# Females smell differently: characteristics and significance of the most common olfactory sensilla of female silkmoths

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**SUPPLEMENTARY INFORMATION**
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**Supplementary material and methods**

*Headspace collection and analysis*

We collected headspace of mulberry leaves and silkmoths. A humidified, charcoal-filtered airflow (0.3 l/min) was pumped into a glass bottle containing leaves from three different mulberry trees or two female or male silkmoths. The odour-enriched air left the bottle through a volatile collection trap (Porapak-Q 25 mg, https://www.volatilecollectiontrap.com). After 24 h, traps were removed and eluted with 300 µl hexane. For SSR, headspace of mulberry leaves was used pure, while headspace of silkmoths was concentrated under a gentle stream of nitrogen to 50 µl. Meconium of silkmoths (female and male separately) was freshly collected before starting SSR and diluted in methanol (1:2). Faeces from silkworms ($5^{th}$ instar) fed on fresh mulberry leaves were collected on four consecutive days and stored at -20°C. On experimental days, one pellet of faeces was defrosted at room temperature for 15 min and dissolved in 1 ml hexane. For the chemical analysis of compounds emitted by silkmoths, meconium and silkworm faeces, a solid phase micro extraction (SPME) fibre (50/30 µm divinylbenzene/Carboxen on polydimethylsiloxane fibre, Supelco) was inserted for 30 min through a septum into a glass vial containing one female or male silkmoth, meconium or silkworm faeces. The fibre was analysed by GC-MS (7890B GC System, 5977A MSD, Agilent Technologies, https://www.agilent.com) with a polar column (HP-INNOWAX, 30 m long, 0.25 mm inner diameter, 25 µm film thickness; Agilent) with helium as carrier gas. The same instrument was used to analyse headspace of mulberry leaves by injection of 1 µl of eluted headspace into the GC-MS. The temperature of the GC oven was held at 40°C for 2 min, and then increased by 15°C/min (SPME fibre) or 10°C/min (liquid injection) to 260°C. This final temperature was kept for 10 min. The MS transfer line was held at 260°C, the MS source at 230°C, and the MS quad at 150°C. Mass spectra were taken in electron ionization mode (70 eV) in the range from m/z 29 to 350. GC-MS data were processed with the MDS-ChemStation Enhanced Data Analysis software (Agilent).

For identification of compounds, we compared their mass spectra with those from a reference library (National Institute of Standards and Technologies) and a database built in our laboratory with synthetic standards using the same GC-MS instrument. Compounds yielding a match of mass spectra above 90% were rated as identified. If no clear match was found, the compound was labelled as unidentified.

*Whole mount fluorescent immunohistochemistry (WM-FIHC)*

Experiments followed protocols described for the desert locust (1). All incubation steps were conducted on an overhead shaker in 0.2 ml reaction tubes. Side branches of *B. mori* antenna were dissected from the antennal stem and transferred into 4% paraformaldehyde in phosphate-buffered saline (PBS, 145 mM NaCl, 1.4 mM $KH_2PO_4$, 8 mM $Na_2HPO_4$) pH 7.4 supplemented with 0.5% Triton X-100. After incubation overnight at 4°C, samples were washed 3 x 10 min in PBS with 0.1 % Triton X-100. After 10 min treatment with 50 mM $NH_4Cl$ in PBS, samples were incubated in blocking solution (10% normal goat serum, 0.5% Triton X-100 in PBS) overnight at 4°C. Samples were then treated with a rabbit-anti-ORco antibody generated against moth ORco (2) diluted 1:500 in blocking solution. Following incubation for 48 h at 4°C, samples were washed 3 x 10 min with blocking solution and subsequently incubated for two days at 4°C with goat-anti-rabbit Alexa Fluor 488-conjugated antibodies (Jackson ImmunoResearch, Ely, Great Britain) diluted 1:1000 in blocking solution. Then, samples were washed 3 x 10 min with PBS, briefly rinsed with $dH_2O$, mounted with mowiol solution on microscope slides, and covered with coverslips.

