Pheromone evolution and sexual behavior in Drosophila are shaped by male sensory exploitation of other males

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sexual behavior over evolutionary time.

Animals exhibit a spectacular array of traits to attract mates. Understanding the evolutionary origins of sexual features and preferences is a fundamental problem in evolutionary biology, and the mechanisms remain highly controversial. In some species, females choose mates based on direct benefits conferred by the male to the female and her offspring. Thus, female preferences are thought to originate and coevolve with male traits. In contrast, sensory exploitation occurs when expression of a male trait takes advantage of preexisting sensory biases in females. Here, we document in Drosophila a previously unidentified example of sensory exploitation of males by other males through the use of the sex pheromone CH503. We use mass spectrometry, high-performance liquid chromatography, and behavioral analysis to demonstrate that an antiphrodisiac produced by males of the melanogaster subgroup also is effective in distant Drosophila relatives that do not express the pheromone. We further show that species that produce the pheromone have become less sensitive to the compound, illustrating that sensory adaptation occurs after sensory exploitation. Our findings provide a mechanism for the origin of a sex pheromone and show that sensory exploitation changes male sexual behavior over evolutionary time.

Significance

Sexual selection is widely regarded as an important mechanism for the origin of new traits and species. Darwin first proposed that the elaboration of male secondary sexual traits is driven by female preferences (1, 2). This concept has been refined by models suggesting that females select male traits that indicate genetic quality or confer direct reproductive benefits (3–7). In contrast, sensory exploitation occurs when expression of a male trait takes advantage of preexisting sensory biases in females (8). In this case, female preference does not coevolve with the male trait but rather precedes it. In one of the first examples documenting sensory exploitation, female Physalaemus coloradorum frogs were shown to prefer male calls that contain a low-frequency “chuck” component despite the absence of this feature in calls from conspecics. The sensory bias for chucks was shown to have its mechanistic basis in the tuning properties of the inner ear, a physiological feature that predated the appearance of chucks (9). Similarly, female platyfish exhibit a preference for males with swordtails despite the absence of swordtails in male platyfish. Females consistently chose to spend more time with conspecific males exhibiting an artificially attached plastic sword (10). In both these examples, female preference predated expression of the trait. Sensory exploitation has since been documented for numerous other visual cues, across a diversity of taxa (11–14). In each case, females prefer traits that are not found naturally in their own species but appear in males of other species. Moreover, both the sensory bias and behavioral response to the trait already were present before expression of the trait.

Pheromones are taste and olfactory cues that, in many species, play an important role in mate selection (15). As with courtship cues detected by other sensory modalities, pheromones are shaped by sexual selection and, thus, may exhibit enormous structural diversity and exquisite stereochemical specificity. In insects, exogenously secreted lipids advertise mating status, availability, and reproductive fitness (16). In some cases, male pheromones serve as a nuptial gift, thus providing direct reproductive benefits to females and offspring in the form of either nutritive or defensive compounds (17). Little is known, however, about the mechanisms underlying the diversification and the origin of chemical specificity. Here, we provide an example of a pheromone that has evolved from sensory exploitation. In Drosophila melanogaster, CH503 [formally, (3R,11Z,19Z)-3-acetoxy-11,19-octacosadien-1-ol; Fig. 1] functions as an antiphrodisiac (18). The pheromone is secreted in the anogenital region, is transferred to females during mating, and suppresses courtship from males. Our findings indicate CH503 evolved from males exploiting the preexisting sensory biases of other males to gain mating advantage by limiting access to females. Moreover, the use of CH503 has altered male sexual behavior over evolutionary time such that males have adapted by becoming less sensitive to the pheromone.

