A mosaic of endogenous and plant-derived courtship signals in moths

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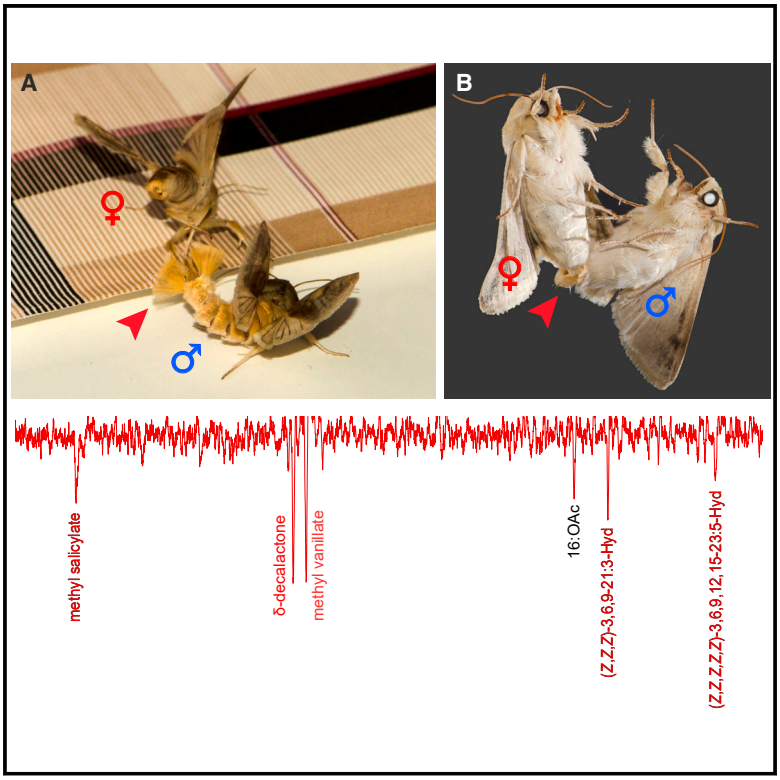
Graphical abstract



Highlights

- The courtship pheromone of male *Chloridea virescens* moths was re-examined
- The pheromone constitutes a mosaic of type I, type II, and plant-derived compounds
- Methyl salicylate (MeSA) is an aphrodisiac that improves mating success
- Two olfactory receptors contribute to high olfactory sensitivity of females to MeSA

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In brief

Liu et al. identify novel chemicals, including methyl salicylate (MeSA), that male moths use during courtship to increase mating success. Two olfactory receptors on female antennae detect MeSA. The male sex pheromone is a mosaic blend that includes fatty acid derivatives, unsaturated hydrocarbons, and sequestered plant-derived compounds.

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Report

A mosaic of endogenous and plant-derived courtship signals in moths

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SUMMARY

Insects rely on olfaction to guide a wide range of adaptive behaviors, including mate and food localization, mate choice, oviposition site selection, kin recognition, and predator avoidance.[1] In nocturnal insects, such as moths[2] and cockroaches,[3] mate finding is stimulated predominantly by long-range species-specific sex pheromones, typically emitted by females. During courtship, at close range, males in most moth species emit a blend of pheromone compounds from an everted, often large, pheromone gland. While long-distance communication with sex pheromones has been remarkably well characterized in thousands of moth species,[2,4] close-range chemosensory sexual communication remains poorly understood. We reveal that in the moth *Chloridea virescens*, the male pheromone consists of three distinct classes of compounds: *de novo* biosynthesized alcohols, aldehydes, acetates, and carboxylic acids that resemble the female's emissions; newly identified compounds that are unique to the male pheromone, such as aliphatic polyunsaturated hydrocarbons; and sequestered plant secondary compounds and hormone derivatives, including methyl salicylate (MeSA). Thus, males employ a mosaic pheromone blend of disparate origins that may serve multiple functions during courtship. We show that two olfactory receptors in female antennae are tuned to MeSA, which facilitates female acceptance of the male. Because MeSA is emitted by plants attacked by pathogens and herbivores,[5] the chemosensory system of female moths was likely already tuned to this plant volatile, and males appear to exploit the female's preadapted sensory bias. Interestingly, while female moths (largely nocturnal) and butterflies (diurnal) diverged in their use of sensory modalities in sexual communication,[6] MeSA is used by males of both lineages.

RESULTS AND DISCUSSION

New sex pheromone components

Moth sex pheromones are usually emitted by stationary, relatively concealed "calling" females, attracting males over meters to kilometers. Research on this paradigm of females as signalers and males as receivers has been valuable for understanding the partitioning of chemical communication channels, evolution of sexual communication,[7,8] and olfactory neuroethology,[9,10] and for generating innovative tactics for pest detection, monitoring, and suppression.[4,11] However, the emphasis on this mate-finding strategy has largely ignored other important features of moth sexual communication, namely close-range chemosensory interactions, which include the emission of male sex pheromones, female assessment of the male's pheromone blend, and ultimately acceptance or rejection of the male,[12,13] in some cases in concert with acoustic sexual signaling.[14] Male sex pheromones are usually emitted from male-specific organs—hairpencils (HPs) in moths and androconial scales in butterflies.

