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ORIGINAL ARTICLE

A small number of male-biased candidate pheromone receptors are expressed in large subsets of the olfactory sensory neurons in the antennae of drones from the European honey bee *Apis mellifera*

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Abstract In the European honey bee (*Apis mellifera*), the olfactory system is essential for foraging and intraspecific communication via pheromones. Honey bees are equipped with a large repertoire of olfactory receptors belonging to the insect odorant receptor (OR) family. Previous studies have indicated that the transcription level of a few OR types including OR11, a receptor activated by the queen-released pheromone compound (2*E*)-9-oxodecenoic acid (9-ODA), is significantly higher in the antenna of males (drones) than in female workers. However, the number and distribution of antennal cells expressing male-biased ORs is elusive. Here, we analyzed antennal sections from bees by *in situ* hybridization for the expression of the male-biased receptors OR11, OR18, and OR170. Our results demonstrate that these receptors are expressed in only moderate numbers of cells in the antennae of females (workers and queens), whereas substantially higher cell numbers express these ORs in drones. Thus, the reported male-biased transcript levels are due to sex-specific differences in the number of antennal cells expressing these receptors. Detailed analyses for OR11 and OR18 in drone antennae revealed expression in two distinct subsets of olfactory sensory neurons (OSNs) that in total account for approximately 69% of the OR-positive cells. Such high percentages of OSNs expressing given receptors are reminiscent of male-biased ORs in moths that mediate the detection of female-released sex pheromone components. Collectively, our findings indicate remarkable similarities between male antennae of bees and moths and support the concept that male-biased ORs in bee drones serve the detection of female-emitted sex pheromones.

Key words chemosensory; odorant receptor; olfaction; pheromone detection; sensilla placodea

Introduction

The eusocial European honey bee (*Apis mellifera*) lives in colonies that comprise tens of thousands of individuals, including drones (reproductive males), workers (sterile females), and a queen (reproductive female). The complex organization of the honey bee society with its sophisticated division of labor and task allocation is largely accomplished through elaborate chemical communication between the members of the colony (Le Conte & Hefetz, 2008; Trhlin & Rajchard, 2011; Bortolotti & Costa, 2014). Honey bees utilize extensive pheromone communication in order to trigger and control worker sterility, care of the brood, defense behavior, nestmate recognition, foraging, and the establishment of a social

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hierarchy (Melathopoulos *et al*., 1996; Hoover *et al*., 2003; Slessor *et al*., 2005; Katzav-Gozansky *et al*., 2006; Le Conte & Hefetz, 2008; Trhlin & Rajchard, 2011; Bortolotti & Costa, 2014). In drones, pheromones play a crucial role for mating behavior (Gary, 1962; Gary & Marston, 1971; Brockmann *et al*., 2006). For mating, the drones fly out of the nest on warm and sunny afternoons in late spring or summer and gather 10–40 m above ground in so-called drone congregation areas with a diameter of approximately 30–200 m. Drone congregation areas can contain thousands of drones originating from a large number of different colonies (Zmarlicki & Morse, 1963; Ruttner, 1966; Baudry *et al*., 1998; Reyes *et al*., 2019). Shortly after the drones, virgin queens leave their hive and fly to the vicinity of a drone congregation area (Koeniger *et al*., 1979; Lensky & Demter, 1985; Koeniger & Koeniger, 2004). As soon as a virgin queen approaches such an area, drones are chemically attracted to her, leading to subsequent copulation in flight. It is commonly assumed that attraction of drones during mating flights largely relies on the pheromonal substance (2*E*)-9-oxodecenoic acid (9-ODA), a major component of the queen mandibular gland secretions (Callow & Johnston, 1960; Gary, 1962; Butler, 1971; Gary & Marston, 1971; Boch *et al*., 1975; Gries & Koeniger, 1996; Brandstaetter *et al*., 2014).

While several pheromonal compounds have been identified in *Apis mellifera* (Slessor *et al*., 2005; Le Conte & Hefetz, 2008; Trhlin & Rajchard, 2011; Bortolotti & Costa, 2014), little is known about the molecular processes mediating the detection of pheromones in honey bees. In insects, pheromones (and other odorants) are received via olfactory sensory neurons (OSNs) residing in cuticular structures on the antennae named sensilla. The antennae of honey bees comprise a long scape, a short pedicel, and a flagellum with 11 (drones) or 10 (workers) segments (Fig. 1A, B) (Slifer & Sekhon, 1961; Esslen & Kaissling, 1976). According to morphological criteria, olfactory sensilla of insects are divided into several categories (Steinbrecht, 1996; Stocker, 2001). In honey bees, sensilla placodea (poreplates) as well as the hair-like sensilla trichodea and the cone-shaped sensilla basiconica are considered as olfactory (Lacher & Schneider, 1963; Lacher, 1964; Esslen & Kaissling, 1976; Akers & Getz, 1993; Getz & Akers, 1993). Unlike other insect species, the poreplate sensilla represent the most abundant olfactory sensillum type in honey bees. This applies in particular to drones that harbor approximately 19 000 sensilla placodea per antenna, whereas their antenna comprises no sensilla basiconica and only approximately 400 olfactory trichoid sensilla (sensilla trichodea A) (Esslen & Kaissling, 1976).

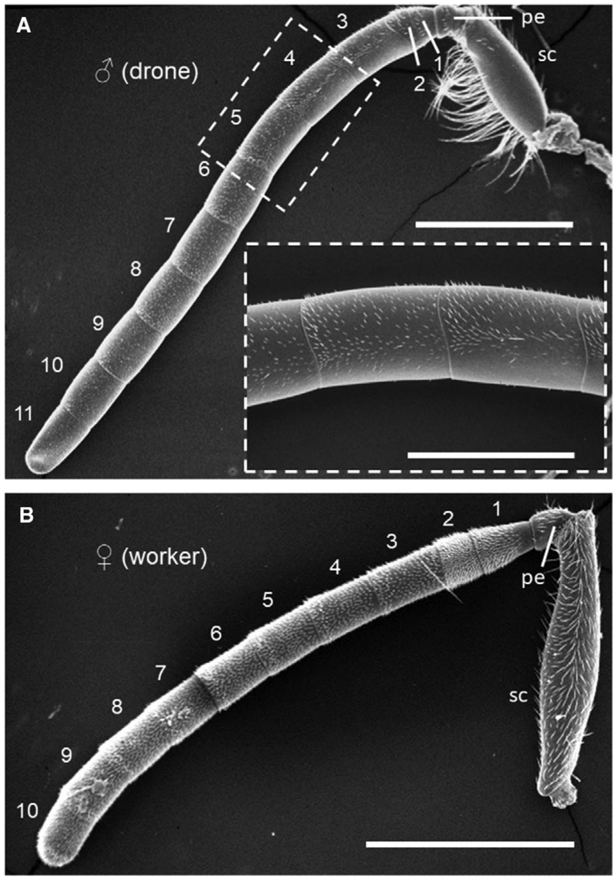

Fig. 1 Antennae of drone and worker honey bees. (A, B) Scanning electron micrographs of antennae from a drone (A) and a worker (B). The scape (sc), the pedicel (pe), and the 11 (drone) or 10 (worker) segments of the flagellum are indicated. The inset in (A) shows a higher magnification of the boxed area. The antennae of workers are shorter and thinner than those of males. Scale bars: A, B = 1 mm; inset in A = 500 μm.

