Drosophila melanogaster females restore their attractiveness after mating by removing male anti-aphrodisiac pheromones

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Males from many species ensure paternity by preventing their mates from copulating with other males. One mate-guarding strategy involves marking females with anti-aphrodisiac pheromones (AAPs), which reduces the females’ attractiveness and dissuades other males from courting. Since females benefit from polyandry, sexual conflict theory predicts that females should develop mechanisms to counteract AAPs to achieve additional copulations, but no such mechanisms have been documented. Here we show that during copulation Drosophila melanogaster males transfer two AAPs: cis-Vaccenyl Acetate (cVA) to the females’ reproductive tract, and 7-Tricosene (7-T) to the females’ cuticle. A few hours after copulation, females actively eject cVA from their reproductive tract, which results in increased attractiveness and re-mating. Although 7-T remains on those females, we show that it is the combination of the two chemicals that reduces attractiveness. To our knowledge, female AAP ejection provides the first example of a female mechanism that counter-acts chemical mate-guarding.

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Polyandry creates evolutionary conflicts between males and females. For most species, one mating provides females with enough sperm to produce offspring, and males with the assurance of paternity. When females remate, however, these extra copulations benefit females by increasing fecundity and offspring genetic diversity\(^1,2\), but come at a cost to males by reducing the number of offspring they sire\(^3\). This creates a conflict between males and females in optimal female mating rate. Sexual conflict theory\(^4\) predicts that males should attempt to reduce or block polyandrous behaviour. Indeed, a variety of mate-guarding strategies are seen across taxa occurring both before\(^5,6\) and after copulation\(^7,8\). One such tactic, chemical mate-guarding involves males marking females with pheromones rendering them unattractive, and thus decreasing their chances of re-mating\(^9,10\). As a response to this, females should defly these ploys and develop mechanisms to overcome or remove the imposed reproductive restraints. Surprisingly, no examples of mechanisms for female liberation have been identified.

In *Drosophila melanogaster*, males pay a high cost to female polyandry as the last male she mates with sires most of her offspring through a phenomenon called last-male sperm precedence\(^11,12\). Accordingly, there is evidence that males employ chemical mate-guarding strategies: the changes to the females’ pheromonal profile via mating are well documented\(^13–17\) and these changes reduce attractiveness as mated females, or their pheromonal extracts, elicit less courtship compared to virgins or extracts of virgin females, respectively\(^15,17,18,19\).

Two main pheromones, *cis*-vaccenyl acetate (cVA) and (Z)-7-Tricosene (7-T), have been associated with chemical mate-guarding in *D. melanogaster*. cVA is a male-specific pheromone produced in the ejaculatory bulb of the male reproductive tract and transferred to females within the ejaculate\(^20-23\). cVA reduces male courtship and delays mating when it is either present in the environment\(^24\) or artificially applied to the female cuticle\(^13,14,24,25\). Two odorant receptor neurons in the antennae\(^26,27\) that express either *Or67d\(^28\) or *Or65a\(^29\) respond to cVA and both are involved in the cVA-induced male courtship inhibition\(^15,24\). The other pheromone, 7-T, is a cuticular hydrocarbon (CHC) produced in subepidermal abdominal cells called oenocytes. Although it is produced by both sexes, 7-T is found in much higher quantities on males compared to females. Genetic modification of CHC production in males produces flies that have significantly reduced levels of various CHCs, including 7-T. These flies are also courted significantly more than their genetic controls\(^30,31\). As male–male courtship inhibition can be rescued with application of 7-T on these CHC-reduced flies\(^32,33\), 7-T has been regarded as a compound that inhibits male courtship. At least one gustatory receptor, Gr32a, responds to 7-T, and the behavioural defects of this mutant support the hypothesis that 7-T also functions to reduce courtship towards mated females\(^32\). One missing piece of the puzzle that has not been identified is the female response to these chemical constraints and possible mechanisms to remove these AAPs.

In *D. melanogaster*, females have a variety of post-mating behaviours including the recently documented ejection of unsorted sperm a few hours after mating\(^12,33–35\), which has been linked to the end of the sperm storage process\(^32\) and marking of egg-laying sites\(^36\). Here we investigated the possibility of female ejection functioning to remove mate-guarding AAPs, resulting in increased post-mating attractiveness. Indeed, we show that the male-derived AAP cVA, but not 7-T, is lost by mated females a few hours after mating through the process of sperm ejection. We demonstrate that the reduction in attractiveness of a mated female in comparison to a virgin is not due to the action of cVA or 7-T alone, but by their interaction. We further show that sperm ejection elevates female attractiveness and dramatically increases the likelihood of re-mating. Finally the results from our experiments suggest that sperm ejection is under active control by the female as the timing of sperm ejection can be modulated by changing her social context or by artificially activating her doublesex-expressing cells. Taken together, our data show that females can alter their own attractiveness via sperm ejection, undermining the interaction of cVA and 7-T, demonstrating for the first time to our knowledge a mechanism that allows females to counter-act chemical mate-guarding strategies.