Results and Discussion

Evolutionary Origin of CH503 Expression. To determine the evolutionary origins of CH503, we examined eight species of Drosophila...
Drosophila willistoni, D. yakuba, and D. sechellia, and Drosophila ananassae (Fig. 1A). A signal for cis-vaccenyl acetate (cVA), another known antiaphrodisiac, also was found in these species (19). In contrast, no signal for CH503 could be detected from the anogenital region of Drosophila willistoni, Drosophila mojavensis, or Drosophila virilis (Fig. 1A). To determine the double-bond geometries and absolute configuration of CH503 from melanogaster group flies, chemical derivatization and high-performance liquid chromatography (HPLC) separation were used to compare the retention times of the derivative of naturally occurring CH503 with synthetic standards of the eight possible derivatized stereoisomers. Each of the derivatized stereoisomers could be differentiated based on their distinct retention times (Fig. 1B). D. melanogaster previously was shown to express (3R,11Z,19Z)-CH503 [hereafter abbreviated as (R,Z,Z)-CH503] (20). HPLC analysis of derivatized thin-layer chromatography-purified fractions revealed a single peak corresponding to (R,Z,Z)-CH503, indicating that as with D. melanogaster, D. simulans, D. yakuba, and D. sechellia express a single stereo-isomer (Fig. 1B). Although a compound with a mass signal corresponding to (R,Z,Z)-CH503 was observed by UV-LDI MS analysis in D. ananassae, the retention time did not match any of the eight stereoisomers. Taken together, chemical profiling with UV-LDI MS and HPLC reveals that all tested drosophilids of the melanogaster subgroup express (R,Z,Z)-CH503. In contrast, other drosophilids representing outgroup taxa from Sophophora (D. willistoni, D. ananassae) and Drosophila (D. virilis, D. mojavensis) do not express (R,Z,Z)-CH503 or any other stereoisomer. The phylogenetic distribution of the trait suggests a single origin in the melanogaster group of Drosophila.

**Conserved Function of CH503 as an Antiaphrodisiac.** We next tested whether the function of CH503 as an antiaphrodisiac is conserved across the different species. Socially isolated virgin males were placed with a virgin female perfumed with various amounts of CH503. Surprisingly, all Drosophila species tested suppressed courtship initiation in a dose-dependent manner in response to CH503, although the pheromone is produced only by a subgroup of these species. Male courtship behavior was significantly inhibited in D. melanogaster, D. simulans, D. yakuba, and D. sechellia, all species that produce CH503 (Fig. 24). The latency to courtship initiation also increased significantly in a dose-responsive manner to increasing amounts of CH503 (Fig. S1). Notably, D. ananassae, D. willistoni, D. mojavensis, and D. virilis, none of which expresses CH503, also responded to the compound by suppressing courtship behavior and delaying courtship initiation (Fig. 2A, Fig. S1, and Table S1). These findings indicate that the behavioral response to CH503 predates the expression of CH503. We hypothesize that males of the ancestral species that gave rise to the melanogaster subgroup used sensory exploitation to inhibit courtship from male competitors.

To test whether production of CH503 might provide an advantage in male–male competition for access to females, we examined the effect of introducing (R,Z,Z)-CH503 into the mating system of species that do not produce the pheromone. D. ananassae and D. virilis males were given a choice of mating with CH503-perfumed or solvent-perfumed females. In both species, males showed a significant aversion to courting the former (Fig. 2F). Thus, the use of a potent antiaphrodisiac by males could significantly shift courtship choice of rival males. By limiting access to mated females, male producers of the pheromone might potentially gain a mating advantage by reducing sperm competition.
is particularly striking in between the strength of the natural vs. unnatural pheromone stereoisomer, (R,Z,Z)-CH503 functions as an antiaphrodisiac both in species that produce the natural pheromone (A) and in species that do not (B). Doses (in nanograms) indicated represent the approximate amount on the cuticular surface following perfuming. The courtship initiation percentage for each dose is observed in all tested species. In species that produce CH503 (C), the artificial stereoisomer is more potent than the natural pheromone. In species that do not produce CH503 (D), the artificial stereoisomer and the natural pheromone are effective over a similar range of doses. Statistical analysis was performed as in A. (E) in courtship choice assays, D. ananassae and D. virilis males spent a greater amount of time courting nonperfumed females over (R,Z,Z)-CH503-perfumed females. D. melanogaster males preferentially courted nonperfumed females over females perfumed with (S,Z,Z)-CH503. In the presence of both stereoisomers, males preferentially courted females perfumed with the natural pheromone. No effect on courtship was observed when females were perfumed with an equivalent dose of a CH503 analog (TB-CH503). The ends of the box plot represent the spread of data between the 25th (bottom) and the 75th percentile (top), whereas the horizontal line represents the median and + represents the mean. Courtship vigor within each pairing was compared using a Wilcoxon rank-sum test. ****P < 0.0001; **P = 0.0032; *P = 0.0397; n = 21–29.