For the detection of odorous and pheromonal ligands, antennal OSNs of insects usually express members of the two major olfactory receptor families, the heptahelical odorant receptors (ORs) and the ionotropic receptors (IRs) (Fleischer *et al*., 2018; Yan *et al*., 2020). Hitherto, most of the characterized insect pheromone receptors (PRs) belong to the OR family (Goes van Naters, 2014; Zhang & Löfstedt, 2015; Fleischer & Krieger, 2018). In various moth species, OR types serving as PRs for female-released sex pheromone compounds are exclusively or predominantly expressed in the antennae of males (Krieger *et al*., 2004; Sakurai *et al*., 2004; Krieger *et al*., 2005; Nakagawa *et al*., 2005; Grosse-Wilde *et al*., 2006; Grosse-Wilde *et al*., 2007; Wang *et al*., 2011; Bastin-Heline *et al*., 2019). Analogously, in honey bees, ORs with a male-biased expression might function as PRs mediating the perception of queen-emitted sex pheromones in drones. Honey bees possess a large repertoire of approximately 170 genes encoding potential ORs (Robertson & Wanner, 2006). Based on quantitative polymerase chain reaction (qPCR), RNA sequencing, and microarray analyses, the honey bee OR types OR11, OR10, OR18, and OR170 were previously reported to have a male-biased expression in the antenna (Wanner *et al*., 2007; Jain & Brockmann, 2020). In addition, OR11 was found to be activated in a heterologous expression system (*Xenopus* oocytes) by the pheromone compound 9-ODA. Consequently, OR11 has been proposed as a putative PR (Wanner *et al*., 2007), although it remains unclear whether this receptor indeed mediates the sensitive detection of queen-released 9-ODA in the antennae of drones during nuptial flights. While the above-mentioned observations have rendered OR10, OR11, OR18, and OR170 prime candidates for serving as PRs involved in the detection of queen-emitted pheromone compounds attracting drones, their ligand repertoire is largely unknown (Wanner *et al*., 2007). Moreover, the number and distribution of the cells expressing these male-biased receptors in the antennae of honey bees has not been analyzed. Thus, unlike the male-biased OR types in moths that are expressed in higher numbers of OSNs in males (Sakurai *et al*., 2004), possibly allowing a more sensitive detection of the cognate ligands, it is currently unclear whether the male-biased transcript levels determined for some ORs in honey bees are associated with an increased number of antennal cells expressing these receptors in drones versus workers. Alternatively, drones and workers could have similar cell numbers expressing these ORs but the relevant mRNA levels per cell might be increased in drones compared to workers due to an enhanced transcriptional rate and/or a reduced degradation of the corresponding mRNA species. Before this background, in the present study, we set out to visualize the expression of male-biased ORs in the antennae of drones and workers by *in situ* hybridization experiments, thus allowing an initial characterization as well as a quantitative comparison of cells expressing these OR types in both sexes which might facilitate evaluating their importance in the olfactory system.

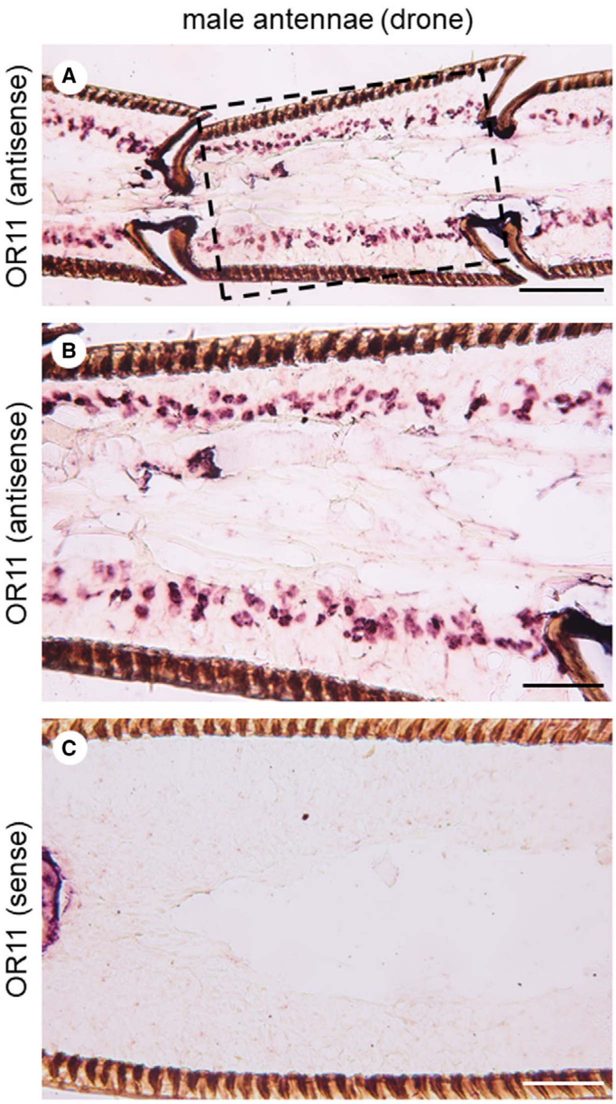

Fig. 2 Visualization of OR11-expressing cells in the antenna of drones. (A) Chromogenic *in situ* hybridization with an antisense riboprobe for OR11 on a longitudinal section through the median plane of the antenna of a drone. In segments of the antennal flagellum, numerous cells were stained, indicating expression of OR11. (B) High-magnification image of the area circumscribed by the broken lines in (A). (C) In control experiments with antennal sections from drones incubated with the corresponding sense riboprobe for OR11, no staining was detectable. The figure exemplarily shows a high-magnification image of a longitudinal section through the central plane of a male antenna. Scale bars: A = 100 μm; B, C = 50 μm.

Materials and methods

Animals

Apis mellifera drones, workers, and virgin queens were taken from hives located in Halle/Saale (Germany) that belonged to the Department of Zoology of the Martin Luther University Halle-Wittenberg or from the apiary of the Institute for Bee Protection (Braunschweig, Germany). The bees were collected between May and September 2019 as well as from April to July 2020.

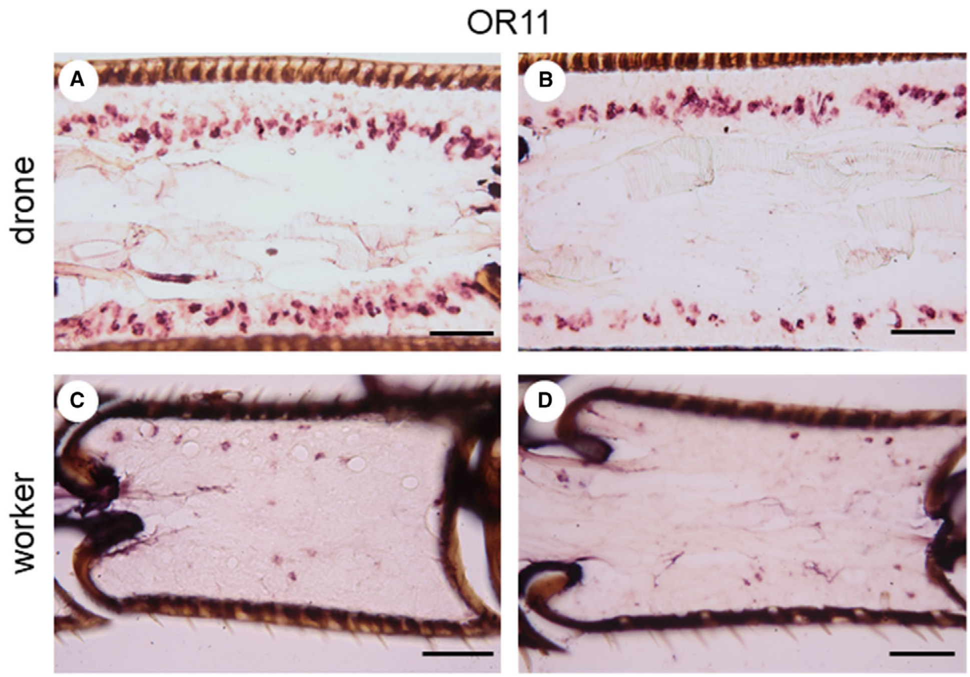

Fig. 3 Comparison of the number of OR11-positive cells in the antenna of drones and workers. (A–D) Longitudinal sections through the median plane of flagellar segments from drone (A, B) and worker (C, D) antennae hybridized with the OR11-specific probe. While a large number of cells in the antenna of drones express OR11, only rather few cells are positive for this receptor type in the antenna of workers. On sections through the median plane of the antenna, in both drones and workers, OR11-expressing cells mostly resided in a zone of the antennal tissue that was separated from the cuticle by a rather thin layer of non-labeled cells. The images shown in (A–D) are representative of five independent experiments with antennae from different drones and workers. Scale bars: A–D = 50 μm.

Scanning electron microscopy (SEM)

Antennae from freshly killed adult workers and drones were carefully removed from the head and fixed for 15 min in a modified Carnoy's solution (60% ethanol, 20% chloroform, 20% acetic acid). The samples were then dehydrated in a graded ethanol series of 60%, 70%, 80%, 90%, and 100% for at least 15 min in each solution, followed by 5 min in hexamethyldisilazane. The samples were placed onto a filter paper and left to dry overnight. Afterwards, they were mounted onto aluminum specimen stubs with double-sided adhesive pads. The following day, the samples were sputter-coated with gold for 145 s at 20 mA using a Balzers SCD 004 sputter coater (BAL-TEC, Balzers, Liechtenstein). The samples were examined with a Hitachi SEM S-2400 scanning electron microscope (Hitachi, Krefeld, Germany) at 12–18 kV and images were captured on ILFORD FP 4 black-and-white film (Harman Technology, Mobberley, UK).