*Whole mount fluorescent in situ hybridization (WM-FISH), and combined WM-FISH and FIHC*

For WM-FISH experiments, digoxigenin-labelled antisense RNAs were transcribed from pCR II-TOPO plasmids (Thermo Fisher Scientific, Waltham, Massachusetts, USA) containing cDNA sequence of the respective *B. mori* OR/IR sequences using the T7/Sp6 RNA transcription system (Merck, Darmstadt, Germany) as recommended by the manufacturer. WM-FISH was conducted as described earlier (3) with few modifications. Antennal samples were prepared as described above for WM-FIHC. All incubation steps were conducted in 0.2 mL PCR tubes and rotated on an overhead shaker unless stated otherwise. Samples were fixed in 4% paraformaldehyde in 0.1M $NaHCO_3$, pH 9.5 overnight at 4°C, followed by washes at room temperature for 1 min in PBS pH 7.1, 10 min in 0.2 M HCl and 2 min in PBS pH 7.1 with 1% Triton

X-100. Afterwards, samples were prehybridized at 55°C for at least 6 h in whole mount hybridization solution [50% formamide, 5×SSC (750 mM NaCl, 75 mM sodium citrate, pH 7.0), 1×Denhardt's reagent (50 µg/mL yeast RNA, 1% Tween 20, 0.1% Chaps, 5 mM EDTA), pH 8.0] followed by hybridization at 55°C for 48-72 h in the same solution containing the digoxigenin-labelled antisense RNA probes. Next, samples were washed 4 x 15 min in 0.1 X SSC with 0.03% Triton X-100 at 60°C on an orbital shaker and subsequently incubated in 1% blocking reagent (Merck) in TBS (100 mM Tris, 150 mM NaCl, pH 7.5) with 1% Triton X-100 overnight at 4°C. Samples were then treated with an anti-digoxigenin AP-conjugated antibody (Merck) diluted 1:500 in 1% blocking reagent in TBS for 48 h at 4°C. For combined WM-FISH/FIHC-experiments, the anti-ORco-antibody was added to this solution at a concentration of 1:500. After washing 5 x 10 min in TBS with 0.05% Tween 20 (TBST) and 1 x 5 min in 150 mM Tris-HCl, pH 8.3, digoxigenin-labelled RNA probes were visualized using the Vector Red alkaline phosphatase substrate kit (Vector Laboratories, Burlingame, CA, USA) as recommended by the manufacturer with an overnight incubation at 4°C. In WM-FISH experiments, samples were then washed 3 x 10 min with TBST and mounted on microscope slides as described earlier. In the case of combined WM-FISH/FIHC with the anti-ORco antibody, samples were incubated with anti-rabbit-Alexa Fluor 488 antibody diluted 1:500 in TBST overnight at 4°C. For immunostaining of neurons, samples were instead treated overnight at 4°C with anti-HRP (horseradish peroxidase) Alex Fluor 647-conjugated antibody (Jackson Immuno Research) diluted 1:200 in TBST. Finally, samples were washed 3 x 10 min with TBST, rinsed briefly with $dH_2O$, mounted on microscope slides with mowiol, and covered with coverslips.
WM-FIHC and WM-FISH were analysed with a confocal laser scanning microscope (LSM 880, Carl Zeiss Microscopy, Jena, Germany). Confocal image stacks of the fluorescence and transmitted-light channels were used to generate either pictures representing single optical planes or projections of optical planes applying the ZEN software (Carl Zeiss Microscopy). Pictures were not altered except for adjusting the brightness or contrast for a uniform tone within a single figure.


**Figure S1. Portraits of male and female silkmoth, *Bombyx mori*.** Size and structure of the branched antenna is similar in both sexes of the domesticated silkmoth (photos by Benjamin Fabian).