**Results**

**Females remove cVA acquired from males by sperm ejection.** Females acquire chemicals such as cVA and a host of CHCs from males during mating, which reduce their attractiveness\(^13–17\). cVA is transferred with the ejaculate and stored in the female reproductive tract\(^21,22,36\). Given that females eject unsorted sperm within 6 h after mating\(^12,33–35\), we investigated whether sperm ejection by mated females can remove male AAPs. We quantified the CHC and cVA profiles of virgin females (‘Virgin’), recently mated females (‘Mated recent’), and recently ejected females (‘Ejected’). We observed major changes in the female chemical profile correlated with mating and sperm ejection, the most drastic involved two male pheromones: cVA and 7-T (Fig. 1a; Supplementary Table 1). Strikingly, over 80% of the cVA transferred by males to females during mating was lost after sperm ejection (Fig. 1a\(_1\)), while the extra 7-T acquired by females through mating was only slightly reduced (Fig. 1a\(_2\)). Although virgin females lost small amounts of female-specific CHCs after mating, the amounts of these CHCs, including 7,11-HD, were not dramatically different in Mated versus Ejected females indicating that sperm ejection mainly affects male compounds (Fig. 1a\(_3\); Supplementary Table 1). Given that sperm ejection occurs on average 6 h after mating\(^12,33,35\), we performed two experiments to investigate the possibility that changes in the chemical profile of Ejected females were due to the passage of time and not causally connected to sperm ejection. First, we quantified the chemical profile of mated females at two time points: 1 h after the start of mating (ASM), referred to as ‘Mated recent’, and ~6 h ASM (at the same time point as an Ejected female) referred to as ‘Mated matched’. Although no sperm ejection occurred in these females, cVA levels were slightly reduced (Fig. 1a\(_1\)) and 7-T levels were slightly lower (Fig. 1a\(_2\)) during this 5 h interval. Given that cVA levels increased over time in mated females, the reduced amount of cVA in Ejected compared to Mated matched females must be explained by sperm ejection itself. However, 7-T levels dropped slightly over time, irrespective of sperm ejection, possibly being lost through grooming and/or evaporation. Second, we examined the chemical profile of mated females that were artificially prevented from ejecting sperm. We prevented sperm ejection by conditionally activating the thermosensitive cation channel *Drosophila* TRP1A1 (dTTrpA1) in doublesex (dsx)-expressing cells in females\(^33\). At 29 °C, dsx-expressing cells were continuously activated for 11 h ASM, blocking sperm ejection in 95% of females, while 100% of control females ejected (Fig. 1b\(_1\)). This effect is connected to the activation of dTrpA1 since at 22 °C, a temperature at which dTrpA1 is not active\(^37\), most females expressing dTrpA1 in dsx cells ejected sperm (Fig. 1b\(_1\)). When sperm ejection was inhibited in these blocked flies, cVA levels remained at mated levels while it was clearly reduced in control females who ejected (Fig. 1b\(_2\); Supplementary Table 2). 7-T levels were elevated by threefold in blocked females compared with controls (Fig. 1b\(_3\)), which is surprising given that sperm ejection does not affect 7-T (Fig. 1a\(_2\)). However, this was not due to an overall increase in CHC levels in blocked females, since the
Figure 1 | Mating and sperm ejection change female chemical profile. (a1–3) Mean amount of cVA, 7-T and 7,11-HD, respectively, on virgin females and females of the following mating statuses: 1h after the start of mating (ASM; Mated recent), time matched to an ejected female (Mated matched), and mated females who ejected sperm (Ejected). Differences between groups were determined by One-way ANOVA followed by Tukey’s post-hoc test. Different letters indicate groups that are significantly different from each other. For full statistical analysis see Supplementary Table 1. (b1) Percentage of females of indicated genotype who had ejected sperm 11 h ASM. Differences between groups were determined by One-way ANOVA followed by Tukey’s post-hoc test. For full statistical analysis see