Isolation of RNA and reverse transcription

Antennae of 15–20 freshly killed honey bees (drones as well as workers) were removed, pooled, and frozen in liquid nitrogen. Next, antennae were homogenized on ice for 10–15 min in 1 mL of Trizol reagent (Thermo Fisher Scientific, Waltham, MA, USA) utilizing a "micro pestle" and a "micro homogenizer". Following a 5-min incubation at room temperature, total RNA was isolated according to the recommendations of the manufacturer of the Trizol reagent. The air-dried RNA pellet was resuspended in 20 μL of RNase-free H_2O. Two microliters were used to determine the concentration and purity of the RNA with an Epoch microplate spectrophotometer (BioTek, Winooski, VT, USA). The remaining 18 μL were subjected to a treatment with 4 units DNase I (New England Biolabs, Ipswich, MA, USA) at 37 °C for 30–40 min. Subsequently, poly(A)$^+$ RNA was isolated utilizing the Dynabeads mRNA purification kit (Thermo Fisher Scientific) following the protocol of the supplier. Ultimately, poly(A)$^+$ RNA samples were eluted with 22 μL H_2O before the purity of the isolated RNA was verified using an Epoch microplate spectrophotometer.

For first-strand cDNA synthesis, 10 μL of isolated poly(A)$^+$ RNA were supplemented with 12 μL RNase-free H_2O, 2 μL 10 mmol/L 2′-deoxynucleoside 5′-triphosphate (dNTP) solution mix (New England Biolabs), and 2 μL 50 μmol/L oligo(dT)$_{20}$ primer (Thermo

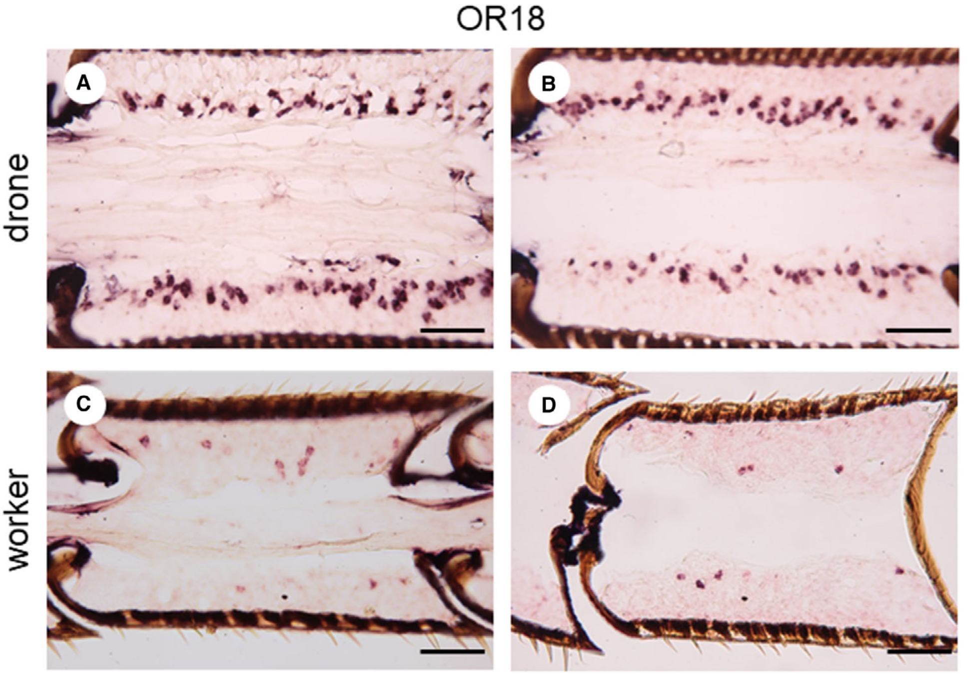

Fig. 4 Expression of OR18 in antennal cells of drones and workers. (A–D) Chromogenic *in situ* hybridization with the OR18-specific probe on longitudinal sections through the median plane of flagellar segments from antennae of drones (A–B) and workers (C–D). Compared to the numerous OR18-positive cells in the antenna of drones, only a relatively low number of antennal cells were stained in workers. The images depicted are representative of five independent experiments using antennae from different drones and workers. Scale bars: A–D = 50 μm.

Fisher Scientific). Following 5 min at 65 °C, 8 μL 5× SSIV Buffer, 2 μL 100 mmol/L 1, 4-dithiothreitol, 2 μL RNaseOut (Thermo Fisher Scientific), and 1 μL Superscript IV reverse transcriptase (Thermo Fisher Scientific) were added on ice. Synthesis of cDNA was conducted for 50 min at 52 °C followed by a 10-min incubation at 80 °C.

PCR amplification and molecular cloning

To generate ribonucleotide probes for *in situ* hybridization experiments, sequences encoding OR10 (GenBank accession number: NM_001242961.2), OR11 (NM_001242962.1), OR18 (XM_003250678.4), OR170 (NM_001242993.1), and the odorant receptor coreceptor Orco (KF911087.1; also designated as OR2) were amplified from antennal cDNA of honey bees utilizing the following oligonucleotide primers: OR10: 5′-ATG GTCCAAATTAGAAACGCGAAAG-3′ and 5′-CCACT TCAATGCAATAAATGCCTGC; OR11: 5′-ATGGTCC AAATTAGAAACGCGAAAG-3′ and 5′-TTACGTAA CCGTACGTAACATATTC-3′; *OR18*: 5′-ATGAACGC GGAAAAGTTGATGATCG-3′ and 5′-TTAGGTTGT GAATGTTCGTAGCATA-3′; OR170: 5′-GACCAATA TAAATGAGAAATTGTCG-3′ and 5′-AACATACCGA ATATGCTTGATTTAG-3′; *Orco*: 5′-ACAAGGGCTAA TCGCCGACCTGATG-3′ and 5′-ACCATGAAGTA GGTAACCATAGCTC-3′.

In PCR reactions, 40.5 μL H_2O were mixed with 5 μL 10× Titanium Taq PCR Buffer (Takara Bio, Saint-Germain-en-Laye, France), 1 μL 10 mmol/L dNTP solution mix, 0.5 μL of a 100 μmol/L stock solution of each primer, 2 μL first-strand cDNA, and 0.5 μL 50× Titanium Taq DNA Polymerase (Takara Bio). For PCR amplification, the following conditions were used: 1 min at 97 °C followed by 35 cycles with 97 °C for 30 s and 3 min at 68 °C. The final cycle was succeeded by an additional incubation at 68 °C for 3 min. PCR products were visualized after agarose gel electrophoresis by ethidium bromide staining. PCR fragments of the predicted molecular size were excised from gels, purified with the Monarch DNA gel extraction kit (New England Biolabs) and cloned into the pGEM-T easy plasmid (Promega, Madison, WI, USA). The identity of the insertion was verified by sequencing.

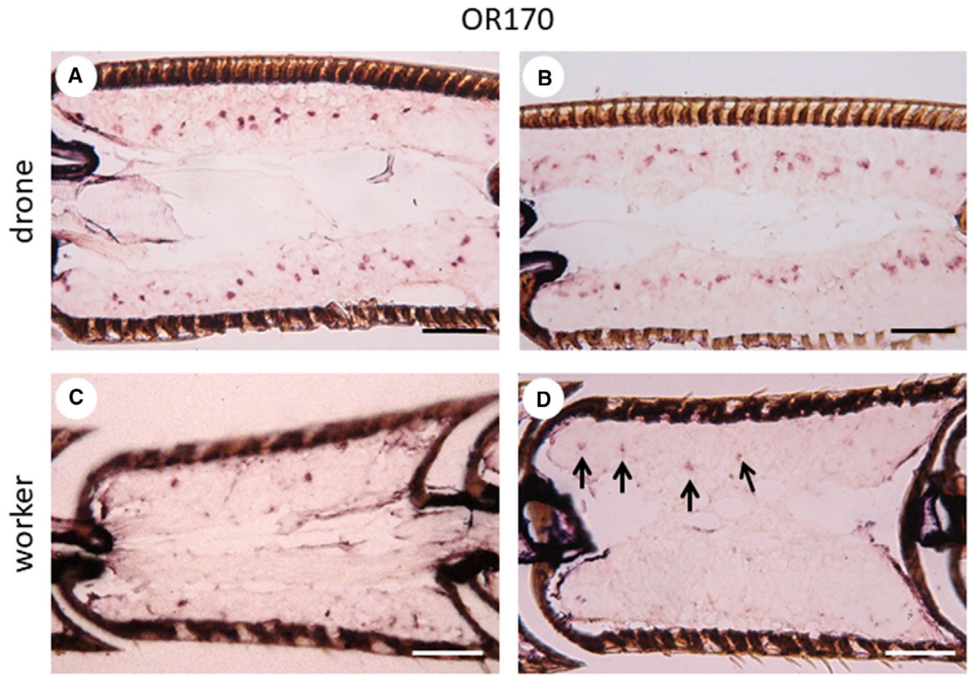

Fig. 5 Expression of OR170 in cells of the antennae of drones and workers. (A–D) Expression of OR170 was visualized by *in situ* hybridization with a specific antisense riboprobe on longitudinal sections through the median plane of flagellar segments from drone (A, B) and worker (C, D) antennae (some of the OR170-positive cells on the antennae of workers are denoted by arrows). The pictures shown are representative of four independent experiments with antennae from different drones and workers. Scale bars: 50 μm.