Male sex pheromones play important roles in various steps of the courtship and mating sequence. In some species, they attract females over a short distance,[15,16] but in many species male courtship pheromones function as "aphrodisiacs," elevating the female's receptivity and increasing her likelihood to copulate.[17] This function is most directly confirmed through experimental ablation of the male's scent disseminating structures, a procedure that has been shown to decrease the probability of mating, as in the yellow peach moth, for example.[18] In some moth species, male courtship pheromones also inhibit female calling, presumably to suppress attraction of rival males,[19] while in others it directly antagonizes attraction of rival males, thus lessening intrasexual competition from conspecific males and representing a form of chemical mate-guarding.[19–21] In some butterflies and moths, male pheromones have dual functions as both female aphrodisiacs and male anti-aphrodisiacs.[6]

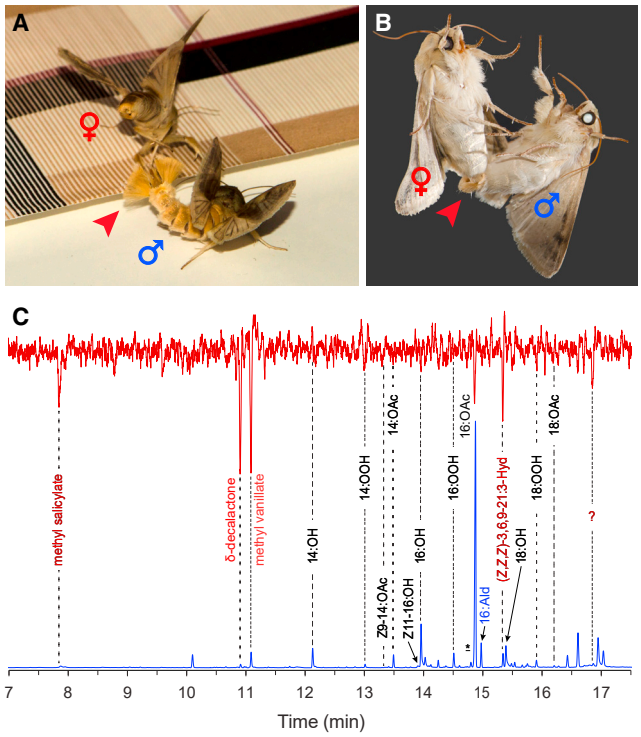

Figure 1. Male *C. virescens* hairpencil display during courtship and chemistry of the hairpencils

(A) Male hairpencil display showing the elaborate everted hairpencils (red arrow).

(B) During mating, the hairpencils are seen (red arrow), enveloping the female's external genitalia.

(C) GC-EAD responses of *C. virescens* female antennae to male hairpencil extract. The flame ionization detector (FID) trace of the male extract is on the bottom (blue), and a representative female EAD response is on top (red). Of the previously identified peaks, only 16:OAc, which occurs at approximately 200 ng, elicited consistent (14 out of 16 female antennae) but relatively low EAD responses. Only 2 of 16 female antennae responded to 14:OAc, 5 of 16 to 16:OH, 8 of 16 to 16:OOH, 6 of 16 to Z7-16:OAc, 2 of 16 to 18:OH, 5 of 16 to 18:OOH, and 1 of 16 to 18:OAc, but all were substantially smaller than the response to 16:OAc. Z7-16:OAc, Z9-16:OAc, and Z11-16:OAc are represented by an asterisk (*). Newly identified compounds that elicited strong EAD responses are indicated in red and include methyl salicylate (Kovats index [KI] on DB-5 = 1,202; 13 out of 16 female antennae responded), δ-decalactone (KI = 1,505; 16 of 16 antennae responded), methyl vanillate (KI = 1,526; 14 of 16 responded), (*Z*,*Z*,*Z*)-3,6,9-heneicosatriene (KI = 2,078; 16 of 16 responded), and an unknown at 16.85 min that we tentatively identified as (*Z*,*Z*,*Z*,*Z*,*Z*)-3,6,9,12,15-tricosapentaene (KI = 2,276; 15 of 16 antennae responded). 16:Ald (in blue) is a newly identified compound that elicited small EAD responses in only 2 of 16 preparations. Photos: Jan van Arkel.

See also Data S1A.

The sex pheromones of moths can be divided into five types according to their chemical structures and biosynthetic origin.[22] Most sex pheromones of moths are type I pheromones; they are *de novo*-produced aliphatic fatty acid derivatives with a terminal alcohol, acetate, or alcohol group, similar to female sex pheromone components.[22,23] Type II pheromones include polyunsaturated hydrocarbons and epoxides derived from linoleic acid, linolenic acid, and other polyunsaturated fatty acids. Type III pheromone compounds are short-chain methyl-branched hydrocarbons, while type 0 pheromones are short-chain secondary alcohols and their corresponding methyl ketones.[22] The last group of sex pheromones are modified from sequestered plant-produced alkaloids; for example, hydropyrrolizines are derived from pyrrolizidine alkaloids that the male also use as nuptial gifts and for protection of his mate and her eggs.[12,24,25]

Chloridea (formerly *Heliothis*) *virescens* (*C. virescens*) is a generalist herbivore of a wide variety of agricultural crops in several plant families.[26] Typical of many moth species, the female biosynthesizes a blend of type I saturated and unsaturated 14- and 16-carbon aldehydes and alcohols.[27] During courtship, the male displays elaborate HPs (Figure 1A), which contain a related but even more complex blend of 14 saturated and unsaturated 14-, 16-, and 18-carbon acetates, alcohols, and acids (Data S1A).[28] Although the emission and function of each component are unclear, generally 7 of these components have been considered as the male sex pheromone.[28,29] The male HP pheromone affects the mating system in several ways. First, high concentrations of male HP extracts inhibited female sex pheromone emission in field studies.[30] Second, HP extract and some of its single components had aphrodisiac effects, increasing the female's acceptance behaviors; the probability of mating decreased after removal of the HPs, but it could be partially restored after the addition of HP extract.[29] Third, the female preferred conspecific male pheromone blends over those of closely related species, showing that the species-specific male HP pheromone blend can function in species recognition.[29] Fourth, in flight-tunnel experiments, two *C. virescens* HP components—16:OAc and 18:OAc—suppressed the navigational responses of conspecific males, suggesting that during courtship the male sex pheromone may function as an antagonist to rival male responses.[31] During copulation (Figure 1B), the HPs "perfume" the female with male pheromone, which functions as an anti-aphrodisiac in the female's subsequent sexual interactions.[21]