Supplementary Table 2. (c1–2) Mean amount of cVA and 7-T in reproductive tracts and on the carcasses of Virgin, Mated, and Ejected females. Non-ejected Mated females were time matched to females that Ejected. Differences between groups were determined by two-way ANOVA followed by Bonferroni test. For full statistics see Supplementary Table 3. (d1–3) Mean amount of cVA, 7-T and 7,11-HD found on virgin females (Virgin), females mated to control males (mC), and females mated to Oe− males (mOe). CHC were extracted 1h ASM. Differences between groups were determined by One-way ANOVA followed by Tukey’s post-hoc test. For full statistical analysis see Supplementary Table 4. Error bars indicate s.e.m. Number of replicates is between brackets. (NS: non-significant; *p<0.05; **p<0.01; ***p<0.001).
produced cVA but no CHCs including 7-T.30. We quantified the ejaculate20 and we show that it is almost entirely contained within the amount lost from the reproductive tract, thus demonstrating (Fig. 1c1–2; Supplementary Table 3). Mating caused an increase in and 7-T were present in different parts of post-mated females, compared the pheromonal profiles, and found that cVA both within the female reproductive tract and on the carcass (Virgin versus Mated). Although copulation caused an increase of 7-T both within the reproductive tract and from the rest of the body (carcass) in parallel. We performed this dual extraction on Virgin, Mated and Ejected females, compared the pheromonal profiles, and found that cVA and 7-T were present in different parts of post-mated females (Fig. 1c1–2; Supplementary Table 3). Mating caused an increase in cVA both within the female reproductive tract and on her carcass (Virgin versus Mated), with 94% of cVA in the reproductive tract (Fig. 1c1). We found that the reproductive tract of Ejected females contained nearly 300 ng less than Mated females (Fig. 1c1) showing that the vast majority of cVA is contained within the reproductive tract and is removed via ejection (Fig. 1c1; Supplementary Table 3). To support this conclusion, we collected sperm after it had been ejected by females and quantified its chemical content. Together with several CHCs, we measured ~300 ng of cVA in the ejected sperm (Fig. 1c1; Table 1) matching the amount lost from the reproductive tract, thus demonstrating that sperm ejection is the route of cVA removal. cVA is clearly transferred from males to females via the ejaculate20 and we show that it is almost entirely contained within the female reproductive tract (Fig. 1c1). However, the source of 7-T acquired by females through mating remains unclear. Although copulation caused an increase of 7-T both within the reproductive tract and on the carcass (Virgin versus Mated; Fig. 1c2), most of the pheromone was on the carcass and did not significantly decrease after ejection (Mated versus Ejected; Fig. 1c2) suggesting that 7-T is acquired through cuticular contact between the male and female. To test this hypothesis, we used transgenic males with ablated oenocytes (Oe−) who produce cVA but no CHCs including 7-T.30. We quantified the pheromonal profile of virgin females, and females mated to either males lacking oenocytes (mOe) or their genetic control (mC; Fig. 1d1–3; Supplementary Table 4). Although mC females had increased amounts of both cVA and 7-T, similar to females who mated with wild-type males (Fig. 1d1–2), mOe females gained only 7-T (Fig. 1d1–2). This indicates that male-derived 7-T on a mated female originates from the oenocytes of the male abdomen and is thus likely acquired through abdominal contact during mating. Moreover, the similar increase in cVA in chemical profiles from mOe or mC females demonstrates that cVA is not contributed through the oenocytes (Fig. 1d1). Finally, female-specific CHCs, such as 7,11-HD, were slightly reduced in females mated to either mOe or mC showing, as expected, that the male oenocytes are not required for this phenomenon (Fig. 1d1; Supplementary Table 4). Overall, our findings establish that females gain 7-T on their cuticle via contact with the male and cVA within the reproductive tract via the ejaculate, explaining why ejaculation results in the loss of cVA but not 7-T.