**Adaptive Response to Sensory Exploitation.** It is well established that the biological activity of pheromones can be highly dependent on the stereocstructure (21). To determine whether the stereocstructure of CH503 is important for its function as an antiaphrodisiac, we tested the effect of a synthetic stereoisomer of CH503, (S,Z,Z)-CH503, a stereoisomer not known to be expressed naturally by drosophilids (Fig. S2). Unexpectedly, D. melanogaster, D. simulans, D. yakuba, and D. sechellia exhibited stronger courtship suppression and a longer latency to initiate courtship in the presence of the artificial stereoisomer compared with the natural pheromone at equivalent doses (Fig. 2C, Fig. S2, and Table S2). When D. melanogaster males were given a choice of females perfumed with (S,Z,Z)-CH503 or a nonperfumed female, males preferred to court the latter (Fig. 2E). Even in the presence of a female perfumed with an equivalent amount of the wild-type stereoisomer, (R,Z,Z)-CH503, males continued to show a stronger aversion to the unnatural pheromone (Fig. 2E). The disparity between the strength of the natural vs. unnatural pheromone is particularly striking in D. simulans, in which nearly 15 times the amount of the natural pheromone is needed to achieve the same level of courtship inhibition produced by the artificial stereoisomer. In contrast, males of D. ananassae, D. willistoni, D. virilis, and D. mojavensis exhibited an equivalent or greater responsiveness to the wild-type form of CH503 compared with the artificial stereoisomer (Fig. 2D, Fig. S2, and Table S2). Thus, species that produce the pheromone exhibit a weaker behavioral response to the natural compound, whereas nonproducers of the pheromone are more sensitive to the pheromone (Fig. 3).

The lowered sensitivity exhibited by CH503-producing species to the natural pheromone may be the result of sensory adaptation or habituation from exposure to the pheromone from conspecifics. However, adaptation likely is not a contributing factor because males were individually housed during the late pupal stage and remained isolated until testing at 4–5 d old. Furthermore, the response of D. melanogaster males collectively raised in groups was not significantly different from that of individually housed flies (Fig. S3). Thus, even in the presence of heightened preexposure to CH503, the differential sensitivity exhibited by
*D. melanogaster* to the natural and artificial pheromone remained unchanged.

Courtship suppression in the presence of *(R,S,Z,Z)*-CH503 might simply be the result of aversion to a foreign chemical or masking of endogenous female pheromones. To address this possibility, males of all species were tested with females perfumed with an equivalent dose of modified *(S,Z,Z)*-CH503 or *(R,Z,Z)*-CH503 analogs bearing either two triple bonds (instead of two double bonds) or a single double bond only. In each case, no significant courtship suppression was observed (Tables S1 and S2; Fig. 2E). These findings indicate that first, *melanogaster* subgroup males have evolved partial resistance to the antiaphrodisiac effects of the natural pheromone, and second, the behavioral response is specific to the stereochemistry of *(R,S,Z,Z)*-CH503.

Our findings support the hypothesis that in *Drosophila*, an antiaphrodisiac pheromone expression evolved as a result of sensory exploitation. All tested species, regardless of their endogenous pheromone expression, suppressed courtship in response to the natural pheromone, *(R,Z,Z)*-CH503. Because *(R,Z,Z)*-CH503 originates only in the *melanogaster* subgroup, response to the pheromone predates expression of the pheromone. In contrast to previous findings that identified attractive superstimulants, these results show that preexisting sensory systems for highly aversive cues also may be exploited and that sensory exploitation also takes place between males.

In *D. melanogaster*, multiple pheromones, such as cVA and *(TZ)*-tricosene, and accessory gland proteins are used to suppress female mating frequency (22–25). We speculate that initial use of CH503 helped reinforce courtship inhibition from rival males because, in contrast to other male-transferred cues, CH503 persists on female cuticles for at least 10 d (18). The low vapor pressure of the pheromone and its behavioral potency would place strong selective pressure for males to become less sensitive to the pheromone to benefit from increased mating opportunities. Our results are consistent with this prediction: members of the *(R,Z,Z)*-CH503–expressing *melanogaster* subgroup have adaptively evolved to become less sensitive to the natural pheromone. In this way, a trait that originated from sensory exploitation drives the evolution of sensory pathways. A similar phenomenon occurs in Goodeidae splitfin fish, where a visual cue originating as a female sensory trap induced sensory adaptation (14). Interestingly, we did not observe sensory adaptation to cVA. Responsiveness to the courtship inhibition properties of the pheromone showed no correlation with whether the species produced the pheromone (Table S3). The multiple functions of cVA as an aggregation cue (26) and aphrodisiac for females (27) likely prevent males from completely losing sensitivity to the pheromone despite the costs of courtship suppression.