Generation of riboprobes for in situ hybridization

Antisense and sense riboprobes for OR10, OR11, OR18, OR170, and Orco were synthesized using pGEM-T easy plasmids containing insertions for relevant coding sequences. To generate riboprobes labeled with either digoxigenin or fluorescein, the T7/SP6 RNA transcription system (Sigma-Aldrich, St Louis, MO, USA) was used as recommended by the manufacturer.

Preparation, fixation, and acetylation of tissue sections for in situ *hybridization experiments*

Antennae of drones, workers, and virgin queens were removed and embedded in Tissue-Tek O.C.T. Compound (Sakura Finetek, Alphen aan den Rijn, the Netherlands). Embedded antennae were stored at −80 °C until use. Longitudinal and transverse sections (10-μm thick) through antennae were prepared with a Cryostar NX50 cryostat (Thermo Fisher Scientific) at −20 °C. Sections were thaw-mounted on Superfrost Ultra Plus adhesive slides (Thermo Fisher Scientific) and immediately utilized for *in situ* hybridization experiments. Next, sections were fixed in a staining trough with 4% paraformaldehyde in 0.1 mol/L $NaHCO_3$ (pH 9.5) and acetylated with 0.25% acetic anhydride freshly added in 0.1 mol/L triethanolamine as described previously (Pregitzer *et al*., 2017). Finally, sections were washed three times for 3 min in 1× phosphate-buffered saline (0.85% NaCl, 1.4 mmol/L KH_2PO_4, 8 mmol/L Na_2HPO_4, pH 7.1) and incubated at 4 °C in prehybridization solution (5× SSC [0.75 mol/L NaCl, 0.075 mol/L sodium citrate, pH 7.0] and 50% formamide) for 15 min.

Chromogenic in situ hybridization

Each slide with tissue sections was covered with 130 μL hybridization solution 1 (50% formamide, 25% H_2O, 25% Microarray Hybridization Solution version 2.0 [GE Healthcare, Freiburg, Germany]) supplemented with the labeled riboprobe. After placing a coverslip, slides were incubated overnight at 65 °C in a box that contained filter paper soaked with 50% formamide. The next day, slides were washed three times for 30 min each in 0.1× SSC at 65 °C in a staining trough. Subsequently, sections were treated for 30 min at room temperature with 1% blocking reagent (Roche Diagnostics, Mannheim, Germany) in

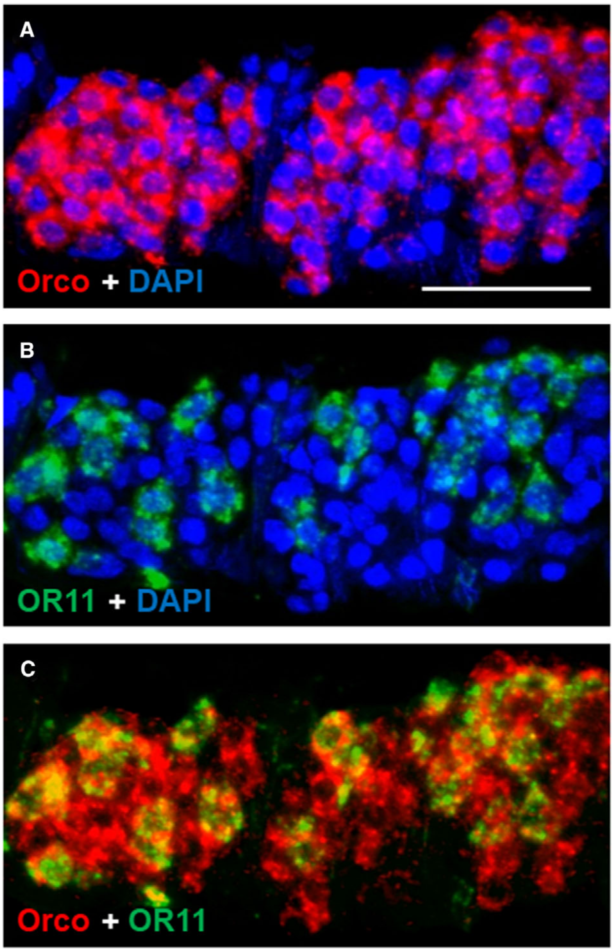

Fig. 6 OR11 is expressed in a subset of Orco-positive olfactory sensory neurons (OSNs) in the antenna. (A–C) High-magnification images of a two-color fluorescence *in situ* hybridization (FISH) experiment on a longitudinal section from an antenna of a drone incubated with antisense RNA probes for Orco (A, red) and OR11 (B, green). The section was counterstained with DAPI (blue). The overlay of the red and the green fluorescence channel in C demonstrates expression of OR11 in a substantial proportion of the Orco-positive cells. The images depict projections of confocal Z-stacks. Scale bar: 25 μm.

Tris-buffered saline (TBS) (100 mmol/L 2-amino-2-[hydroxymethyl]propane-1,3-diol [Tris], 150 mmol/L NaCl, pH 7.5) supplemented with 0.3% Triton X-100. This incubation was conducted in a box containing filter paper soaked with water (henceforth designated as humidity box). Then, 130 μL of anti-digoxigenin alkaline phosphatase-conjugated antibody (Roche Diagnostics, catalog number 11093274910) diluted 1 : 750 in TBS with 0.3% Triton X-100 and 1% blocking reagent was spread on each slide and a coverslip was placed on top for a 30-min incubation at 37 °C in a humidity box. After washing twice in TBS for 15 min each, slides were briefly rinsed in DAP buffer (100 mmol/L Tris, pH 9.5, 100 mmol/L NaCl, 50 mmol/L $MgCl_2$) before visualization of hybridization signals was carried out by incubating at 37 °C in a staining trough filled with DAP buffer containing 0.0225% NBT (nitroblue tetrazolium) and 0.0175% BCIP (5-brom-4-chlor-3-indolyl phosphate). Finally, tissue sections were mounted using Vectamount (Vector Laboratories, Burlingame, CA, USA) and analyzed with a Leica DMLB microscope (Leica Microsystems, Wetzlar, Germany) and a Canon EOS 700D camera (Canon, Tokyo, Japan).

Table 1 Percentage of Orco-positive OSNs expressing OR11 or OR18.

	OR11/ Orco	OR18/ Orco
Specimen 1 (cell numbers)	15/38	30/106
Specimen 2 (cell numbers)	27/75	32/83
Specimen 3 (cell numbers)	33/100	33/92
Specimen 4 (cell numbers)	28/75	17/56
Specimen 5 (cell numbers)	36/124	33/101
Specimen 6 (cell numbers)	29/75	44/124
Total cell numbers	168/487	189/562
Percentage of OR-positive OSNs in relation to Orco-expressing OSNs	34.5%	33.6%

Based on two-color fluorescence *in situ* hybridization (FISH) experiments on longitudinal sections from drone antennae with antisense probes for Orco in combination with OR11 or OR18, the percentage of Orco-positive olfactory sensory neurons (OSNs) expressing these receptors was determined. Clusters of Orco-positive cells (specimens 1–6) from three male individuals were randomly chosen and the number of Orco-expressing cells as well as the cells positive for the relevant receptor (OR11 or OR18) were counted in each cluster (see also Fig. S5–S6). For instance, out of the 38 Orco-expressing cells found in a cluster of OSNs on specimen 1, 15 co-expressed OR11 (Fig. S5).