**Sperm ejection increases female attractiveness and re-mating.** To directly determine whether changes in pheromonal profile after copulation and sperm ejection are associated with changes in female attractiveness, we compared the courtship behaviour of naïve males towards Virgin, Mated or Ejected females. Since mated females exhibit rejection behaviour such as ovipositor extensions,38,39 and decreased sexual receptivity compared with virgins,40 we used decapitated females, who remain alive and are courted but do not display rejection behaviour.41 This excludes the contribution of rejection behaviour from our examination of the effect of male-derived AAPs on female attractiveness. We found that males courted Virgins significantly more than Mated females but not significantly differently than Ejected females; ejected females are thus intermediate in terms of attractiveness (Fig. 2a). To directly test whether ejected females are more attractive than non-ejected females, we decapitated Mated and Ejected females and placed one of each within the same arena, and determined the courtship preference of a naïve male. We constructed two types of arenas, one closed with a plastic lid allowing saturation of the air with pheromones, the other open (topped with a nylon net to prevent flies from escaping) preventing saturation of the air with pheromones. Although we found no courtship preference in the closed arena, we found that males courted Ejected females significantly more than Mated females in the open arena (Fig. 2b) providing direct evidence that sperm ejection increases female attractiveness independently of behavioural modification.

As cVA is located within the reproductive tract of mated non-ejected females (Fig. 1c1), the ovipositor extrusions displayed by mated females may expose higher concentrations of cVA to courting males, further inhibiting courtship. As decapitated females do not display ovipositor extrusion, we investigated whether courtship towards intact females with a full post-mating behavioural repertoire followed the same pattern as courtship towards decapitated females. We observed the courtship behaviour of naïve males towards intact Virgin, Mated, or recently Ejected females for a 10-minute period. Again, we found that males courted intact Virgins more vigorously than Mated but not significantly differently than Ejected females, showing that ejection also impacts the attractiveness of normal females (Fig. 2c). In the light of sexual conflict theory, we predicted that the increased attractiveness following ejection functions to increase the likelihood of female re-mating. To test this, we let the courting pairs interact for an additional 20 minutes to monitor the occurrence of copulations. Within the 30 minute window, all the Virgin females mated, and strikingly so did nearly half of the Ejected females; however, no Mated (non-ejected) females mated (Fig. 2d). Taken together, our data indicate that ejection confers the potential for female re-mating, possibly due to increased attractiveness as a result of sperm ejection. Although chemical mate-guarding has been established for decades in *Drosophila*13–16, a female response predicted by sexual conflict

| # | Compound name | Abbreviation | Amount (ng) |
|---|---------------|-------------|-------------|
| 1 | n-Heneicosane | n-C21       | 0.40 ± 0.53 |
| 2 | cis-Vaccenyl Acetate | cVA | 318.20 ± 96.88 |
| 3 | 7-Tricosene | C23:1(7) | 18.39 ± 6.26 |
| 4 | n-Tricosane | n-C23 | 11.67 ± 3.27 |
| 5 | n-Tetracosane | n-C24 | 15.18 ± 1.62 |
| 6 | 9-Pentacosene | C25:1(9) | 2.44 ± 2.25 |
| 7 | 7-Pentacosene | C25:1(7) | 2.47 ± 1.27 |
| 8 | n-Pentacosane | n-C25 | 5.88 ± 2.98 |
| 9 | 7,11-Heptacosadiene | C27:2(7,11) | 24.52 ± 8.82 |
| 10 | 2-methyl-Hexacosane | 2MeC26 | 7.51 ± 2.54 |
| 11 | 7,11-Nonacosadiene | C29:2(7,11) | 1.91 ± 2.34 |

Peak numbers (#) correspond to the elution order of each compound shown in the chromatograph in Fig. 1c2. The mean amount ± s.e.m. of each compounds is indicated.

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data. Table 1 Major compounds detected in ejected sperm.
or placed in groups of 6 females until ejection. Start of mating of females mated in either pairs or groups, and then isolated (***

that the timing of re-mating is significantly faster in groups than pairs either held in pairs (blue) or groups (red). Bootstrap analysis reveals (...

period when placed with a naive male (...

were determined by One-way ANOVA followed by Tukey’s tests. Error bars indicate s.e.m. For full statistics see Supplementary Table 6. (b) Courtship preference index of naive wild-type male with two females: one Mated but not yet ejected, the other just Ejected. Assays were performed within an open or a closed mating arena. Error bars indicate minimum and maximum data points. Significant preference for the ejected female is indicated in each box plot by asterisks as determined by a two-tailed exact Wilcoxon signed rank test (NS: non-significant; *p < 0.05).

Letters to the right of the box plots indicate statistical comparisons between groups determined by a logistic regression model. For full statistics see Supplementary Table 5. (c) Mean CI of 1 naive wild-type male with one wild-type female of indicated mating status. Differences between groups were determined by One-way ANOVA followed by Tukey’s post-hoc tests. Error bars indicate s.e.m. For full statistics see Supplementary Table 6. (d) Proportion of females that copulated within a 30-minute observation period when placed with a naive male. (χ²-test. χ² = 46.0387 p < 0.00001). (e) Time between virginal mating and second mating (re-mating) for flies either held in pairs (blue) or groups (red). Bootstrap analysis reveals that the timing of re-mating is significantly faster in groups than pairs (***p < 0.001). (f) Mean (± s.e.m.) time to female sperm ejection after the start of mating of females mated in either pairs or groups, and then isolated or placed in groups of 6 female until ejection.