The robust behavioral response of *melanogaster* subgroup species to *(S,Z,Z)*-CH503 suggests future opportunities for sensory exploitation. A subtle change in stereochemistry due to allelic variations in genes underlying pheromone production might allow for expression of a stereoisomer variant that would circumvent male adaptation. Behavioral experiments are consistent with this prediction: use of the unnatural pheromone results in significantly greater courtship aversion, even in the presence of the natural pheromone. It will be interesting to consider whether persistent use of *(S,Z,Z)*-CH503 over the long term may contribute to physiological desensitization and also result in disadvantages for the population, such as lowered mating frequency, resulting in reduced offspring fitness (28).

Several mechanisms underlying the evolution of antiaphrodisiacs have been proposed, arguing that chemical diversity in pheromone structures arises as a result of male–female cooperation (23, 29), male–female sexual conflict (30), or male–male competition (31). Our study indicates that sensory exploitation is another mechanism for the evolution of antiaphrodisiacs and traits used in male–male competition. A similar phenomenon whereby synthetic nonnatural stereoisomers elicited a behavioral response stronger than that of the natural pheromone was reported previously in the German cockroach, indicating that sensory exploitation may underlie the evolution of other insect pheromone systems (32). These findings contrast with an alternative mechanism shown in *Nasonia* wasps, in which novel pheromone compounds appear before a preexisting response (33). Currently, the chemosensory receptor(s) and underlying neural circuits mediating CH503 detection are unknown. Once they are identified in *D. melanogaster*, it will be possible to correlate evolution of the receptor(s) structure with behavioral responses of various species and in this way, to understand the underlying neurophysiological mechanisms. Furthermore, determining
molecular markers for the relevant sensory pathways will provide a way to map the evolutionary origins of predisposed sensory biases and the shaping of pheromone structural specificity.

Materials and Methods

*Drosophila* Stocks and Husbandry. Flies were obtained from the Bloomington Stock Centre and Ehime University Stock Center, and maintained on cornmeal agar food at 25°C, 60% humidity, with a 12:12-h light:dark cycle. *D. mojavensis* were wild-caught from Las Bocas, Sonora, Mexico (in March 2009) by W. J. Etges (University of Arkansas) and raised on standard cornmeal agar food supplemented with banana (~110 g per 20 half-perfumed bottles) and cactus powder (~2.3 g per 20 half-perfumed bottles). Adult flies used in courtship assays were isolated at the pupal stage or collected within 4 h after pupal eclosion. Male virgins were raised individually in a test tube containing 2 mL of cornmeal agar food, whereas females were kept in groups of 10 per vial. For grouped-male conditions, adult males were collected at the pupal stage and kept in groups of 10 per vial for 7 d. Flies were tested at the following ages, corresponding to sexual maturity: *D. melanogaster* and *D. simulans*, 4–7 d; *D. sechellia* and *D. yakuba*, 5–7 d; *D. ananassae*, 7 d; *D. virilis* and *D. mojavensis*, 10 d; and *D. willistoni*, 15 d.

**Pheromone Profiling of Drosophila Species Using UV-LDI MS Analysis.** UV-LDI MS analysis and the procedures for preparing the flies were described in detail previously (18). Measurements were performed on a Q-Star Elite (AB SCIEX) orthogonal time-of-flight mass spectrometer equipped with an intermediate pressure oMALDI2 source and an Nd:YAG laser (~337 nm, 40-Hz repetition rate, 200-μm beam diameter, 3-ns pulse duration). Ions were generated in a buffer gas (He or Ne) or a nitrogen gas stream. At least two dozen flies were attached to a coverslip with adhesive tape and mounted onto a custom-built sample plate. During data acquisition, the anogenital region was irradiated for 30 s, corresponding to 1,200 laser shots. For each species, 10–15 socially naïve male flies were measured. Mass accuracy for the mass spectrometer was ~20 ppm. More details about instrumentation conditions and analytical parameters important for the chemical imaging of insects are provided in ref. 34.