Using gas chromatography (GC) and GC-mass spectrometry (GC-MS), we detected all 14 compounds that were previously identified in male HPs.[28] However, using antennal electrophysiological recordings (GC-electroantennogram detection [EAD]), only 16:OAc elicited consistent responses at a dose of 0.5 male-equivalent (i.e., ~100 ng 16:OAc) (Figure 1C). In addition, GC-EAD analyses revealed several new electrophysiologically bioactive peaks in HP extracts. Methyl salicylate (MeSA), δ-decalactone, methyl vanillate, and (*Z*,*Z*,*Z*)-3,6,9-heneicosatriene were unambiguously identified based on their retention indices, MS spectra, and co-injection with synthetic standards on both non-polar and polar columns. These compounds were barely detectable in GC analysis, but they elicited much larger EAD responses than 16:OAc, the main pheromone component (Figure 1C). In addition, *C. virescens* female antennae were highly responsive to a peak that we tentatively identified as (*Z*,*Z*,*Z*,*Z*,*Z*)-3,6,9,12,15-tricosapentaene, but its structure remains to be unambiguously elucidated. MeSA, δ-decalactone, and methyl vanillate represent compounds sequestered from plants, whereas (Z,Z,Z)-3,6,9-heneicosatriene and the tentatively characterized (Z,Z,Z,Z,Z)-3,6,9,12,15-tricosapentaene constitute type II pheromones. Thus, unlike all previously described moths, *C. virescens* males use a unique mosaic of pheromone components of different types and different biosynthetic origins in their close-range communication with females.

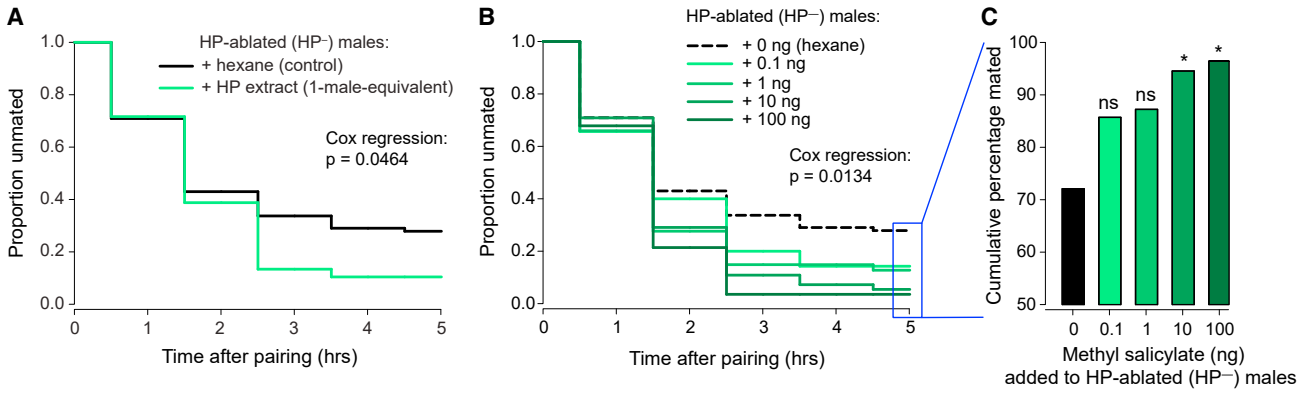

Figure 2. Role of methyl salicylate in *C. virescens* mating

(A) Cumulative mating rate of HP-ablated (HP^-) 2-day-old *C. virescens* males during a 5-h observation period. A group of HP^- control males was treated with the hexane vehicle (n = 86) and another group of HP^- males (n = 67) received 1 male equivalent of an HP extract. Survival analysis of the time course of mating was conducted using the Cox regression model: beta (± SE) = 0.36 ± 0.18; hazard ratio (95% CI) = 1.440 (1.0–2.06); Wald test statistic = 3.97; p = 0.0464.

(B) The time course of mating of HP^- males (treated with 0 ng hexane) with normal female *C. virescens* can be rescued by the addition of MeSA.

(C) The respective cumulative responses at 5 h. Sample sizes for 0, 0.1, 1, 10, and 100 ng methyl salicylate were 86, 35, 47, 55, and 28 pairs. Survival analysis of the time course of mating was conducted using the Cox regression model shown in (B), followed by Dunnett's multiple comparisons relative to the 0 ng treatment, shown in Table S1.

See also Figures S1–S4.

Role of HPs in mating

To conduct behavioral assays with ablated HPs, it was imperative to determine where the putative pheromone components were localized (Figures S1A and S1B) and thus minimize damage to the male while maximizing the thoroughness of the ablation. Both 16:OAc and MeSA were similarly distributed across the six HP structures, with the bilateral long hairs (structure-2) containing significantly more of these two compounds than any other structures (Figures S1E and S1F). GC analysis confirmed that ablation of structure-2 (Figures S1C and S1D) significantly reduced the titer of 16:OAc by 77% (Figures S1G and S1H). Therefore, we ablated only the structure-2 HPs to minimize damage to the male. Also, because MeSA and δ-decalactone peaked in the HPs of 2-day-old males (Figure S2), we used 2-day-old virgin *C. virescens* females and males to investigate the time course of mating.