Figure 2 | Sperm ejection increases female attractiveness and re-mating. (a) Mean courtship index (CI) in pairs consisting of 1 naive wild-type male with one decapitated female of the indicated mating status. Differences between groups were determined by one-way ANOVA followed by Tukey’s test. Error bars indicate s.e.m. For full statistics see Supplementary Table 6. (b) Courtship preference index of naive wild-type male with two females: one Mated but not yet ejected, the other just Ejected. Assays were performed within an open or a closed mating arena. Error bars indicate minimum and maximum data points. Significant preference for the ejected female is indicated in each box plot by asterisks as determined by a two-tailed exact Wilcoxon signed rank test (NS: non-significant; *p < 0.05).
question how much of the cVA is available to males? Although this remains unclear, it is known that the odorant receptor neurons tuned to cVA respond to a dose as little as $10^{-4}$ ng (ref. 29), which is several orders of magnitude smaller than then 400 and 25 ng located within and on a mated female, respectively (Fig. 1c). Taken together, these data suggest that males may be able to sense cVA stored in the female reproductive tract, but that this compound is not sufficient to inhibit courtship at natural concentrations.

Since the largest increase in CHC after mating was in levels of 7-T and this pheromone has known effects on courtship30,31, we found that females mated to perfumed Oe wild-type levels of 7-T. We found that females mated to perfumed Oe males ($m^{7-T}$) had elevated amounts of 7-T ($183.24 \pm 4.16$ ng) comparable to females mated to wild-type males (see Fig. 1a). As well as the normal amount of cVA. As a result of this manipulation, these females were courted significantly less than virgin females (Fig. 3a) suggesting that the combination of cVA and 7-T can inhibit male courtship behaviour when presented together within biologically relevant ranges.

To directly test an interaction effect of cVA and 7-T in reducing male courtship, we perfumed decapitated virgin females with various amounts and combinations of 7-T and cVA. As both cVA and 7-T are volatile28, we reasoned that the chemical profile of one female may influence the attractiveness of other nearby females. To control for this effect, we determined the courtship behaviour of males towards perfumed virgin females both within a no-choice (one female) and choice assay (containing a benchmark virgin control female and a perfumed virgin female). No dose of 7-T applied onto a virgin female affected her attractiveness in either a no-choice (Fig. 3b) or choice assay (Fig. 3c), even at a dose 90-fold higher than that found on a mated female. This unambiguously shows that 7-T is not sufficient to reduce male courtship. Unlike 7-T, the influence of cVA on male courtship was dose- and context-dependent (Fig. 3d,e). In a no-choice assay, cVA had little to no influence at concentrations normally found in mated females with inhibition only occurring at 5,000 ng, 10 times higher than the 400 ng naturally transferred from males to the reproductive tract of females (Fig. 3d). It did, however, decrease courtship at doses at and above 400 ng perfumed on the external part of the females in a choice assay (Fig. 3e). Since males discriminated against females perfumed with cVA at lower concentrations in the choice assay, we proceeded with this paradigm to test the combined effects of cVA and 7-T at physiological levels. Given that our perfuming protocol applies compounds on the carcass of the flies and not on the inside, we perfumed amount of 7-T and cVA naturally found on the carcass (as measured in Fig. 1c1–2) We applied ~100 ng of 7-T (Fig. 1c2) and 120 ng of cVA, which is four times the level of cVA found on the carcass of mated female (Fig. 1c1). The presence of either 7T or cVA individually at those