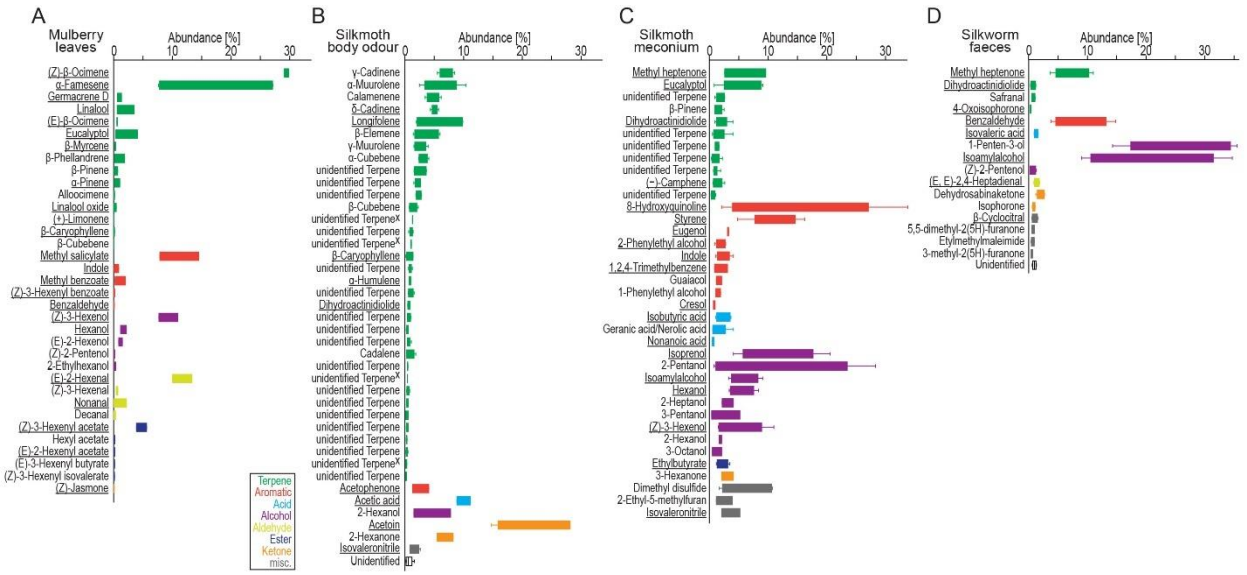


**Figure S2. Relative abundance of chemical compounds in the headspace of silkmoth-related natural sources.** *Boxes*, interquartile range of relative abundance for each compound; *whiskers*, range; *underlined odour names*, tested in SSR. **A**. Compounds (n=35) in the headspace of mulberry leaves (3 samples) collected with SuperQ-filters and eluted with hexane. Only compounds that occurred in each sample are shown. **B**. Compounds (n=41) in the headspace of silkmoths (3 females, 2 males) collected with a grey SPME fibre. Only compounds that occurred at least in two animals are shown; $^{x}$, unidentified terpenes found only in females. **C**. Compounds (n=37) in the headspace of silkmoth meconium (3 females, 3 males) collected with a grey SPME fibre. Only compounds that occurred at least in two animals are shown; no sex-specific compounds were found. **D.** Compounds (n=17) in the headspace of silkworm faeces (4 samples) collected with a grey SPME fibre. Only compounds that occurred in each sample are shown.

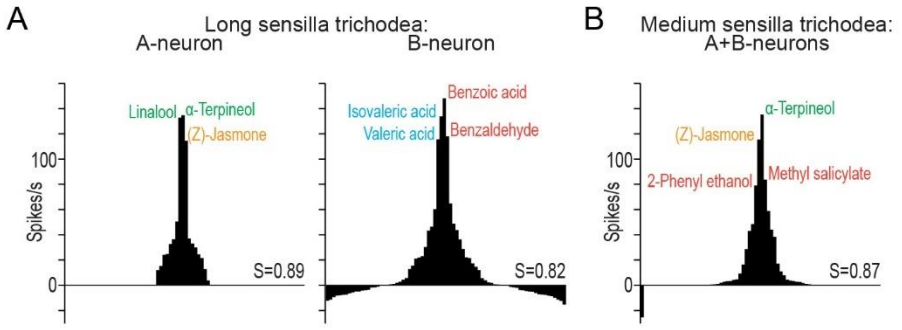


**Figure S3. Tuning width of female sensilla trichodea.** Tuning curves of long (**A**) and medium (**B**) trichoids, showing the distribution of median responses to a panel of 76 monomolecular compounds, ordered along the x-axis according to the median response (data from Fig. 1B, E). Odorant names depict ligands that elicited more than 50% of the maximal response. S, lifetime sparseness value calculated as in (Bhandawat et al. 2007), illustrating the tuning breadth of a neuron with a value of 0=non-selective and 1=maximally selective.