Two-color fluorescence in situ hybridization (FISH)

For two-color FISH, sections were prepared, fixed, acetylated, prehybridized, hybridized, washed, and blocked as described for chromogenic *in situ* hybridization. Yet, a different hybridization buffer (50% formamide, 2× SSC, 10% dextran sulphate, 0.2 mg/mL yeast tRNA [Sigma-Aldrich], 0.2 mg/mL sonicated herring sperm DNA [Sigma-Aldrich]) was used, and sections

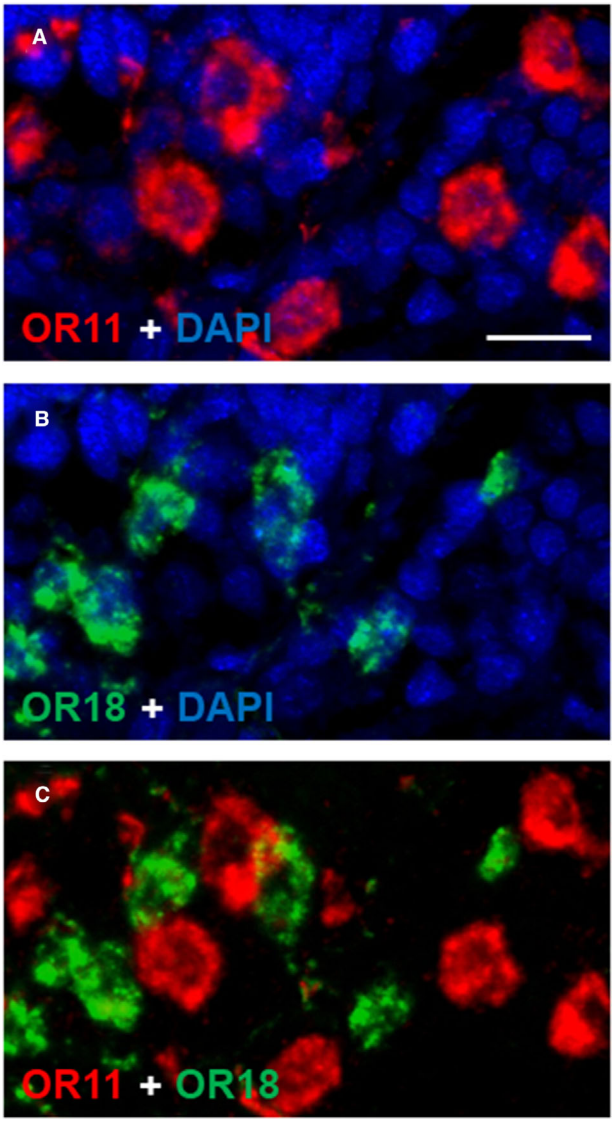

Fig. 7 Expression of OR11 and OR18 by distinct subsets of cells in the antenna of drones. (A–C) Two-color fluorescence *in situ* hybridization (FISH) on a longitudinal section through the antenna of a drone hybridized with antisense riboprobes for OR11 (A, red) and OR18 (B, green). Counterstaining was conducted with DAPI (blue). The overlay of the red and the green fluorescence channel (C) shows that cells positive for OR11 lack expression of OR18 and vice versa. The pictures represent projections of Z-stacks of confocal images; they are representative of five independent experiments with antennae from different drones. Scale bar: 10 μm.

were simultaneously hybridized with digoxigenin- and fluorescein-labeled probes. After blocking, each slide was covered with 130 μL TBS supplemented with 0.3% Triton X-100, 1% blocking reagent, anti-digoxigenin alkaline phosphatase-conjugated antibody (1 : 500,

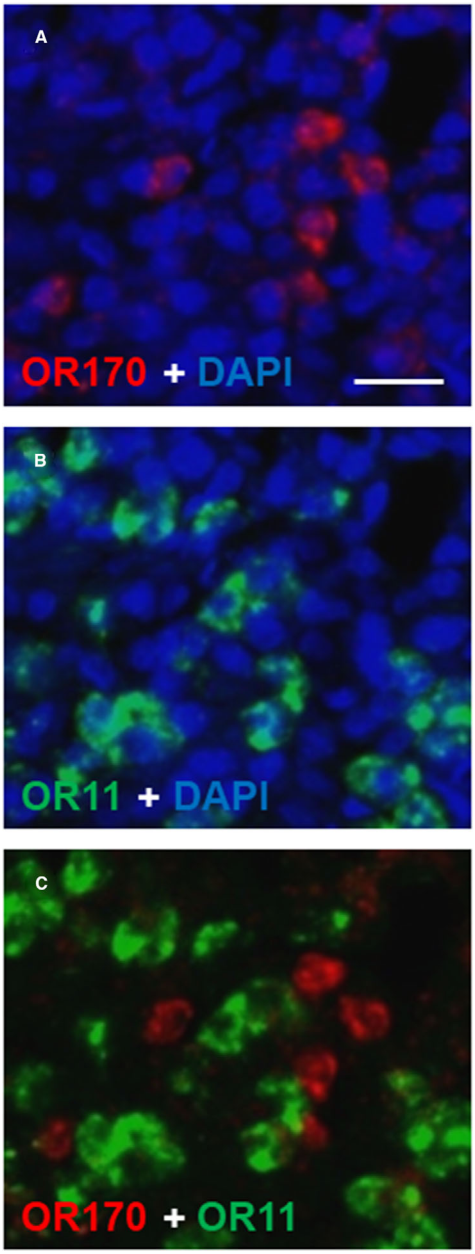

Fig. 8 OR170 and OR11 are expressed by different cells in the antenna of drones. (A–C) A longitudinal section of a male antenna was incubated in a two-color fluorescence *in situ* hybridization (FISH) experiment with antisense probes for OR170 (A, red) and OR11 (B, green) prior to counterstaining with DAPI (blue). The merged image (C) of the red and the green fluorescence channel reveals that OR170 and OR11 are expressed by different subpopulations of cells in the male antenna. The images depict projections of confocal Z-stacks; they are representative of five independent experiments using antennae from different drones. Scale bar: 10 μm.

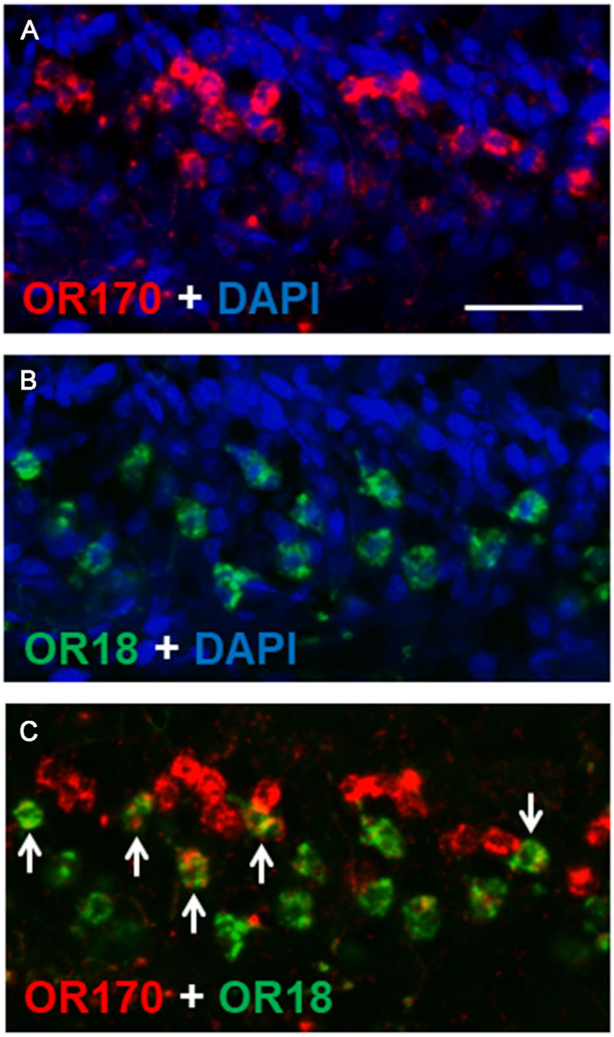

Fig. 9 Partial co-expression of OR170 and OR18 in a subset of antennal cells in drones. (A–C) Antisense probes for OR170 (A, red) and OR18 (B, green) were used for two-color fluorescence *in situ* hybridization (FISH) on a longitudinal section through the antenna of a drone. The section was counterstained with DAPI (blue). The merged image (C) of the red and the green fluorescence channel discloses that the receptors OR170 and OR18 are mostly expressed by distinct cells. Yet, a subset of the OR170-positive cells co-express OR18 (indicated by arrows) in the male antenna. The pictures represent projections of Z-stacks of confocal images. They are representative of five independent experiments using antennae from different drones. Scale bar: 20 μm.