Mating frequency of unoperated males increased from 27.3% after 1 h to 100% after 4 h (Figure S3A). Mating in sham-operated males (HP^+) increased from 27.8% (1 h) to 94.4% (5 h), which was not significantly different from the unoperated males (CoxHP: HR = 0.93, Z = −0.217, p = 0.828). On the other hand, mating success in HP-ablated males (HP^-) was 20% at 1 h and rose to only 70% at 5 h, significantly less than in the combined HP-non-ablated group (CoxHP: HR = 0.49, Z = 5.1, p = 0.024) (Figure S3A).

When we supplemented HP-ablated males with 1 male equivalent of HP extract, their mating rate increased significantly from 28.4% at 1 h to 89.6% at 5 h, significantly more than in the control group (hexane added to HP-ablated males) in which it increased from 29.1% (1 h) to 72.1% (5 h), like the HP-ablated-only treatment group (Figure 2A).

Of the newly identified compounds in the HP extract, we chose to investigate the role of MeSA in courtship and mating, because this compound is a common pest- and pathogen-induced plant volatile that is generally detectable by many insect herbivores.[5]

The addition of 0.1 to 100 ng MeSA to HP-ablated males elevated their mating rate in a dose-dependent manner (Figure 2B). Low doses (0.1 and 1 ng) raised mating success, but not significantly above the control treatment (0 ng). However, supplementation of HP-ablated males with 10 and 100 ng of MeSA significantly restored mating success (Cox regression and Dunnett's multiple comparisons relative to 0 ng) (Figure 2C; Table S1), achieving levels comparable to the addition of the whole HP extract (Figure 2A).

MeSA sequestration from diet

Males reared in the laboratory on a synthetic diet had low amounts of MeSA, whereas males freshly collected in a soybean field had significantly more MeSA in their HPs (Figure 3A). When male larvae were fed soybean leaves or artificial larval diet spiked with MeSA, significantly elevated amounts of MeSA were detected in the HPs of the adult males (Figures 3B and 3C). The most effective means to increase MeSA in the HP gland was allowing adult males *ad libitum* access to MeSA-supplemented sugar water (Figure 3D). By using both MeSA-fed and sugar-fed control males in mating assays, we showed that when males were stimulated to vigorously court females, the amount of MeSA in their HP glands declined significantly (Figure 3E), indicating that MeSA, a highly volatile semiochemical, was emitted during courtship. Notably, males that did not evert their HPs in courtship displays in the presence of a calling female retained significantly more MeSA in their HPs, suggesting mobilization of MeSA from internal storage sites into the HPs.

Olfactory receptors tuned to MeSA

In our previous work on ORs in *Helicoverpa armigera*, a phylogenetically related species to *C. virescens*, we identified 44 ORs involved in detection of plant volatiles, 2 of which—HarmOR27 and HarmOR43—also responded to MeSA.[32] We cloned these two orthologous ORs (CvirOR27 and CvirOR43) from the

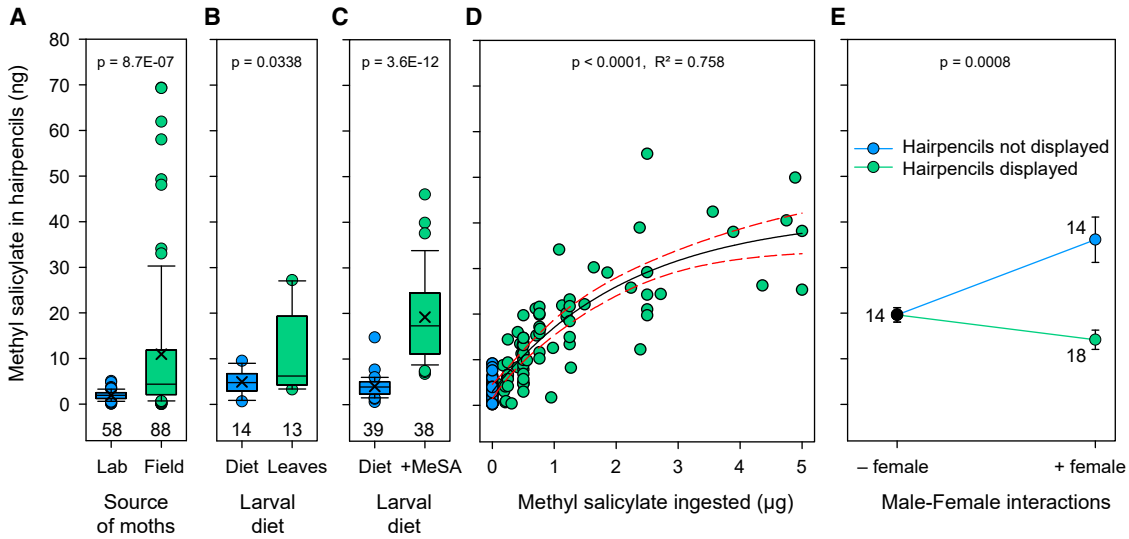

Figure 3. Methyl salicylate sequestration and its mobilization in hairpencils of *C. virescens* males

(A–C) Methyl salicylate is significantly more abundant in hairpencils of (A) field-collected males and (B) in males fed as larvae on soybean leaves or (C) on artificial diet supplemented with 1 μg MeSA/g of wet diet.

(D) Adult males fed ~50 μL sugar water containing various amounts of MeSA.