**Purification of CH503 from *Drosophila* Species.** Purification of CH503 by thin layer chromatography (TLC) was performed using the conditions described by Yew et al. (18). Approximately 800–1,000 males and females of *D. melanogaster*, *D. simulans*, *D. yakuba*, *D. sechellia*, and *D. ananassae* were extracted with hexane (10 mL) for 20 min at room temperature. The extract was concentrated to dryness using a stream of N2. The residue was dissolved in hexane before separation by TLC. TLC separation was performed on glass-backed silica gel plates (10 × 10 cm, coated with 0.2 mm of silica gel 60; Merck) using a running solvent consisting of hexane/diethyl ether/acetatic acid (66:33:1, each by volume). The major component of the isolated fraction was confirmed as CH503 by Direct Analysis in Real Time (DART) mass spectrometry. The DART ion source was operated in positive-ion mode with helium gas, with the gas heater set to 200 °C. The glow discharge needle potential was set to 3.5 kV. Electrode 1 was set to +150 V, and electrode 2 (grid) was set to +250 V.

**HPLC Analysis.** The separation of eight stereoisomers was determined using a previously described method of derivatization with (1R,2R)- or (1S,2S)-2-[3,3′,4′-antrachendicarboximido)cyclohexanecarboxylic acid and HPLC (20, 35). HPLC separation was performed using a Tosoh DP-8020 pump equipped with a Rhenodyne 7125 sample injector, Jasco FP-920 fluorescence detector, and column heater (Cryocool CC100 II). Data analysis was performed with Chromatocorder 21 (System Instruments).

**Synthesis of CH503 Analogs.** CH503 analogs (R)- and (S)-3-acetoxy-11,19-octacosadiyn-1-ol (triple bond or TB-CH503), and (3R,11Z)- and (3S,11Z)-3-acetoxy-11-octacosan-1-ol (11Z)-dihydro-CH503 were synthesized according to published procedures (36).

**Single-Fly Courtship Behavior Assay.** (R,Z,Z)- and (S,Z,Z)-CH503 were synthesized previously by Mori et al. (37). Perfuming of females with synthetic (R,S,8,Z)-CH503 was performed by gently vortexing live females in a glass vial coated with 2–100 ng of CH503, as previously described (18, 38). The lowest dose tested was 83 ng, corresponding to the approximate amount expressed by males from laboratory Canton-S stocks (37). The same procedures were used for perfuming control flies, except that the vials were coated with hexane alone and the solvent was allowed to evaporate before vortexing. Courtship assays were performed at 23.3 °C, 60% humidity. Perfumed female flies were decapitated and placed in 16 × 9-mm or 35 × 10-mm courtship chambers containing moistened filter paper to maintain humidity. Control trials with hexane-perfumed females were conducted in parallel with experimental trials. For *D. sechellia* and *D. mojavensis*, live females were necessary to induce male courtship behavior. Males were aspirated into the chambers, and features of courtship behavior (latency to initiate courtship, orienting, wing extension and vibration, attempted copulation, and copulation (for live females)) were scored for 30 min. Courtship initiation percentage is defined as the number of trials in which males displayed continuous courtship behavior for at least 1 min. For *D. mojavensis*, 20 s was used as the threshold because of the rapid copulation time (usually between 1 and 2 min). Courtship latency is defined as the amount of time elapsed between aspiration of the male into the chamber and the first display of courtship behavior sustained for at least 1 min. When no courtship is observed during the 30-min trial, the maximum score of 1,800 s is given.

**Two-Choice Courtship Behavior Assays.** A single CH503-perfumed female and a single hexane-perfumed female were decapitated and placed 10–15 mm apart in a 35 × 10-mm courtship chamber. A socially naïve male then was aspirated into the chamber, and courtship behavior was scored for 30 min. Courtship vigor was calculated by normalizing the amount of time spent displaying courtship behaviors toward one target to the total time males spent courting either target. Trials in which courtship lasted for less than 10 s were not considered.

**Statistical Analysis.** Courtship percentages were compared using a Holm–Bonferroni corrected Fisher exact test. For courtship latencies, a Kruskall–Wallis test followed by a Wilcoxon rank sum test was applied. All analyses were performed using GraphPad Prism 6.