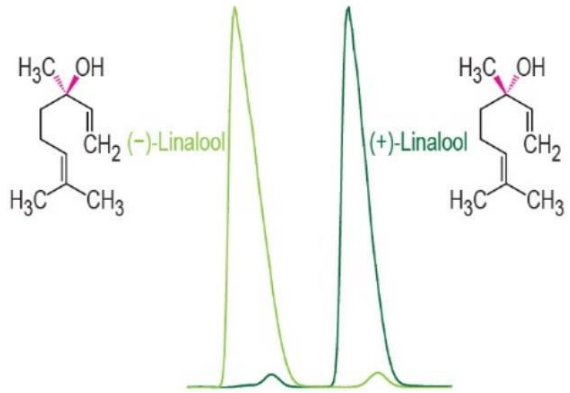


**Figure S4. Impurity of linalool enantiomers.** Samples of (+)-linalool and (−)-linalool were tested with a chiral column in GC-MS. Both odours showed a 2%-contamination with the opposite enantiomer.

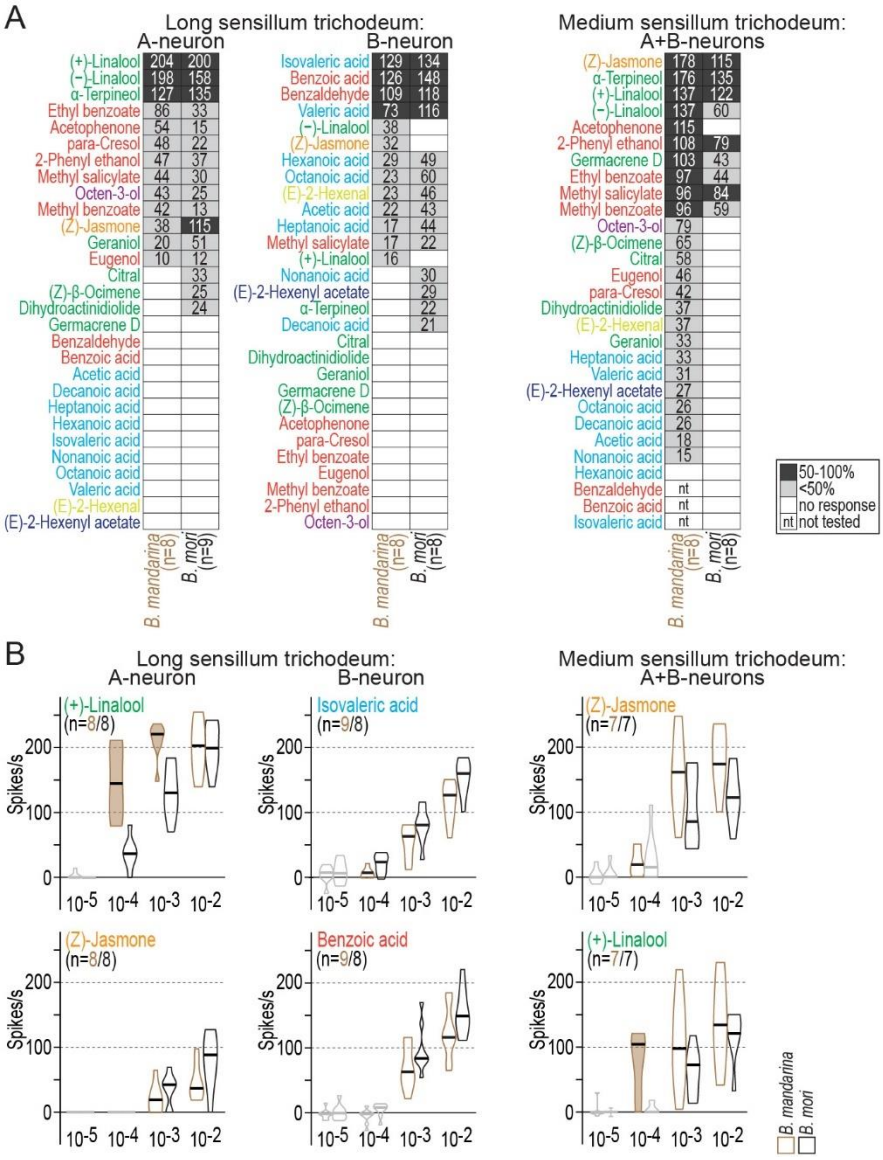


**Figure S5. Comparison of domesticated and wild silkmoths. A.** Responses of OSNs in sensilla trichodea of female *B. mandarina* and *B. mori*. Data for *B. mori* are from Fig. 1B, E. Heatmaps show normalized median, solvent-subtracted maximum spike frequencies to ligands identified in *B. mori*. Stimuli sorted according to their response in *B. mandarina*. *Filled cells*, data differ from zero ($p<0.05$, Wilcoxon rank sum test); *values in