(E) Two-day-old males were fed MeSA-supplemented sugar water (1 μg MeSA/male). The following day the males were placed in an arena (A) without a female (– female), so no courtship displays were performed and the hairpencils were never everted, or (B) with a female (+ female) whose abdomen was skirted so she could emit pheromone, but not mate. Males were separated into those that courted the female, everting the hairpencils, or ones that were near the female but did not display the hairpencils. Males were allowed 3 h in the arena, then removed, and their hairpencils were extracted 30 min later.

antennal cDNA of *C. virescens* to clarify their function in MeSA detection. CvirOR27 and CvirOR43 contain the complete open reading frames (ORFs) of 1,281 and 1,194 bp, which encode 426 and 397 amino acid residues, respectively. The phylogenetic tree of CvirOR27 and CvirOR43, along with 18 previously reported ORs in *C. virescens* and 65 ORs identified in *H. armigera*, provides strong support for orthologous ORs in these 2 species. All 20 ORs in *C. virescens* cluster with their orthologs in *H. armigera* and form separate clades (Figure S4A). CvirOR27 clustered with HarmOR27, and CvirOR43 with HarmOR43, and both clades clustered separately from the Orco and pheromone receptors' clades. CvirOR27 shares 89.7% amino acid sequence identity with HarmOR27 (Data S2A), and CvirOR43 shares 85.1% sequence identity with HarmOR43 (Data S2B).

We co-expressed CvirOR27 and CvirOR43 with CvirOrco in *Xenopus* oocytes and functionally characterized them, using the voltage-clamp recording system with 66 plant volatile odorants (Data S1B). CvirOR27 responded (mean response > 100 nA) to 6 odorants (Figures S4B and S4C), and in dose-response studies, the half-maximal effective concentration (EC_{50}) of MeSA was 8.203×10^{-5} M (Figure 4A). CvirOR43 responded to at least 25 odorants (Figures S4B and S4C), and its EC_{50} to MeSA was 5.854×10^{-5} M (Figure 4B).

Wider perspectives

MeSA is derived from the plant hormone salicylic acid and has been detected in the headspace of many insect-infested plants.[5] It is an important mediator of tritrophic interactions, because it can attract predators and parasitoids of herbivores and thus serves as an active signal for indirect plant defense. Moreover, MeSA has been shown to suppress oviposition on some plant species,[5] yet it stimulates oviposition in others.[33] A variety of insects has been shown to be attracted to MeSA,[34] and in some species the addition of MeSA to synthetic sex pheromone lures significantly increases male trap catches in the field.[35] It is important to note, however, that in these insects MeSA is not a natural part of their pheromone component blends and represents an environmental cue. Our results suggest that MeSA is sequestered either during larval development or in the adult stage from flower nectar and serves as a close-range aphrodisiac pheromone component during courtship, promoting mating success in *C. virescens*. MeSA might also fine-tune species recognition in *C. virescens*, as do other secondary components of pheromone blends.[29] Because females have the sensory, perceptual, and behavioral capacity to detect and respond to MeSA, the use of MeSA by *C. virescens* males as a sexual signal appears to be a case of "sensory bias."[36] Females are likely preadapted to detect MeSA as a plant volatile (kairomone) within their olfactory landscape. Therefore, males might have evolved sexual signals that match the pre-existing olfactory bias of the female's chemosensory system and thus gain an advantage in courtship.

MeSA might also convey to the female the male's larval metabolic fitness, represented by his ability to cope with MeSA-induced plant defenses and to sequester MeSA. Since flowers and nectar are often rich in MeSA, its sequestration and use as a sex pheromone component (i.e., ornament) might also constitute a display of his foraging ability as an adult. Finally, MeSA serves as a strong repellent for some insects.[37] In the polyandrous butterfly *Pieris napi*, MeSA is biosynthesized by males, transferred to the female during copulation, and it ensures the

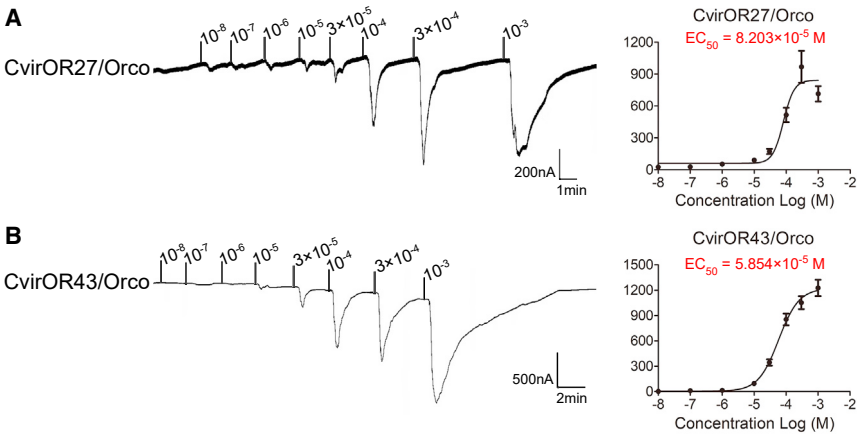

Figure 4. Responses of *Xenopus* oocytes co-expressing CvirOR27/Orco and CvirOR43/Orco to methyl salicylate

(A) Inward current responses of CvirOR27/Orco- and (B) CvirOR43/Orco-expressing *Xenopus* oocytes exposed to various concentrations of methyl salicylate. The respective dose-response curves of CvirOR27/Orco- and CvirOR43/Orco-expressing *Xenopus* oocytes and their EC_{50} values are shown. Error bars indicate SEM.

See also Figures S4B and S4C and Data S1 and S2.

male's paternity by serving as a mate-guarding anti-aphrodisiac that repels males and delays female re-mating.[38] It remains to be determined whether MeSA serves multiple functions in *C. virescens*.