**Mapping of CH503 Expression and Behavioral Response onto the Phylogeny.** Character mapping was performed with Mesquite 2.75 (39) using a linear parsimony model. Presence or absence of CH503 expression was treated as an unordered character state. The behavioral response to natural CH503 was represented in terms of a courtship suppression index. The index is a measure of the behavioral response elicited by the natural pheromone (R,Z,Z)-CH503 relative to the artificial stereoisomer (S,Z,Z)-CH503, and was calculated as follows:

\[
R_{cr} = \frac{R_{cr}^{SCP} - R_{cr}^{SCP}}{R_{cr}^{SCP}}
\]

where \(R_{cr}\) is the courtship initiation percentage for (R,Z,Z)-CH503 and \(R_{cr}^{SCP}\) is the courtship initiation percentage for (S,Z,Z)-CH503 at the lowest or second lowest effective dose. To determine whether there is significant phylogenetic signal from the behavioral response to the natural pheromone, the terminal taxa of the tree were shuffled 10,000 times. The likelihood of the character distribution was greater than expected by chance alone (P = 0.017), indicating that the courtship suppression index exhibits significant phylogenetic signal. *Drosophila* phylogeny was reconstructed based on information from FlyBase (40, 41).

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Supporting Information

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Fig. S1. Courtship latency in the presence of the natural isomer (3R,11Z,19Z)-CH503 [(R,Z,Z)-CH503]. (A and B) Courtship assays reveal that (R,Z,Z)-CH503 increases males’ latency to initiate courtship in a dose-responsive manner for both (R,Z,Z)-CH503–producing species (A) and nonproducing species (B). In trials in which no courtship was observed, 1,800 s (maximum length of the trial) was attributed as latency. Courtship latency also is shown, excluding trials in which no courtship was observed (C and D). The ends of the vertical line plot represent the spread of data between the 25th (bottom) and the 75th percentiles (top), whereas the horizontal line represents the median. Courtship latency for each dose is compared with control values using a Kruskal–Wallis test followed by a Wilcoxon rank-sum test. ns, nonsignificant. *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001; unless otherwise stated, n = 24–30 for each dose. Doses are shown in nanograms.

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Fig. S2. Courtship latency in the presence of the artificial isomer (S,Z,Z)-CH503. (A) Chemical structure of (S,Z,Z)-CH503. (B and C) Courtship assays reveal that (S,Z,Z)-CH503 increases males’ latency to initiate courtship in a dose-responsive manner for both (R,Z,Z)-CH503–producing species (B) and nonproducing species (C). In trials in which no courtship was observed, 1,800 s (maximum length of the trial) was attributed as latency. Courtship latency also is shown, excluding trials in which no courtship was observed (D and E). The ends of the vertical line plot represent the spread of data between the 25th (bottom) and the 75th percentiles (top), whereas the horizontal line represents the median. Courtship latency for each dose is compared with control values using a Kruskal–Wallis test followed by a Wilcoxon rank-sum test. ns, nonsignificant; *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001; unless otherwise stated, n = 24–30 for each dose. Doses are shown in nanograms.
Fig. S3. Group-housed and isolated males respond similarly to (R,S,Z,Z)-CH503. Compared with controls, both grouped and isolated males exhibit significant courtship suppression in the presence of 700 ng of (R,Z,Z)-CH503 or 83 ng of (S,Z,Z)-CH503. Grouped and individually housed Drosophila melanogaster males showed similar levels of courtship suppression. Courtship initiation percentage was statistically assessed using a Holm–Bonferroni-corrected Fisher exact test. ns, nonsignificant. Grouped males: **$P = 0.0026$; ***$P = 0.0002$. Isolated males: **$P = 0.0046$ and $P = 0.0017$ for 700 ng of (R,Z,Z)-CH503 and 83 ng of (S,Z,Z)-CH503, respectively; $n = 24–36$ for each dose.
Table S1. Courtship inhibition percentage resulting from various doses of (R,Z,Z)-CH503, modified TB-(R)-CH503, or single-bond (R)-dihydro-CH503