cells*, median spike frequencies, *shades of grey*, strength of response (dark grey, ≥ half maximal response, light grey, < half maximal response. **B.** Dose-response experiments. Data for *B. mori* are from Fig. 1C, F; *violin plots*, net maximum spike frequencies (spikes/s) to five (v/v) odour concentrations corresponding to 60 ng, 600 ng, 6 µg, 60 µg of pure substance on filter paper; *horizontal line*, median; *grey violin plots*, data not different from zero ($p>0.05$, Wilcoxon rank sum test); *filled violin plots*, data are different between the species ($p<0.05$, Mann-Whitney-U test).

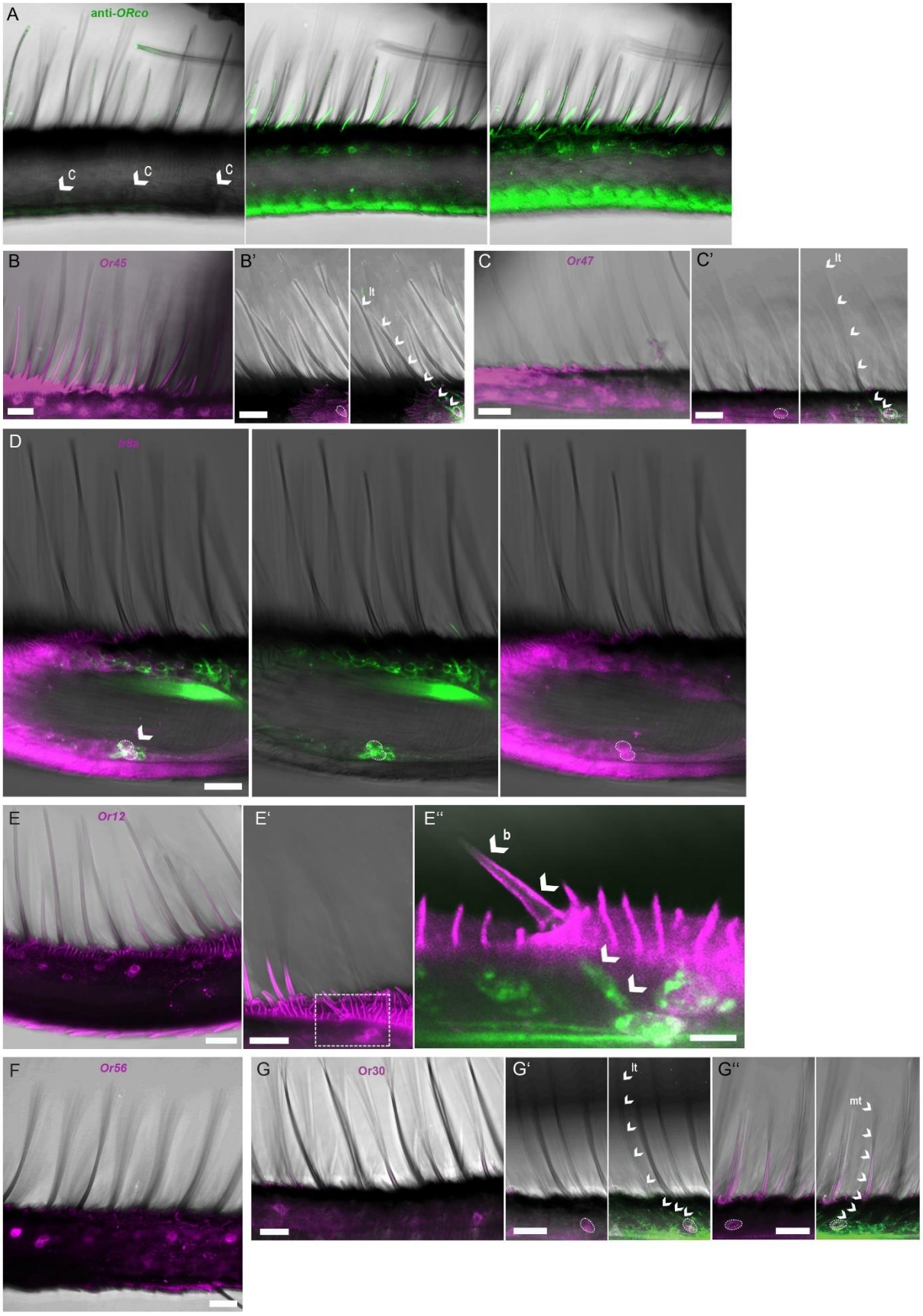
A
anti-ORco
c
c
c
B
Or45
B'
lt
C
Or47
C'
lt
D
Ir8a
E
Or12
E'
E''
b
F
Or56
G
Or30
G'
lt
G''
mt