The ubiquity of MeSA in nature, and its widespread use in tritrophic interactions, might imply a potential conflict in its use in sexual signaling. However, the instantaneous (transient) function of MeSA in close-range sexual interactions is unique from its persistent function as a plant volatile. Therefore, we do not anticipate any sensory conflicts in the use of MeSA in different ecological contexts. Indeed, the use of MeSA by male *C. virescens* might be similar to the use of nonanal, another ubiquitous chemical in nature, by fall armyworm (*Spodoptera frugiperda*) females as a sex pheromone blend component[39] and by *Agriphila aeneociliella* as a male sex pheromone component.[40]

The butterfly and moth lineages diverged ~98 million years ago.[41] In most moths, which are largely nocturnal, females emit volatile sex pheromones that attract males over some distance. Conversely, most butterflies are day-active, and long-range mate finding is mainly visual whereas males emit sex pheromones at close-range courtship interactions. Thus, while female butterflies and moths have greatly diverged in their mate-finding strategies, males appear to share the use of aphrodisiac pheromones in courtship. Interestingly, MeSA has been isolated from some male butterflies,[6,42] and in some it has been shown to function in close-range sexual interactions,[43] as we demonstrate here for *C. virescens* males. Thus, MeSA may represent an evolutionary relic of the common ancestor of moths and butterflies, reflected not only in retaining the same semiochemical for communication but also in the same context during male courtship. It is also possible that male moths and butterflies have independently converged on the use of MeSA in courtship.

Our findings highlight a previously unrecognized dimension of close-range sexual signaling in moths, which are the most important agricultural pests globally. We are unaware of any other system where males or females produce or emit a cocktail of pheromone components representing type I, type II, and sequestered plant compounds. It will be intriguing to know if the compounds we identified serve as pheromone components in other moth species. These new compounds also connect the semiochemistry of moths and butterflies and thus contribute significantly to our general understanding of Lepidoptera evolution, the second most species-rich insect order. Finally, male *C. virescens*, as well as other moth and many butterfly species, invests in rather large HP structures that emit a complex assortment of pheromone compounds in courtship displays. A lingering enigma is why males use such a complex medley of signaling molecules in such short-range interactions. We propose that the hybrid blend of molecules from different biosynthetic origins might represent an effective multifunctional signal for species- and sex-recognition, female acceptance, mate-guarding, mate choice, male-male competition, and as a nuptial gift that the female could use during egg-laying to suppress plant defenses.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.cub.2023.07.010.

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AUTHOR CONTRIBUTIONS

Project design, Y.L., J.J.H., J.B., A.T.G, and C.S.; EAD and behavioral experiments, J.J.H., J.B., M.v.W., and A.W.-K.; expression experiments, Y.L., S.Z., and G.W.; data analysis and visualization, Y.L., J.J.H., S.Z., J.B., M.v.W., and C.S.; writing, all authors wrote and revised the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

INCLUSION AND DIVERSITY

We support inclusive, diverse, and equitable conduct of research.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
methyl salicylate	Sigma-Aldrich	Cat#M6752
δ-decalactone	Sigma-Aldrich	Cat#W236128
methyl vanillate	Sigma-Aldrich	Cat#138126
dimethyl sulfoxide (DMSO)	Sigma-Aldrich	Cat#D8418
n-hexane	Thermo Scientific	Cat#AAL09938AU
pentadecyl acetate	Sigma-Aldrich	Cat#P0385
66 odorants – see Data S1B	N/A	N/A
Critical commercial assays		
mMESSAGE mMACHINE T7 kit	Ambion	Cat#AM1344
TIANprep Mini Plasmid Kit	TIANGEN	Cat#DP103-03
Deposited data		
Raw data	This paper	Available on Dryad: https://doi.org/10.5061/dryad.rbnzs7hhd
CvirOR27	This paper	GenBank: OR134762
CvirOR43t	This paper	GenBank: OR134763
Experimental models: Cell lines		
Trans1-T1 Phage Resistant Chemically Competent Cell	TransGen Biotech	Cat#L2940913
Experimental models: Organisms/strains		
Chloridea virescens	Field-collected in North Carolina, USA	N/A
Oligonucleotides		
Primers for pT7TS expression vector construction, CvirOR27-F: ATCACTAGTGGGCCCGCCACCATG TTGTCAAAGATAAAGAATATTA	This paper	N/A
Primers for pT7TS expression vector construction, CvirOR27-R: CTAGTCAGTCGCGGCCGCTCACT CTTTTTTTCCCAACA	This paper	N/A
Primers for pT7TS expression vector construction, CvirOR43-F: TCAGGGCCCGCCACCATGGGGCT GATGGTGAAGAATG	This paper	N/A
Primers for pT7TS expression vector construction, CvirOR43-R: TCAGCGGCCGCTTATTTCGATTTA TTCCGTTCTTC	This paper	N/A
Recombinant DNA		
pT7TS expression vector	N/A	N/A
Software and algorithms		
Agilent OpenLab CDS	Agilent	Ver. A.01.07
Wiley 7/NIST 05 database	Wiley	N/A
MEGA X	N/A	https://www.megasoftware.net/
pCLAMP 10.2 software	Molecular Devices	https://www.moleculardevices.com/products/axon-patch-clamp-system/acquisition-and-analysis-software/pclamp-software-suite
JMP Pro 17.0.0	SAS	Ver. 622753
R (v. 4.1)	N/A	https://cran.r-project.org/

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Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Other		
cups (arenas)	Fabri-Kal	Cat#KC9OF
Nanoliter 2010 injection system	WPI	https://www.wpiinc.com/blog/post/nanoliter-2010-perfect-for-microinjection
OC-725C voltage clamp	Warner Instruments	https://www.warneronline.com/sites/default/files/2018-09/OC-725C_Oocyte_Clamp_PDF_Rev1.1_0.pdf
Digidata 1440A data acquisition	Axon Instruments	https://www.moleculardevices.com/sites/default/files/en/assets/user-guide/dd/cns/digidata-1440a-low-noise-data-acquisition-system.pdf
5890 GC	Agilent	N/A
6890N GC	Agilent	N/A
7890A GC	Agilent	N/A
5975 MS	Agilent	N/A
EC-5 column	Alltech	Cat#19647
DB-5	Agilent	Cat# 122-5032
EC-Wax column	Alltech	Cat#5121767
HP-5ms	Agilent	Cat# 19091S-433
OpenLab CDS	Agilent	Ver. A.01.07

RESOURCE AVAILABILITY

Lead contact

Further information and requests of resources and reagents should be directed to and will be fulfilled by the lead contact, Coby Schal (coby@ncsu.edu).