<table>
<thead>
<tr>
<th>Species</th>
<th>Control, % (n)</th>
<th>83 ng</th>
<th>166 ng</th>
<th>249 ng</th>
<th>415 ng</th>
<th>581 ng</th>
<th>700 ng</th>
<th>830 ng</th>
<th>996 ng</th>
<th>1,245 ng</th>
<th>1,660 ng</th>
<th>2,075 ng</th>
<th>TB-CH503/ (R)-dihydro-CH503, % (ng; n)†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dmel</td>
<td>85 (60)</td>
<td>88 (24)</td>
<td>—</td>
<td>92 (24)</td>
<td>77 (30)</td>
<td>71 (24)</td>
<td>54** (24; ( P = 0.0046 ))</td>
<td>46*** (24; ( P = 0.0006 ))</td>
<td>42*** (24; ( P = 0.0002 ))</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>67 (830; 24)</td>
</tr>
<tr>
<td>Dsim</td>
<td>80 (59)</td>
<td>79 (24)</td>
<td>—</td>
<td>83 (24)</td>
<td>88 (24)</td>
<td>—</td>
<td>—</td>
<td>71 (24)</td>
<td>—</td>
<td>74 (23)</td>
<td>54 (24)</td>
<td>46** (24; ( P = 0.0037 ))</td>
<td>79 (1,660; 23)</td>
</tr>
<tr>
<td>Dyak</td>
<td>78 (68)</td>
<td>93 (30)</td>
<td>—</td>
<td>70 (30)</td>
<td>60 (30)</td>
<td>63 (24)</td>
<td>—</td>
<td>50** (30; ( P = 0.0086 ))</td>
<td>37*** (30; ( P = 0.0002 ))</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>79 (830; 24)</td>
</tr>
<tr>
<td>Dsec</td>
<td>80 (55)</td>
<td>54* (24; ( P = 0.0284 ))</td>
<td>—</td>
<td>50* (22; ( P = 0.0125 ))</td>
<td>41*** (29; ( P = 0.0006 ))</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>67 (415; 24)</td>
</tr>
<tr>
<td>Dana</td>
<td>98 (61)</td>
<td>75 (24)</td>
<td>—</td>
<td>57** (30; ( P = 0.0017 ))</td>
<td>47**** (30)</td>
<td>—</td>
<td>—</td>
<td>22**** (36)</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>96 (830; 24)</td>
</tr>
<tr>
<td>Dwil</td>
<td>74 (66)</td>
<td>54 (24)</td>
<td>38** (24; ( P = 0.0024 ))</td>
<td>17**** (24)</td>
<td>17**** (24)</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>67 (415; 24)</td>
</tr>
<tr>
<td>Dmoj</td>
<td>94 (49)</td>
<td>60*** (30; ( P = 0.0006 ))</td>
<td>—</td>
<td>45**** (29; ( P &lt; 0.0001 ))</td>
<td>28**** (29)</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>73 (415; 30)</td>
</tr>
<tr>
<td>Dvir</td>
<td>91 (75)</td>
<td>43**** (30)</td>
<td>33**** (24)</td>
<td>21**** (24)</td>
<td>0**** (24; ( P &lt; 0.0001 ))</td>
<td>—</td>
<td>—</td>
<td>0**** (24; ( P &lt; 0.0001 ))</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>91 (166; 22)</td>
<td></td>
</tr>
</tbody>
</table>

*Compared with control values using a Holm–Bonferroni-corrected Fisher exact test. (**P < 0.05; ***P < 0.01; ****P < 0.001; *****P < 0.0001.)

†The dose at which triple-bond (TB)-(R)-dihydro-CH503 was tested is equivalent to the lowest effective dose of (R,Z,Z)-CH503 for each species; Dsec was tested with (R),19Z-dihydro-CH503 and Dmoj was tested with (R),11Z-dihydro-CH503.
Table S2. Courtship inhibition percentage resulting from various doses of (S,Z,Z)-CH503, modified TB-(S)-CH503, or single-bond (S)-dihydro-CH503.