Roche Diagnostics, catalog number 11093274910), and anti-fluorescein horseradish peroxidase-conjugated antibody (1 : 50, Roche Diagnostics, catalog number 11426346910). A coverslip was placed on the slides and following an incubation for 1 h at 37 °C in a humidity box, sections were washed three times for 5 min each with TBS supplemented with 0.05% Tween-20. After rinsing the slides briefly with 150 mmol/L Tris-HCl (pH 8.3) comprising 0.1% Tween-20, the Vector red alkaline phosphatase substrate kit (Vector Laboratories) was used as recommended by the manufacturer to visualize digoxigenin-labeled probes. Accordingly, 2.5 mL of 150 mmol/L Tris-HCl (pH 8.3) containing 0.1% Tween-20 and 80 μL of each Vector red reagent (1, 2, and 3) were mixed before 130 μL of this solution were applied to each slide. After placing a coverslip on top, sections were incubated for 50 min at room temperature in a humidity box. Sections were washed three times for 10 minutes each in TBS supplemented with 0.05% Tween-20. Next, to visualize fluorescein-labeled probes, the TSA fluorescein system kit (Perkin Elmer, Waltham, MA, USA, catalog number NEL701001KT) was utilized. The fluorophore tyramide reagent was reconstituted with dimethyl sulfoxide and diluted 1 : 50 with 1× amplification diluent as recommended by the manufacturer. After spreading 130 μL of this solution on each slide, a coverslip was placed on top. Following a 50-min incubation at room temperature in a humidity box, slides were washed three times for 10 min each in TBS supplemented with 0.05% Tween-20.

To visualize cell nuclei, sections were counterstained with 4′,6-diamidino-2-phenylindole (DAPI). For this purpose, 1 mg DAPI dissolved in 1 mL H_2O was diluted with TBS (1 : 250 or 1 : 500 dilution). One milliliter of DAPI solution was spread on each slide and counterstaining was conducted in a humidity box for 30 min at room temperature. Finally, sections were briefly rinsed with H_2O, air-dried, and mounted in 100 μL Mowiol per slide. For preparing the Mowiol solution, 6.0 g glycerin, 2.4 g Mowiol 4–88, 6.0 mL H_2O, and 12.0 mL 0.2 mol/L Tris (pH 8.5) were stirred for 2 h at room temperature.

Sections were analyzed with a confocal LSM 880 laser scanning microscope (Carl Zeiss Microscopy, Jena, Germany). Confocal image Z-stacks were acquired from antennae in the red, green, and blue fluorescence channel. Images were taken and processed using the ZEN software (Carl Zeiss).

Results

Male-biased expression of different OR types in the antenna

As an initial step to visualize and analyze the cells expressing OR10, OR11, OR18, and OR170, *in situ* hybridization experiments with antisense riboprobes for

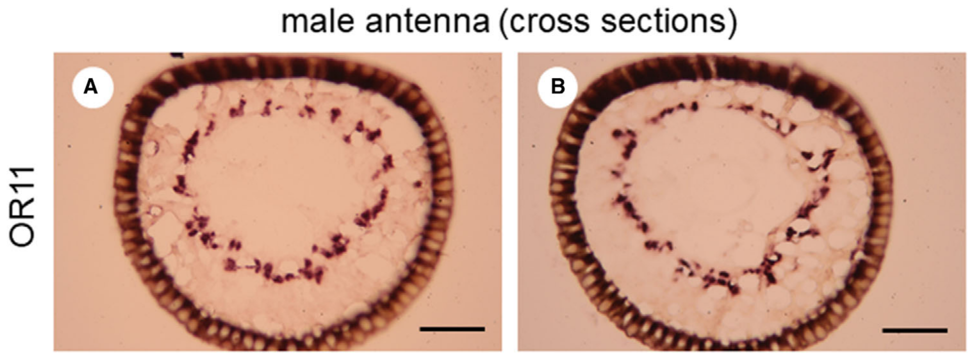

Fig. 10 Distribution of OR11-expressing cells on transversal sections through the antenna of drones. (A, B) Chromogenic *in situ* hybridization on cross sections through flagellar segments derived from antennae of drones using an OR11-specific antisense probe. The OR11-expressing cells are arranged in a ring-shaped manner in the antennal tissue beneath the cuticle. Scale bars: 50 μm.

these receptors were conducted on longitudinal sections through male and female (worker) antennae. Using an antisense RNA probe for OR11, a high number of cells in male antennae were found to express this receptor type (Fig. 2A, B). By contrast, in experiments with the corresponding sense riboprobe for OR11, no labeling of antennal cells was observed (Fig. 2C), substantiating the specificity of the signals obtained with the antisense probe. The OR11-positive cells in the antenna of drones were detectable throughout the different antennal segments except the scape, the pedicel, and the first two proximal segments of the flagellum (data not shown). Large numbers of OR11-positive cells were found for all antennal segments harboring these cells. Comparing the number of OR11-expressing cells in antennae from drones versus workers by *in situ* hybridization revealed clear differences: while numerous cells were stained per segment by the OR11-specific probe in drones (Fig. 3A, B), the number of OR-positive cells in antennal segments of workers was relatively low (Fig. 3C, D). Thus, these results demonstrate that expression of OR11 is male-biased with respect to the number of relevant cells in the antenna.

Next, we investigated the expression of OR18 in the antennae of honey bees. Following hybridization with an OR18-specific antisense riboprobe, a large number of cells were labeled in the antenna of males (Fig. S1A–B), whereas no staining of antennal cells was observed upon incubation with the corresponding sense probe (Fig. S1C). The OR18-positive cells (Fig. S1A–B) were localized in a similar layer of the antennal tissue as the cells expressing OR11 (Fig. 2A, B). Furthermore, the OR18-expressing cells were detectable throughout the antenna of drones except the scape, the pedicel, and the first two proximal segments (data not shown). Similar to OR11, a comparatively large number of OR18-expressing cells was detectable in all segments of the male antenna that comprised such cells. Analyzing the expression of OR18 in antennae of drones versus workers showed that this receptor type is expressed in a rather low number of cells in segments of the workers' antenna (Fig. 4C, D) while it is abundantly expressed in the antenna of drones (Fig. 4A, B).

Examining the expression of OR170, a substantial number of cells on sections through the antennae of drones were labeled upon incubation with an OR170-specific antisense riboprobe, whereas no signals were observed with the corresponding sense probe (Fig. S2). Hybridizing longitudinal sections through the antennae of both drones and workers with the antisense probe for OR170 revealed that this receptor is expressed in a higher number of antennal cells in males (Fig. 5A, B) compared to workers (Fig. 5C, D). However, in drones, the number of cells expressing OR170 appeared to be lower than for OR11 and OR18 (Figs. 3A, B and 4A, B).

In contrast to OR11, OR18, and OR170, *in situ* hybridization experiments on antennal sections from drones and workers with an antisense riboprobe for OR10 revealed no labeled cells (data not shown), although the same experimental conditions were used as for the other receptors tested. Therefore, expression of OR10 could not be analyzed any further.