Materials availability

This study did not generate new unique reagents.

Data and code availability

- All data reported in this paper are available on Dryad: https://doi.org/10.5061/dryad.rbnzs7hhd.
- This paper does not report original code.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

We used the YDK strain of *Chloridea virescens* for our experiments. Moths were originally collected in 1988 from seven tobacco fields in Yadkin County, and one field each in neighboring Stokes and Forsythe Counties, North Carolina, USA.[44] Eggs were briefly rinsed in a 1.3% bleach solution, and after drying eggs were placed individually in 30 ml diet cups (DART, Mason, MI, USA) each containing 7.5 ml of artificial diet[45] and maintained at 26 ± 1 °C with a photoperiod of 14:10 (L: D). Pupae were separated by sex and placed in separate plastic cups. Emerged adults were checked daily and provided with a 10% sucrose solution. Male and female pupae and adults were kept in separate incubators at 26 ± 1 °C and on a reversed photocycle (14 L:10 D) with L-off at 09:00.

METHOD DETAILS

GC-EAD analysis of male HP components

Gas chromatography (GC) coupled to electroantennogram detection (EAD) was performed as described previously.[46] A Hewlett-Packard 5890 Series II GC (Agilent, Folsom, CA, USA), equipped with either a non-polar capillary column (EC-5, 30 m × 0.25 mm ID, 0.25 μm film thickness; Alltech, Deerfield, IL, USA) or a polar capillary column (EC-Wax, 30 m × 0.25 mm ID, 0.25 μm film thickness; Alltech) was used for GC-EAD analyses. The oven temperature was held at 50 °C for 2 min, then increased by 15 °C/min to 250 °C. Injector and detector temperatures were both set to 250 °C. Splitless injection was used with hydrogen as carrier gas at a flow of 1.2 ml/min. The column effluent was split 1:1 to a flame ionization detector (FID) and through a heated transfer line (250 °C) to the EAD.

Moth antennae were excised with micro-scissors from 1-3-day-old virgin females and positioned between two gold wire electrodes within saline-filled glass capillaries. The physiological saline solution was adjusted specifically for *Chloridea* based on Hayashi and Hildebrand[47]: 8.77 mg/ml NaCl, 0.3 mg/ml KCl, 0.67 mg/ml $CaCl_2$, 2.4 mg/ml HEPES, and 0.9 mg/ml D-(+)-glucose were dissolved in diH_2O, and the pH adjusted to ~7 with 1 N NaOH. Aliquots of the saline solution were stored at –30 °C. The electrodes were held in a custom-made acrylic holder that was placed inside a humidified cooling condenser maintained at ~15 °C. Pure humidified air at ambient temperature was continuously blown over the antenna preparation in the cooled condenser. The output signal from the antennae was amplified 10× by a custom high-input impedance DC amplifier and filtered by a high-pass filter with a cutoff frequency of 0.5 Hz.[46] The amplifier output was routed through a signal acquisition board within the GC and displayed along with the FID signal in Agilent OpenLab CDS (version A.01.07).

GC-MS analysis of male HP components

For compound identification we used a 6890N GC coupled with a 5975 mass-selective detector (MS, Agilent, Folsom, CA, USA) operating in electron impact ionization mode (MS ion source set to 230 °C, MS quadruple set to 150 °C). Samples were injected in splitless mode with an initial temperature of 280 °C. Separation of compounds was performed on a non-polar column (HP-5ms, 30 m x 0.25 mm x 0.25 μm (5%-phenyl)-methyl-siloxane stationary phase, Agilent). The temperature program started at 50 °C for 1 min and then increased by 10 °C per min to a final temperature of 320 °C. Compounds were identified according to their Kovats indices, diagnostic ions, and their respective mass spectra. The Wiley 7/NIST 05 database was used for reference, and the identity of novel compounds was confirmed with synthetic standards (Sigma-Aldrich, St. Louis, MO, USA) processed in the GC-MS as well as on the GC-EAD-FID; both non-polar and polar columns were used.

Behavioral assays

Observations of moth mating behavior were conducted in a dark room at 26 ± 1 °C., illuminated with dim red lights. Two-day old adult moths, which are best suited for behavioral assays,[48] were used in all mating behavior experiments. The moths were taken out of the incubators and allowed to acclimate in the dark room 1 hr before L-off. Thirty min before L-off, one male and one female (2-days old) were placed in an observation arena (cylinder, 9 cm diameter x 7.5 cm high) (Fabri-Kal Corp, Kalamazoo, MI, USA) and moths were observed for mating every 30 min for 5 hrs. Moths mate for several hours, so pairs that remained *in copula* for at least 60 min were considered mated.[21]

Effects of HP ablation on mating

Application of gentle pressure to the abdomen caused the hairpencil (HP) complex to extend. HP structures were ablated by removing only the hairs associated with structure-2 (Figures S1A–S1D) with fine forceps. Sham-operated males were squeezed to expose the HPs, but no hairs were removed. All sham-operations and ablations were conducted before the onset of the photophase, about 14 hrs before the behavioral experiments, to give adequate time for the males to recover after the surgery.