Figure 3 | 7-T and cVA interact to inhibit male courtship. (a) Mean courtship index (CI) in pairs consisting of 1 naive wild-type male with one decapitated female who mated with the indicated male: females mated to control male ($m^{C}$), females mated to Os males ($m^{7-T}$) and females mated to Oe males perfumed with 7-T before mating ($m^{7-T}$). CI of wild-type males towards each group of mated females was compared against CI towards virgins using a one-way ANOVA followed by Dunnett’s post hoc test (NS = non-significant; *p < 0.05). (b) Mean CI in pairs consisting of 1 naive wild-type male with 1 decapitated virgin female perfumed with indicated amount of 7-T. Differences between groups were determined by One-way ANOVA followed by Dunnett’s post hoc test comparing CI of each group to the CI of benchmark unperfumed virgin female. (c) Courtship preference of one naive wild-type male with two females: one virgin; and one virgin female perfumed with indicated amount of 7-T. Error bars indicate minimum and maximum data points. Significant preference for the unperfumed female is indicated in each box plot by asterisks as determined by a two-tailed exact Wilcoxon signed rank test (NS = non-significant). (d) Mean CI in pairs consisting of 1 naive wild-type male with one decapitated virgin female perfumed with indicated amount of cVA. Error bars indicate ± s.e.m. Statistical comparisons are as in b. (e) Courtship preference of naive wild-type male with one decapitated virgin female perfumed with indicated amount of cVA. Error bars indicate ± s.e.m. Statistical analysis as in c. For full statistics see Supplementary Tables 5 and 6.
concentrations did not significantly influence the perfumed female's attractiveness when pitted against a non-perfumed virgin female. However, the presence of both chemicals on the female significantly reduced female attractiveness (Fig. 3f) leading us to conclude that these two compounds work in combination to affect female attractiveness. Removal of cVA, but not 7-T, by sperm ejection thus separates the two compounds, neutralizing their combined anti-aphrodisiac effects.

Previous studies have indicated that both cVA and 7-T are AAPs, seemingly contradicting our results. cVA has been found to be sufficient to decrease male courtship54,28,29. However, a closer look at these studies reveals some inconsistencies. For instance, there is evidence that 200 ng of cVA can sometimes result in sperm ejection thus separates the two compounds, neutralizing their combined anti-aphrodisiac effects.

AAPs are sensed by taste and olfactory receptors of males. Having established that 7-T and cVA act in combination as mate-guarding pheromones, we next tested how courting males sense these pheromones. As cVA is detected by odorant receptors Or67d and Or65a24,28,29, we disrupted the functioning of these receptors or neurons and observed their effect on mating status-dependent courtship. We paired a single decapitated Virgin, Mated or Ejected female with a male either deficient in orco and Or67d receptors or neurons and observed their effect on mating status-dependent courtship. We paired a single decapitated Virgin, Mated or Ejected female with a male either deficient in orco and Or67d receptors or neurons and observed their effect on mating status-dependent courtship.

In our experiments with a single AAP, use a combination of pheromones, sensed by a combination of chemosensory receptors to reduce courtship by other males. Females respond to this chemical marking via a behavioural counter-adaptation, sperm ejection, that removes cVA and neutralizes the effect of 7-T. Several traits are thus simultaneously at play, consistent with the idea that sexual conflict triggers the sequential evolution of a series of adaptations and counter-adaptations in males and females56. Our findings support such models as we show that males, rather than using a single AAP, use a combination of pheromones, sensed by a combination of chemosensory receptors to reduce courtship by other males. Females respond to this chemical marking via a behavioural counter-adaptation, sperm ejection, that removes cVA and neutralizes the effect of 7-T. Several traits are thus simultaneously at play, consistent with the idea that sexual conflict triggers the sequential evolution of a series of adaptations and counter-adaptations in males and females56. To make this picture more complex, attempts by males to reduce female promiscuity may be further limited by another function of cVA and sperm ejection in D. melanogaster. cVA has long been known to also act as an aggregation pheromone57, and we have recently shown that sperm ejection is used by mated females to selectively deposit cVA on food and attract males and females58. This aggregation allows communal egg-laying by females, which increases the chance of offspring survival, thereby benefiting both females and males58 and lending a non-sexually antagonistic dimension to the role of cVA and sperm ejection. The proposed model of sexual conflict resolution by the sequential evolution of a series of adaptation and counter-adaptation also suggests, in turn, that the mechanisms underlying mate-guarding pheromones must be complex. Recently, studies of the neuronal circuitry supporting male courtship have revealed its inherent complexity, using both inhibitory and excitatory signals from a variety of
Environmental stimuli that converge on a small population of neurons in the male brain to determine courtship output\(^{9,60}\). This complexity likely explains the difficulties in linking the function of single sex pheromones, even one as well studied as cVA, with a single behavioural function.