Male-biased OR types are expressed in substantial numbers of Orco-positive OSNs

On longitudinal sections through the central plane of antennal segments, the cells expressing the male-biased OR types OR11, OR18, and OR170 were arranged in

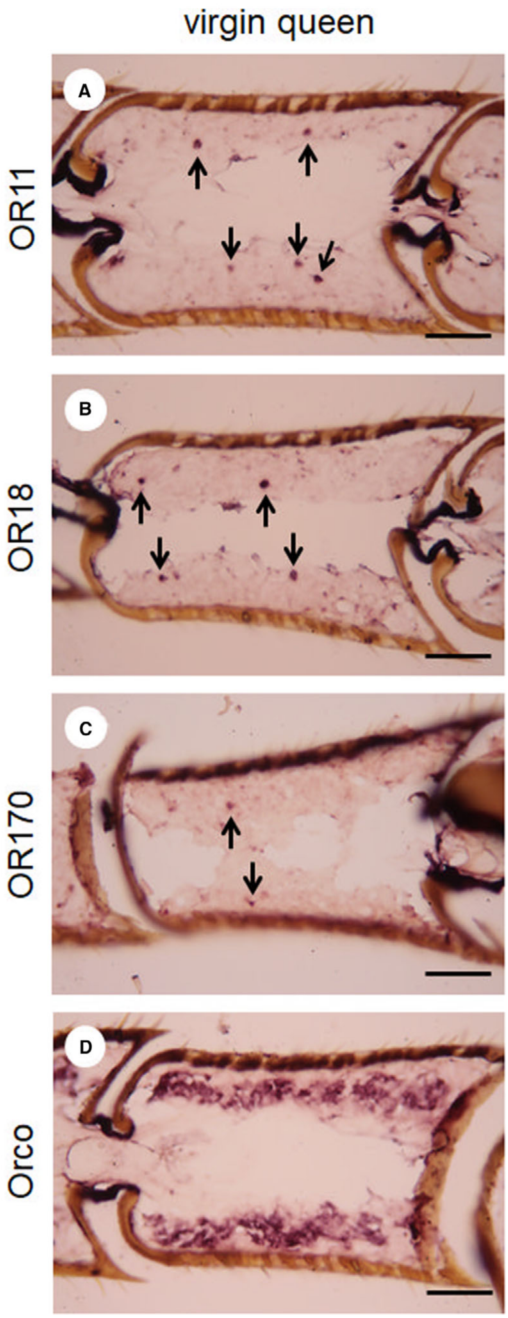

Fig. 11 . Expression of male-biased odorant receptors (ORs) in the antenna of virgin queens. A–D. Longitudinal sections through the antennae of virgin queens that were hybridized with antisense riboprobes for OR11 (A), OR18 (B), OR170 (C), or Orco (D) in chromogenic *in situ* hybridization approaches. Some of the stained cells in (A–C) are indicated by arrows. The micrographs shown in (A–C) are representative of two (OR11 and OR18) to three (OR170) independent experiments with antennae from different virgin queens. Scale bars: 50 μm.

a ribbon-shaped pattern in a tissue layer below the cuticle. Yet, the OR-positive cells were usually separated from the cuticle by a layer of OR-negative cells (Figs. 2A, B, 3, 4, and 5). This distribution of the OR-expressing cells is reminiscent of the expression of the odorant receptor co-receptor Orco in the antennae of honey bees (Fig. S3). Since Orco is generally considered a marker for OR-positive OSNs in insects (Vosshall *et al*., 1999; Vosshall *et al*., 2000; Krieger *et al*., 2003; Larsson *et al*., 2004; Pitts *et al*., 2004; Jones *et al*., 2005), we set out to evaluate if and to what extent the male-biased receptor types OR11, OR18, and OR170 are expressed in Orco-positive OSNs. In two-color FISH experiments using longitudinal sections through the antenna of drones and antisense probes for Orco and OR11, it was found that a substantial number of the Orco-positive OSNs express OR11 (Fig. 6A–C). Likewise, two-color FISH with antisense probes for OR18 and Orco revealed that OR18 is also expressed by a large subpopulation of the Orco-positive OSNs in the antenna of drones (Fig. S4). Regarding OR170, however, two-color FISH experiments with an antisense probe for Orco were not feasible because in contrast to probes labeled with digoxigenin, the fluorescein-labeled probes for both OR170 and Orco did not yield clear staining.

For more detailed quantitative analyses regarding the expression of male-biased receptor types in the antenna of drones, longitudinal antennal sections were concomitantly hybridized with antisense probes for Orco and the relevant ORs to determine the percentage of Orco-positive OSNs expressing OR11 or OR18, respectively. The results of these approaches are exemplarily shown for OR11 in Fig. S5 and for OR18 in Fig. S6. Based on these staining experiments, out of 487 randomly chosen Orco-positive OSNs originating from different cell clusters and sections through the antennae of three distinct male individuals, 168 (~35%) were observed to express OR11 (Table 1). Consequently, approximately one third of the Orco-expressing OSNs in male antennae seem to be endowed with OR11. Similarly, we also found that approximately one third of the Orco-positive OSNs in the antenna of males express OR18. In fact, from a total of 562 Orco-positive OSNs chosen at random from different cell clusters and sections through the antennae of three distinct male individuals, 189 (~34%) expressed OR18 (Table 1). Due to similar percentages of Orco-positive OSNs in the antenna of drones expressing OR11 (~35%) or OR18 (~34%), respectively, we next tested whether OR11 and OR18 might be co-expressed by the same cells. Two-color FISH with antisense probes for OR11 and OR18 demonstrated that these two receptors are expressed by non-overlapping subsets of OSNs in the antenna of drones (Fig. 7). Therefore, it can be concluded that approximately 69% of the Orco-positive OSNs in male antennae either express OR11 or OR18. Of note, OSNs positive for OR11 or OR18 were often found to be located in close vicinity (Fig. 7), suggesting that these receptors are expressed in neurons belonging to the same sensillum.

Investigating a potential co-expression of OR170 with OR11, two-color FISH experiments with sections through the antenna of drones disclosed that these two receptors are expressed by distinct subpopulations of antennal cells (Fig. 8). Similar staining approaches with antisense probes for OR170 and OR18 demonstrated that these two receptors are predominantly expressed in different subsets of OSNs in the antenna of drones (Fig. 9). However, we also found a subpopulation of the antennal cells that were co-labeled by both the OR170 and the OR18 antisense probe (Fig. 9), indicating that OR170 and OR18 are partially co-expressed in certain OSNs.

Assignment of the OR11-expressing OSNs to a sensillum type

In the antennae of bees, poreplate sensilla constitute the most frequent olfactory sensillum type, notably in drones that reveal a substantially increased number of poreplates compared to workers (Esslen & Kaissling, 1976). In addition, drones lack sensilla basiconica and have comparatively few other olfactory sensilla (i.e., sensilla trichodea A) on their antennae (Esslen & Kaissling, 1976). Collectively, these findings imply that ORs present in a high number of cells on the male antenna, such as the male-biased receptor types OR11, OR18, and OR170, are expressed by OSNs of sensilla placodea. Therefore, attempts were made to scrutinize whether OR11 is expressed in OSNs of poreplate sensilla. In this context, it has to be pointed out that the poreplates represent the only olfactory sensillum type that is present on the front and on the back side of the antenna in drones (Esslen & Kaissling, 1976) (supplemental Fig. 7). Hybridizing transverse sections through the antennae of drones with the OR11-specific antisense riboprobe disclosed that OR11-positive cells are arranged annularly and can be found in all quadrants of the antennal tissue beneath the cuticle (Fig. 10), namely OR11 is expressed in cells of the front and the back side of the antenna. Thus, the arrangement of the OR11-expressing OSNs is consistent with the distribution of sensilla placodea, supporting the view that OR11 is expressed in cells of the poreplates.

Expression of male-biased ORs in queens

Unlike the non-mating workers, mating is considered to be the only important function of drones for the colony. Therefore, the elevated expression of OR11, OR18, and OR170 in the male antenna suggests that these OR types might be implicated in the detection of pheromonal compounds, such as 9-ODA, that are critical for drones to find a mating partner (i.e., a virgin queen). With respect to mating and a possible role of OR11, OR18, and OR170 in the detection of sex pheromones, we also assessed the expression of these receptor types in queens. *In situ* hybridization experiments with the relevant antisense riboprobes and longitudinal sections through antennae of virgin queens (Fig. 11A–C) revealed that in marked contrast to Orco (Fig. 11D), OR11 is expressed in a rather low number of cells in the antenna of queens (Fig. 11A). This result demonstrates that OR11 is more abundantly expressed in males (Fig. 3A, B) than in females, namely queens (Fig. 11A) and workers (Fig. 3C, D). Likewise, also OR18 and OR170 were found to be expressed in rather few cells in antennae of queens (Fig. 11B, C). Thus, the number of cells positive for these OR types is also considerably lower in queens than in drones (Figs. 4A, B and 5A, B).

Discussion

In the present study, we have investigated the number and distribution of cells in the antennae of honey bees expressing the receptor types OR11, OR18, or OR170 that are considered male-biased according to previous qPCR and RNA sequencing experiments (Wanner *et al*., 2007; Jain & Brockmann, 2020). Consistent with a function of these ORs as olfactory receptors, our *in situ* hybridization approaches disclosed expression of OR11 and OR18 in antennal cells positive for Orco (Fig. 6 and Fig. S4), a marker for insect OSNs endowed with ORs (Vosshall *et al*., 1999; Vosshall *et al*., 2000; Krieger *et al*., 2003; Larsson *et al*., 2004; Pitts *et al*., 2004; Jones *et al*., 2005). For OR170, co-staining experiments with a probe for Orco were not successful; yet, the partial co-expression of OR170 with OR18 (Fig. 9) indirectly indicates that also OR170 is expressed by OSNs.