**Figure S6. Expression of ORs, *ORco* and *Ir8a* in the female antenna.** Fluorescent RNA *in situ* hybridization (FISH) and fluorescent immunohistochemistry (FIHC) alone or in combination performed with whole mount preparations. Images show fluorescent channels overlaid with the transmitted light channel. *Scale bars*, 20 µm, except in E'' (5 µm). **A.** FIHC with an anti-*ORco* antibody (green) showing different optical planes of a confocal z-stack. Left image, position (arrowheads) of coeloconic sensilla (c); middle and right image, labelling of somata and dendrites of neurons innervating sensilla basiconica and trichodea. **B.** FISH with *Or45*-specific RNA probe (magenta) combined in (**B'**) with FIHC using anti-HRP antibody (green) showing innervation (arrowheads) of a long trichoid by an *Or45*-expressing neuron (encircled soma). **C.** FISH with *Or47*-specific RNA probe (magenta) combined in (**C'**) with FIHC using anti-HRP antibody (green) showing innervation (arrowheads) of a long trichoid by an *Or47*-expressing neuron (encircled soma). **D.** FISH with an *Ir8a*-specific RNA probe (magenta) combined with FIHC using an anti-HRP (horse radish peroxidase) antibody (green) showing *Ir8a*-positive olfactory sensory neurons (encircled somata) innervating a sensillum coeloconicum (arrowhead). **E.** FISH with an *Or12*-specific RNA probe (magenta) combined in (**E', E''**) with FIHC using an anti-HRP antibody (green) showing innervation (arrowheads) of a sensillum basiconicum (b). **E''**. Magnification of the encircled region in **E'**. **F.** FISH with an *Or56*-specific RNA probe (magenta). **G.** FISH with *Or30*-specific RNA probe (magenta) combined in (**G'**, **G''**) with FIHC using anti-HRP antibody (green) showing innervation (arrowheads) of long (G') and medium trichoids (G'') by *Or30*-expressing neurons (encircled somata).

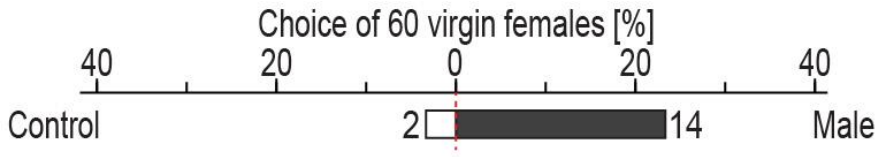


**Figure S7. Male silkmoths attract virgin females in a y-maze.** *Bar graph*, proportion of 60 individually tested virgin females (absolute numbers next to the bars) that chose control arm (empty 100 ml bottle) or test arm (100 ml bottle with one male silkmoth) during a 10 min test duration. Choices were different from a 50:50 distribution (p=0.045, Chi square goodness of fit test).