**Methods**

*Drosophila* stocks and genetics. Flies were reared on food medium containing agar (10 g l\(^{-1}\)), glucose (167 mM), sucrose (44 mM), yeast (35 g l\(^{-1}\)), cornmeal (15 g l\(^{-1}\)), wheat germ (10 g l\(^{-1}\)), soya flour (10 g l\(^{-1}\)), molasses (30 g l\(^{-1}\)), propionic acid and Tegosept; and is referred to as 'fly food' 'food media' in this report. Flies were reared in a 12:12 h light/dark cycle (LD 12:12) at 25°C. Virgins.
were collected using CO₂ anaesthesia. Females were aged in same-sex groups of 20 in vials for 5–7 days before testing. Tester males were aged singly for 5–7 days in glass vials (40 mm × 8 mm Petri dishes) to limit experience before testing as exposure to derived chemicals can modify male courtship. However, males used to generate mated non-jected females from the same mating arena (Mated matched female), as well as a virgin female (Virgin), were immediately anesthetized on ice and used for pheromone extraction.

Sperm ejection chemical analysis. After a female from ‘Mating status chemical analyses’ ejected the mating plug and associated sperm/seminal fluid, the entire ejection was collected from the dishes using fine forceps and transferred into a glass microvial and subjected to CHC as described below.

Neuronal manipulation for sperm ejection inhibition. Females with a temperature-gated calcium ion channel expressed in dsx-1 cells as well as their associated controls were paired with Canton-S males in a 10 × 8 mm Petri dish with a layer of fly food coating the bottom at 22°C. All pairs that did not copulate within 3 h were discarded. Once copulation ended, males were removed and the dish containing the female was placed either in a 20°C incubator or kept at room temperature (22°C). Eleven hours ASM, females were flash frozen with liquid nitrogen, checked for the presence of a mating plug to determine if they ejected, and subjected to CHC extraction.

Chemical analysis of female reproductive tract and carcass. Virgin, Mated and Ejected females were generated as described in ‘mating status chemical analysis’ (note: all non-jected females in this experiment were ‘Mated matched’). To assess the hydrocarbon profile of the reproductive tract and the female carcass, a female was placed on ice under a stereomicroscope and her abdomen was opened using forceps to expose the reproductive organs under dry conditions. The intact reproductive tract (including the ovipositor, bursa/uterus, sperm receptacle, oviducts and ovaries) was extripated from the carcass. The carcass and reproductive tract were placed on separate pieces of Whatman filter paper (5 × 5 mm). These filter papers were subjected to CHC extraction.

CHC extraction and analysis. Each item (whole fly/ejected/female reproductive tract or carcass/filer paper) was placed individually into a glass vial containing 1 ml of hexane (Supelco, USA) and 50 μl of hexane (Supelco, USA) containing 10 ng ml⁻¹ of hexacosane (C26) an internal standard. The vial was then vortexed for 2 min, and the item was removed using a clean metal pin. Vials were placed in °C for 2 h before analysis. The resulting hydrocarbon extracts were analysed using an Agilent 7890 gas chromatograph with a flame ionization detector, an Agilent J&W 1.5% cross-linked 5% phenyl methyl silicone column (Diameter: 0.32 mm × 1.8 μm, film thickness: 0.18 μm) and a splitless injector set at °C with 40 ml/min splitless flow. The injector valve was opened 1.5 min after injection in splitless mode with helium as the carrier gas (flow: 37.2 cm s⁻¹). The oven temperature programme begins at °C for 1.5 min, ramping at °C min⁻¹ to °C, then ramping at °C min⁻¹ to °C and holding for 5 min. ChemStation software (Agilent Technologies) was used to quantify compounds based on peak areas relative to internal standard C26.

Mating status and attractiveness. Females of different mating statuses were generated as described in ‘Mating status chemical analysis’. If experiment involved decapitation (to minimize movement and rejection behaviour21), females were placed on ice and decapitated using a razor blade ~2 h ASM. Females were transferred to an empty Petri dish and monitored for sperm ejection. All females were placed into a courtship arena (a 10 × 8 mm Petri dish layered with 1 ml of fly food) and given 2 min to acclimate. All dishes were video taped using a Canon high definition camcorder (Canon Legria GP M36). For dishes containing an intact female, a naive virgin Canton-S tester male was then introduced and the dish was first observed for 10 min to determine amount of male courtship behaviour elicited by the female, and then observed for an additional 20 min to determine whether copulation occurred. If no courtship was observed during that period of time, the data were not used. Courtship index for Mated and Ejected females was calculated by dividing number of seconds the male courted over total observation time (600 s). However, as all virgins mated within this 10-minute period, courtship index for virgins was calculated by dividing number of seconds the male courted over copulation latency (time from start of assay to start of copulation). For dishes containing a decapitated female, a naive virgin tester male was introduced and the dish was video taped for either 30 (Canton-S tester male) or 45-min period (all other genotypes). Courtship index was determined and 10-min assessment window based on male courtship.