Two groups of male moths were set up: (1) HP^+: Sham-operated *C. virescens* males; (2) HP^-: HP-ablated *C. virescens* males. The mating rate of these two groups of males with 2-day-old virgin female moths was monitored in single pair assays.

Extraction of HP chemicals after HP-ablation

The hairs removed during HP ablation are largely storage and emission sites, whereas their underlying cells presumably biosynthesize the male sex pheromone. To determine whether HP ablation in fact reduced the amount of male sex pheromone, the HPs were extracted, and the quantity of male sex pheromone was determined by GC-FID analysis. In separate groups of males, the structure-2 hairs of the HPs (Figures S1A–S1D) were excised, and either 1 hr or 14 hrs later the whole HP complex was removed and extracted (Figures S1G and S1H). Two extractions were performed. Short (10 sec) extractions were used to represent the chemicals on the surface of the HP gland. Long (20 min) extractions were designed to extract the chemicals from both the surface and interior of the HP glands. Each HP was extracted in 100 μl hexane containing 40 ng pentadecyl acetate (15:OAc) as internal standard. All extracts were kept at −30 °C prior to chemical analysis and analyzed individually on an Agilent 7890A GC equipped with a 30 m × 0.25 mm ID DB-5 capillary column (Agilent). The amount of 16:OAc, the main component of the HPs of *C. virescens*, was quantified in each extract.

Effects of HP extract and MeSA on mating

A large pool of HP extracts was produced by excising the structure-2 hairs of 2–4-day-old *C. virescens* males in 100 μl hexane per male. For behavioral assays, one male-equivalent (1 ME; 100 μl of extract) was applied to a 42.5 mm diameter filter paper disc (Whatman #1, Sigma, St. Louis, MO, USA). The hexane was allowed to evaporate, and the filter paper was added to the behavioral assay arena.

The effect of HP extract and MeSA on mating of *C. virescens* was tested using HP-ablated males and 2-day old virgin females. Hair pencil extract and a range of MeSA doses were loaded onto filter papers and the mating rates in different groups were assessed. Four concentrations of MeSA were tested (100 ng, 10 ng, 1 ng and 0.1 ng) along with a hexane control for a total of six treatment groups: (1) Hexane: 100 μl hexane; (2) HP extract: 100 μl (1 ME) of *C. virescens* HP extract; (3) MeSA 100 (100 ng MeSA: 100 μl of 1 ng/μl); (4) MeSA 10 (10 ng MeSA: 100 μl of 0.1 ng/μl); (5) MeSA 1 (1 ng MeSA: 100 μl of 0.01 ng/μl); (6) MeSA 0.1 (0.1 ng MeSA: 100 μl of 0.001 ng/μl).

Cloning and sequence analysis of MeSA ORs

The full ORF sequences of CvirOR27 and CvirOR43 were obtained in the *C. virescens* antennal transcriptomes based on the basic local alignment search tool (BLAST) using the HarmOR27 and HarmOR43 sequences as queries. Full-length coding sequences of CvirOR27 and CvirOR43 were obtained from the antennal cDNA of *C. virescens* adults by PCR with specific primer pairs for each gene. Phylogenetic analysis was performed using 20 ORs from *C. virescens* (including 18 previously reported ORs and the two ORs we identified in this study) and 65 ORs from *H. armigera*. Amino acid sequences were aligned (Data S2) using ClustalW and neighbor-joining tree was constructed using Poisson model as implemented in MEGA X software.

Functional characterization of ORs

The Functional characterization of individual ORs was performed by heterologous expression in *Xenopus* oocytes combined with a two-electrode voltage-clamp system.[49] Briefly, the full-length gene of each OR and Orco were subcloned into the eukaryotic expression vector pT7TS. cRNAs were generated from linearized expression vectors using the mMESSAGE mMACHINE T7 kit (Ambion, Austin, TX, USA). Then, the cRNA mixture of ORx and Orco (27.6 ng each) was injected (Nanoliter 2010, WPI, Sarasota, FL) into *Xenopus* oocytes. After incubation in nutrient solution at 18 °C for 3–5 days, the response profile of each oocyte to multiple plant odorants was recorded via a two-electrode voltage clamp (OC-725C oocyte clamp, Warner Instruments, Hamden, CT, USA) at a holding potential of -80 mV. Data were acquired by using a Digidata 1440 A and were analyzed by pCLAMP 10.2 software (Molecular Devices, San Jose, CA, USA).

Stock solutions of each odorant were prepared at 1 M using DMSO as a solvent, and each odorant was diluted in 1× Ringer's solution (96 mM NaCl, 2 mM KCl, 5 mM $MgCl_2$, 0.8 mM $CaCl_2$, and 5 mM HEPES, pH 7.6) to the indicated concentrations for electrophysiological recording. Information on the 66 odorants used in this study is listed in Data S1B.

QUANTIFICATION AND STATISTICAL ANALYSIS

All quantification and statistical analysis methods are described in the Figure legends and Table legends. Quantification of compounds in HP extracts was done using Agilent OpenLab CDS software (version A.01.07). The distribution of 16:OAc and MeSA in various structures of the HPs and the effects of extraction duration were assessed using ANOVA followed by Tukey's Honestly Significant Difference test (JMP). The time-course of cumulative mating success was analyzed using survival analysis. A Cox Proportional Hazards model was fitted and the Wald test was used to compare treatments. Dunnett's multiple comparison test was used to compare supplementation with various doses of MeSA to the no-supplementation control (0-ng treatment) (Table S1). For all statistical analyses $\alpha = 0.05$.