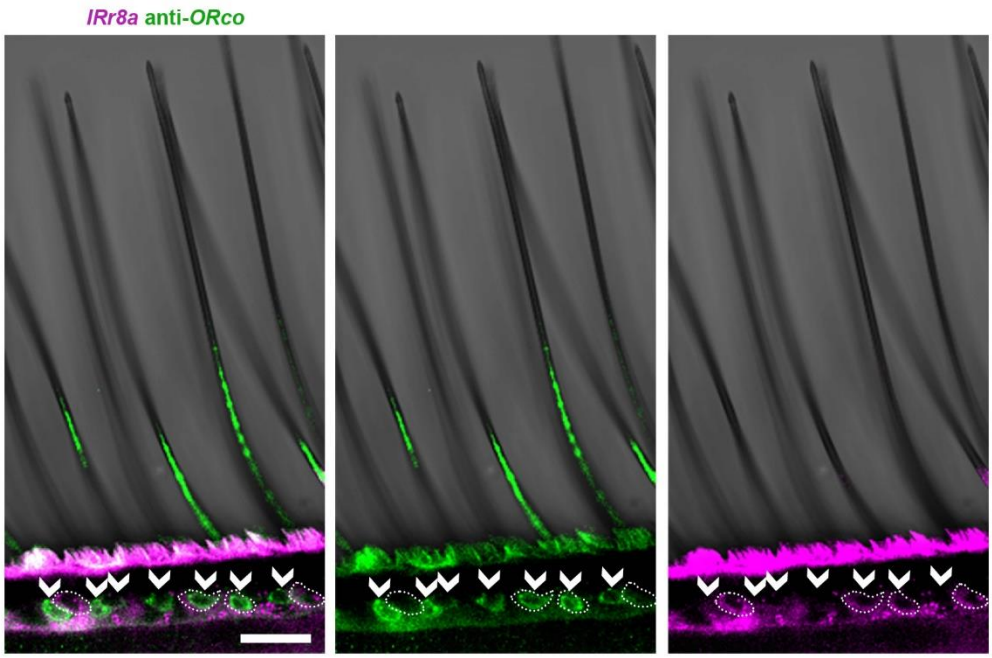


**Figure S8. Expression of *ORco* and *Ir8a* in the antenna of males.** Combined fluorescent RNA *in situ* hybridization and fluorescent immunohistochemistry on whole mount preparations using an *Ir8a*-specific RNA probe (magenta) and an anti-*ORco* antibody (green). Images show fluorescent channels overlaid with the transmitted light channel. *Circles*, somata of *Ir8a*-positive neurons; *arrowheads*, *ORco*-positive neurons; *scale bar*, 20 µm.

**Table S1. List of stimuli for single sensillum recordings.** Symbols mark compounds identified in the headspace of mulberry leaves (*****), silkmoths (**§**), their meconium (**#**) or silkworm frass (&).

| **Odorant name** | **Chemical class** | **CAS number** |
|---|---|---|
| delta-Cadinene§ | Terpene | 483-76-1 |
| (−)-Camphene# | Terpene | 5794-04-7 |
| beta-Caryophyllene*§ | Terpene | 87-44-5 |
| Citral | Terpene | 5392-40-5 |
| Dihydroactinidiolide§#& | Terpene | 17092-92-1 |
| Eucalyptol*# | Terpene | 470-82-6 |
| alpha-Farnesene* | Terpene | 502-61-4 |
| Geraniol | Terpene | 106-24-1 |
| Geranyl acetate | Terpene | 105-87-3 |
| Germacrene D* | Terpene | 23986-74-5 |
| alpha-Humulene§ | Terpene | 6753-98-6 |
| (E)-beta-Ionone | Terpene | 79-77-6 |
| Limonene* | Terpene | 138-86-3 |
| (±)-Linalool* | Terpene | 78-70-6 |
| (+)-Linalool* | Terpene | 126-90-9 |
| (−)-Linalool* | Terpene | 126-91-6 |
| Linalool oxide* | Terpene | 60047-17-8 |
| Linalyl acetate | Terpene | 115-95-7 |
| Longifolene§ | Terpene | 475-20-7 |
| Methyl heptenone# | Terpene | 110-93-0 |
| beta-Myrcene* | Terpene | 123-35-3 |
| (Z)-beta-Ocimene* | Terpene | 3338-55-4 |
| 4-Oxoisophorone& | Terpene | 1125-21-9 |
| alpha-Pinene* | Terpene | 80-56-8 |
| gamma-Terpinene | Terpene | 99-85-4 |
| alpha-Terpineol | Terpene | 98-55-5 |
| Valencene | Terpene | 4630-07-3 |
| Acetophenone§ | Aromatic | 98-86-2 |
| Benzaldehyde*& | Aromatic | 100-52-7 |
| Benzoic acid | Aromatic | 65-85-0 |
| para-Cresol# | Aromatic | 106-44-5 |
| Diethyl toluamide (DEET) | Aromatic | 134-62-3 |
| Ethyl benzoate | Aromatic | 93-89-0 |
| Eugenol# | Aromatic | 97-53-0 |
| (Z)-3-Hexenyl benzoate* | Aromatic | 25152-85-6 |
| Indole*# | Aromatic | 120-72-9 |
| Methyl benzoate* | Aromatic | 93-58-3 |
| Methyl salicylate* | Aromatic | 119-36-8 |
| 2-Phenyl ethanol# | Aromatic | 60-12-8 |
| Styrene# | Aromatic | 100-42-5 |
| 1,2,4-Trimethylbenzene# | Aromatic | 95-63-6 |
| Acetic acid§ | Acid | 64-19-7 |
| Decanoic acid | Acid | 334-48-5 |
| Heptanoic acid | Acid | 111-14-8 |
| Hexanoic acid | Acid | 142-62-1 |
| Isobutyric acid# | Acid | 79-31-2 |