<table>
<thead>
<tr>
<th>Species</th>
<th>Control, % (n)</th>
<th>83 ng†</th>
<th>166 ng</th>
<th>249 ng</th>
<th>332 ng</th>
<th>415 ng</th>
<th>830 ng</th>
<th>1,245 ng</th>
<th>TB(S)-dihydro-CH503, % (ng; n)‡</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Dmel</em></td>
<td>85 (60)</td>
<td>50** (24; (P = 0.0017))</td>
<td>—</td>
<td>46** (24; (P = 0.0006))</td>
<td>—</td>
<td>25**** (24)</td>
<td>—</td>
<td>—</td>
<td>83 (83; 24)</td>
</tr>
<tr>
<td><em>Dsim</em></td>
<td>80 (59)</td>
<td>33**** (30)</td>
<td>—</td>
<td>3**** (30)</td>
<td>—</td>
<td>0**** (24)</td>
<td>—</td>
<td>—</td>
<td>71 (83; 24)</td>
</tr>
<tr>
<td><em>Dyak</em></td>
<td>78 (68)</td>
<td>58 (36; (P = 0.0429))</td>
<td>—</td>
<td>3**** (36; (P = 0.0072))</td>
<td>—</td>
<td>38**** (34; (P = 0.0001))</td>
<td>—</td>
<td>—</td>
<td>79 (415; 24)</td>
</tr>
<tr>
<td><em>Dsec</em></td>
<td>80 (55)</td>
<td>54* (21; 0.0284)</td>
<td>—</td>
<td>4**** (24)</td>
<td>—</td>
<td>9**** (23)</td>
<td>—</td>
<td>—</td>
<td>71 (249; 24)</td>
</tr>
<tr>
<td><em>Dana</em></td>
<td>98 (61)</td>
<td>92 (24)</td>
<td>—</td>
<td>—</td>
<td>79** (24; (P = 0.0062))</td>
<td>63**** (24)</td>
<td>38**** (24)</td>
<td>78 (2,075; 23)</td>
<td></td>
</tr>
<tr>
<td><em>Dwil</em></td>
<td>74 (66)</td>
<td>62 (26)</td>
<td>—</td>
<td>58 (24)</td>
<td>29**** (24; (P = 0.0002))</td>
<td>8**** (24)</td>
<td>—</td>
<td>—</td>
<td>75 (415; 24)</td>
</tr>
<tr>
<td><em>Dmoj</em></td>
<td>94 (49)</td>
<td>77* (30; (P = 0.037))</td>
<td>—</td>
<td>50**** (30)</td>
<td>—</td>
<td>33**** (30)</td>
<td>—</td>
<td>—</td>
<td>77 (415; 30)</td>
</tr>
<tr>
<td><em>Dvir</em></td>
<td>91 (75)</td>
<td>61*** (36; (P = 0.0005))</td>
<td>57**** (24; (P = 0.0006))</td>
<td>25**** (24)</td>
<td>—</td>
<td>21**** (36)</td>
<td>6**** (36)</td>
<td>—</td>
<td>92 (83; 24)</td>
</tr>
</tbody>
</table>

*Compared with control values using a Holm–Bonferroni-corrected Fisher exact test. (**P < 0.05; ***P < 0.01; ****P < 0.001.)

†The dose at which triple-bond (TB)(S)-dihydro-CH503 was tested is equivalent to the lowest effective dose of (S,Z,Z)-CH503 for each species; *Dsec* and *Dmoj* were tested with (S,11Z)-dihydro-CH503.

*Dana*, Drosophila ananassae; *Dmel*, Drosophila melanogaster; *Dmoj*, Drosophila mojavensis; *Dsec*, Drosophila sechellia; *Dsim*, Drosophila simulans; *Dvir*, Drosophila viridis; *Dwil*, Drosophila willistoni; *Dyak*, Drosophila yakuba.
Table S3. Courtship suppression by cVA

<table>
<thead>
<tr>
<th>Species</th>
<th>cVA production</th>
<th>Lowest concentration required for significant inhibition, ng</th>
</tr>
</thead>
<tbody>
<tr>
<td>D. melanogaster</td>
<td>Yes (1,400 ng/ffly) (1)</td>
<td>830</td>
</tr>
<tr>
<td>D. simulans</td>
<td>Yes (8–900 ng/ffly) (2)</td>
<td>83</td>
</tr>
<tr>
<td>D. yakuba</td>
<td>Yes (800 ng/ffly) (3)</td>
<td>83</td>
</tr>
<tr>
<td>D. sechellia</td>
<td>Yes* 250</td>
<td></td>
</tr>
<tr>
<td>D. ananassae</td>
<td>Yes (52 ng/ffly) (4)</td>
<td>625</td>
</tr>
<tr>
<td>D. willistoni</td>
<td>No</td>
<td>665</td>
</tr>
<tr>
<td>D. mojavensis</td>
<td>No</td>
<td>250</td>
</tr>
<tr>
<td>D. virilis</td>
<td>No</td>
<td>830</td>
</tr>
</tbody>
</table>

cVA, cis-vaccenyl acetate.
*Signal detected using UV laser desorption/ionization MS measurements.