copulation and interspecific courtship to take place are detected by some other organ, such as the proboscis.

**Pheromones and Isolation.** Some female-predominant cuticular hydrocarbons have been implicated in the inhibition of interspecific courtship (12, 24). In particular, hydrocarbon transfer experiments with *D. sechellia* females have suggested that 7,11-HD acts as an inhibitor of interspecific courtship in males of *D. simulans* (12) and *D. mauritiana* (24). However, interpretation of these data was made complex by the fact that coated females carried a mixture of native and foreign hydrocarbons. The coating experiments described here do not suffer from this drawback, because hs-tra females are presumably free of all modulatory pheromones.

Our data (Fig. 3; Table 1) show that modulatory pheromones play a decisive role in isolation between the two cosmopolitan species studied here, *D. melanogaster* and *D. simulans*. The substantial reduction in courtship shown by *D. simulans* males with hs-tra + mel females shows the role of *D. melanogaster* female-specific modulatory pheromones (in particular 7,11 dienes) in inhibiting *D. simulans* males. The reduction in courtship by *D. melanogaster* males with hs-tra + sim females shows the role of *D. simulans* modulatory pheromones (in particular 7-T) in inhibiting *D. melanogaster* males.

The behavior of *D. mauritiana* males provides striking confirmation of the stimulatory power of the ur-pheromones and of the role of modulatory pheromones in isolation. When hs-tra females were covered with *D. simulans* hydrocarbons, unexpectedly high levels of interspecific mating were observed (50% within 60 minutes; Table 1); in the presence of *D. melanogaster* female hydrocarbons, however, interspecific mating was abolished. These data suggest that hybridization between these two species is primarily controlled by female cuticular hydrocarbons. One of the main male courtship signals, the modal interpulse interval of male courtship song, is extremely similar in these two species (25), which probably explains why *D. melanogaster* females will accept *D. mauritiana* males.

**Why Are Hydrocarbons Eliminated in hs-tra Females?** Overexpression of the UAS-tra transgene for 60 minutes in female flies at a critical period in the fly’s development (at 6 hours old) led to the elimination of 93% of all hydrocarbons, including all known cuticular pheromones. A series of controls was carried out to ascertain whether the effect was indeed because of overexpression of UAS-tra (data not shown). Although overexpression of UAS-tra produced the largest reduction in hydrocarbon levels (\( \approx 93\% \)), heat shock of females carrying a single copy of hsp70-GAL4 reduced hydrocarbons by \( \approx 70\% \), and heat shock of control Cs females reduced hydrocarbons by \( \approx 15\% \). These data suggest that the effect described here is produced by a combination of factors expressed at a particular moment in development: heat-shock, activation of the hsp70-GAL4 gene, and overexpression of UAS-tra. The complexity of this effect, and our limited understanding of *Drosophila* hydrocarbon biosynthesis pathways and its interaction with sex-determination genes is such that it is not possible to put forward even a speculative hypothesis to explain why hydrocarbon levels decline radically in hs-tra females (4, 8, 26). The complexity of hydrocarbon biosynthesis is further indicated by the fact that when UAS-tra was overexpressed in male flies, a substantial reduction of hydrocarbon levels similar to that in hs-tra females, was very occasionally accompanied by a slight feminization of the remaining cuticular substances (data not shown).

**Identifying the Novel Pheromones.** Washing experiments suggest that the fundamental pheromones revealed here are cuticular hydrocarbons. The most likely candidates are some or all of the five substances identified on the cuticle of hs-tra females (see Fig. 1 legend). One of these substances, 7-H, can probably be excluded. 7-H induces minimal wing vibration by *D. melanogaster* males in doses \( > 500 \text{ ng} \) (5) (40 times the levels of 7-H observed in hs-tra females) and has no significant effect on courtship when studied in a natural blend (6). Furthermore, it is absent from some *D. simulans* females (including the strain studied here) and from most males in the *D. melanogaster* complex apart from *D. sechellia* and some strains of *D. simulans* (18).

Of the four remaining substances, the levels of n-pentacosane (2–4%) and n-heptacosane (1–4%) are very similar in the seven strains representing the four species tested here, whereas 2-methylhexacosane (3–15%) and 2-methylheptacosane (3–18%) show a greater variation. The relative simplicity of these four substances, the fact that they show no sexual dimorphism, and the fact that they are resistant to manipulation by UAS-tra supports the hypothesis that one or more of them may constitute an ancestral form of attractive, sexually monomorphic pheromone. They do not correspond to the substances that were initially suggested to be responsible for courtship in *D. melanogaster* (27).

Although the effect of the fundamental pheromones could be removed by washing, when the extract that presumably contained these substances was placed on a dead fly, there was no effect (Fig. 2). This may be because these substances are hydrocarbons that undergo chemical modification during extraction and are thereby rendered inactive. There is an intriguing precedent for this finding. Young *D. melanogaster* flies of both sexes are highly attractive to conspecific adult males (28) and *D. sechellia* males (11). The attractive power of the
young flies is lost after killing and washing, but covering dead flies with a hexane wash of young flies does not produce reproducible results (29). It is also notable that one of the four candidates for the role of the ur-pheromone, 2-methyloctacosane, makes up 30% of the cuticle of young flies of both sexes and could account for the high levels of courtship shown toward young D. melanogaster flies of both sexes and toward mature hs-tra females.

These data could also be taken as support for an alternative hypothesis as to the nature of the copulation-inducing pheromones; they could be proteins that are denatured by hexane washing and thus rendered inactive and that cannot be restored to their previous conformation by subsequent covering with a hexane wash. This possibility requires further investigation.

Finally, we cannot rule out that the hs-tra females produce an inhibitory substance that accounts for the difference in courtship between species, and hence perhaps in their communication by using a wider range of sensory modalities than is currently suspected. Analyzing the interaction between the sexes and the interchange of sensory information will be a major challenge for the future.

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