| | | |
|---|---|---|
| Isovaleric acid[&] | Acid | 503-74-2 |
| Nonanoic acid[#] | Acid | 112-05-0 |
| Octanoic acid | Acid | 124-07-2 |
| Palmitic acid | Acid | 57-10-3 |
| Stearic acid | Acid | 57-11-4 |
| Valeric acid | Acid | 109-52-4 |
| Bombykol | Alcohol | 765-17-3 |
| Hexanol*[#] | Alcohol | 111-27-3 |
| (Z)-3-Hexenol*[#] | Alcohol | 928-96-1 |
| Isoamylalcohol[#&] | Alcohol | 123-51-3 |
| Isoprenol | Alcohol | 763-32-6 |
| Octen-3-ol | Alcohol | 3391-86-4 |
| Bombykal | Aldehyde | 63024-98-6 |
| Butyraldehyde | Aldehyde | 123-72-8 |
| (E, E)-2,4-Heptadienal[&] | Aldehyde | 4313-03-5 |
| (E)-2-Hexenal* | Aldehyde | 6728-26-3 |
| Nonanal* | Aldehyde | 124-19-6 |
| Ethyl butyrate[#] | Ester | 105-54-4 |
| Ethyl hexanoate | Ester | 123-66-0 |
| (E)-2-Hexenyl acetate* | Ester | 2497-18-9 |
| (Z)-3-Hexenyl acetate* | Ester | 3681-71-8 |
| Methyl palmitate | Ester | 112-39-0 |
| Methyl stearate | Ester | 112-61-8 |
| Acetoin[§] | Ketone | 513-86-0 |
| (Z)-Jasmone* | Ketone | 488-10-8 |
| Benzothiazole | Sulphur containing | 95-16-9 |
| Dimethyl trisulfide | Sulphur containing | 3658-80-8 |
| Isovaleronitrile[§#] | Nitrogen containing | 625-28-5 |
| Tetradecane | Alkane | 629-59-4 |
| Tricosane | Alkane | 638-67-5 |
| beta-Cyclocitral[&] | Apocarotenoid | 432-25-7 |
| 8-Hydroxyquinoline[#] | Aromatic heterocycle | 148-24-3 |

**Supplementary references**


1. Cassau S, Degen A, Krueger S, Krieger J. The specific expression patterns of sensory neuron membrane proteins are retained throughout the development of the desert locust *Schistocerca gregaria*. Current Research in Insect Science. 2023;3:100053.
2. Nolte A, Gawalek P, Koerte S, Wei H, Schumann R, Werckenthin A, et al. No evidence for ionotropic pheromone transduction in the hawkmoth *Manduca sexta*. PLoS ONE. 2016;11(11):e0166060.
3. Krieger J, Grosse-Wilde E, Gohl T, Breer H. Candidate pheromone receptors of the silkmoth *Bombyx mori*. European Journal of Neuroscience. 2005;21(8):2167-76.