To determine courtship preference of Canton-S tester males for Mated versus Ejected females, two females (decapitated Mated and Ejected female) were transferred into a larger courtship arena (90 in diameter × 8 mm deep) with either a plastic lid allowing saturation of the air with pheromones called ‘closed arena’ or a nylon net in place (the plastic lid preventing saturation of the air with pheromones called ‘open arena’) and dishes were placed in a fume hood. Preference index was determined by dividing the time a male spent courting a female of the indicated mating status over total time male spent courting within a 10-minute assessment window (no. of seconds male spent courting specific female/no. of seconds male spent courting non-specific female) and holding for 5 min.
extension/vibration, or attempted copulation (note: orientation was only used as an indication of courtship if wing extension/vibration, and attempted copulation followed). A male was considered to exhibit courtship if he courted females for at least 30 s within the 600 s observation period. Proportion of males that displayed courtship behaviour was determined by dividing the amount of males that courted by the total number of males tested. If a ‘Mated’ female ejected during this observation time, the dish was discarded. For Canton-S males, dishes were discarded and data not used if the testing time exceeded 45 min. A single wild-type Canton-S test male was transferred to the dish and courtship behaviour was filmed, and either a courtship index or preference index was calculated for perfumed females in a no-choice or choice assay, respectively. Oe– males were perfumed with 7-T. After perfuming, females were given 1 h to recover and then placed with females in group choice assays. If any males mated to Oe– females perfumed with 7-T were then used singly into a 10 × 8.5 mm Petri dishes layered with 1 ml of fly food. A single wild-type Canton-S test male was transferred to the dish and courtship behaviour was filmed and courtship index was assessed; or female was used in chemical analysis to determine chemical profile.

**Statistical analysis.** To estimate variance in time to re-mating (Fig. 2e), a bootstrap analysis was performed using the Matlab software (MathWorks). The first six re-mating times if male failed to mate to the first time the first re-mating of a given replicates in the group experiments were randomly rearranged with those of the other replicates. This generated pseudo-replicates made up of the observed re-mating times but in random rankings. This process was iterated 10,000 times and represents all possible combinations of ranks of time to re-mating. The variance in re-mating times of these pseudo-replicates was compared to the variance observed in pairs using the SPSS software (IBM, Inc.) to estimate the degree of between samples variability in the two social contexts.

For analysis of the timing of sperm ejection in a group versus pair (Fig. 2f), the unit of replication was the mating arena. Effects of social context during and after mating on the timing of sperm ejection were determined using a standard least square mixed effect model in which variables were continuous and normally distributed. Pre- and post-mating conditions as well as their interactions were modelled as fixed effects, and mating arenas as random effects. The model was run using JMP v. 9.0 for Mac.

Statistical analysis of chemical profile data was performed using GraphPad Prism 5 (GraphPad Software, Inc., USA). To analyse differences in amount of chemicals across groups (Virgin, Mated recent, Mated matched, and Ejected; or females expressing dTrapA1 in dsx+ cells and their associated controls), we first checked the distribution of the data with a Kolmogorov-Smirnov test (with Dallal–Wilkinson–Lillie for p value) for normality. If those tests was not used to confirm the amount of pheromone(s) transferred and the 6 others used for courtship assays. Wild-type virgin females were either perfumed with (with Dallal–Wilkinson–Lillie for p value) for normality.

**Pheromone bioassay.** Virgin males and females were perfumed as described in Billeter et al.33 with the modification that virgin males were anaesthetized using CO2. Females were perfumed with 7-T, or a Mann–Whitney test; comparisons across groups were done with with a one-way ANOVA (normally distributed data) or a Kruskal–Wallis test followed by either a Tukey’s Multiple Comparison test, a Bonferroni Multiple Comparison test, or a Dunn’s Multiple Comparison test. The data for preference index within a choice assay did not follow a Gaussian distribution, so exact two-tailed Wilcoxon signed-rank test was used to test whether courting males preferred one female over the other. Preference data were tested against the null hypothesis that males did not make a choice, with no preference. To determine differences in propensity to mate, the number of males displaying courtship behaviour was compared to the number of males that failed to show at least 30 s of courtship behaviour using a chi-square test (p < 0.05). To determine differences in courtship latency to females with different mating statuses (Virgin, Mated recent, Mated matched, and Ejected) we first checked the distribution of the data with a Kolmogorov-Smirnov test (with Dallal–Wilkinson–Lillie for p value) for normality and analysed data with a Kruskal–Wallis test.

**Data availability.** All relevant data are available from the authors on request.

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