Comparing the expression of OR11 in the antennae of males (drones) and females (workers and queens) revealed that OR11 was expressed in considerably higher numbers of cells in the antennae of drones (Figs. 3 and 11). This increased number of OR11-positive OSNs in males most likely accounts for the male-biased transcript level of OR11 in the antenna that was observed in previous qPCR and RNA sequencing experiments (Wanner *et al*., 2007; Jain & Brockmann, 2020). However, it remains elusive why OR11 is expressed at such different levels in males versus females. In this context, it is noteworthy that OR11 is activated by 9-ODA (Wanner *et al*., 2007), the major component of the queen mandibular pheromone (Barbier & Lederer, 1960; Callow & Johnston, 1960). 9-ODA has been reported to have multiple functions for pheromone communication in honey bees (Le Conte & Hefetz, 2008; Trhlin & Rajchard, 2011; Bortolotti & Costa, 2014); most notably, virgin queens utilize 9-ODA to attract drones during nuptial flights (Gary, 1962; Gary & Marston, 1971; Boch *et al*., 1975). Moreover, as an essential compound of the queen mandibular and the queen retinue pheromone, 9-ODA (in combination with some other synergistically active components of these pheromone blends) is supposed to affect the ovary development and the behavior of workers, including attracting workers to the queen (Le Conte & Hefetz, 2008; Trhlin & Rajchard, 2011; Bortolotti & Costa, 2014). Thus, detection of 9-ODA is important for both sexes. However, while drones are apparently capable of long-range detection of 9-ODA (Gary, 1962; Pain & Ruttner, 1963; Butler & Fairey, 1964; Loper *et al*., 1993), this chemical and other queen-released pheromonal substances are transmitted in the hive via retinue bees or other workers through direct contact (Naumann *et al*., 1991), indicating that it is not necessary for workers to detect 9-ODA over larger distances. Consequently, in drones, the number of OR11-expressing OSNs could have been considerably elevated in order to sensitively detect minute quantities of 9-ODA to locate virgin queens entering a drone congregation area, whereas in workers, these cells are less abundant since workers might not rely on ultrasensitive reception of this compound that is present at higher concentrations in the hive. In fact, approximately one third of the Orco-positive OSNs in the antenna of drones express OR11 (Table 1 and Fig. S5). This finding for drones is reminiscent of the substantial percentage of Orco-positive OSNs in the antennae of male *Bombyx mori* silkworm moths expressing the OR types BmOR1 (43%) or BmOR3 (48%) that mediate ultrasensitive and specific reception of the female-emitted sex pheromone compounds bombykol and bombykal, respectively (Nakagawa *et al*., 2005). This analogy to pheromone receptors from *Bombyx mori* further supports the notion that receptor OR11 from honey bees serves in drones the detection of a sex pheromone component emitted by queens, most likely 9-ODA.

In various insects, including moths, *Drosophila* flies, locusts, ants, and beetles, pheromone-sensitive olfactory neurons, notably OSNs expressing male-biased PRs, are typically housed in sensilla trichodea or sensilla basiconica (Keil, 1989; Meng *et al*., 1989; Ljungberg *et al*., 1993; Hallberg *et al*., 1994; Clyne *et al*., 1997; Ochieng' & Hansson, 1999; Krieger *et al*., 2004; Sakurai *et al*., 2004; Krieger *et al*., 2005; Pophof *et al*., 2005; Alvarez *et al*., 2015; McKenzie *et al*., 2016; Ghaninia *et al*., 2017). By contrast, our *in situ* hybridization experiments disclosed a circular arrangement of OR11-expressing OSNs (Fig. 10), demonstrating expression of OR11 in sensilla on the front and on the back side of the male antenna. This arrangement of the OR11-positive cells is perfectly in line with the distribution of poreplates on the antennal flagellum in drones (Esslen & Kaissling, 1976), indicating that OR11 is expressed in OSNs of sensilla placodea. In addition, because other olfactory sensilla on the male antennae are rather rare (Esslen & Kaissling, 1976), the huge number of OR11-positive cells in drones (Figs. 2, 3, Fig. S5, and Table 1) strongly argues for an expression of this 9-ODA-activated receptor in OSNs of poreplates. In accord with this notion, 9-ODA-responsive OSNs in the antennae of drones apparently reside in sensilla placodea as shown by an early electrophysiological study (Kaissling & Renner, 1968). From a more comprehensive perspective, our findings support the concept that the expression of PRs in insects is not confined to a particular sensillum type.

Like OR11, the receptor types OR18 and OR170 are expressed by considerably higher numbers of cells in the antenna of drones as compared to females (Figs. 4–5 and Fig. 11). Because mating is the only obvious task drones have to perform for honey bee colonies, our findings for OR18 and OR170 imply that also these receptors could be implicated in the reception of pheromones critical for mating. Yet, in heterologous expression experiments using *Xenopus* oocytes, OR18 and OR170 were not activated by components of the queen mandibular pheromone and a number of further queen-emitted pheromonal substances (Wanner *et al*., 2007). However, it could not be excluded that activation of these OR types by the pheromonal compounds tested failed due to technical reasons; for example, insufficient receptor expression in the heterologous system (Wanner *et al*., 2007). Alternatively, it is conceivable that OR18 and OR170 respond to yet unknown queen-released compounds. In any case, in particular the substance(s) activating OR18 can be considered of high relevance for drones since this receptor is expressed by approximately one third of the Orco-positive OSNs in the antennae of males (Table 1 and Fig. S6), similar to OR11. With respect to the expression of OR18 and OR170, we have observed that these two receptors are partially co-expressed by the same cells (Fig. 9). Although OSNs are commonly thought to express only a single OR type, exceptions to this tenet have been reported for *Drosophila* flies, mosquitoes, and moths (Dobritsa *et al*., 2003; Couto *et al*., 2005; Goldman *et al*., 2005; Koutroumpa *et al*., 2014; Karner *et al*., 2015). The functional implications of the co-expression of ORs are unknown, but it has been proposed that OR co-expression might broaden the ligand spectrum of the relevant cells and/or may allow them to signal the coincidence of two distinct chemical cues (Goldman *et al*., 2005).

In the olfactory system of insects, sexual dimorphisms are not confined to the number of OSNs expressing given ORs but have also been described for the antennal lobe in which the axonal terminals of antennal OSNs endowed with a given OR type converge onto a single out of numerous round-shaped neuropil structures termed glomeruli (Vosshall *et al*., 2000; Couto *et al*., 2005; Sakurai *et al*., 2014). The dimension of a glomerulus is supposed to be correlated with the number of OSNs that express the relevant OR in the antennae (Grabe *et al*., 2016). Consequently, male moths with an exceptionally vast number of OSNs expressing OR types dedicated to the reception of different female-released sex pheromone components comprise several enlarged glomeruli (designated as macroglomeruli) that form the so-called macroglomerular complex (Hansson *et al*., 1992; Christensen & Hildebrand, 2002; Berg *et al*., 2014). Male-specific macroglomeruli also exist in other insects, including cockroaches, ants, and bees (Arnold *et al*., 1985; Boeckh & Tolbert, 1993; Hansson & Anton, 2000; Hoyer *et al*., 2005; Sandoz, 2006; Galizia & Rössler, 2010). In honey bee drones, four macroglomeruli have been found that are absent in workers (Arnold *et al*., 1985). Intriguingly, this number perfectly matches with the four male-biased OR types (OR10, OR11, OR18, and OR170) that have been reported for this species (Wanner *et al*., 2007; Jain & Brockmann, 2020). In view of the correlation between the number of OSNs expressing a given OR type and the volume of the glomerulus formed by the axonal terminals of these cells (Grabe *et al*., 2016), our findings that OR11, OR18, and OR170 are expressed in vast numbers of OSNs in males (Figs. 3–5) strongly suggest that the axons of the antennal neurons expressing these three receptors converge onto macroglomeruli in the antennal lobe. In accordance with this notion, one of the macroglomeruli in honey bee drones is specifically activated following exposure of the antennae to the queen-released pheromonal substance 9-ODA that activates OR11 (Sandoz, 2006; Wanner *et al*., 2007). Likewise, male-specific macroglomeruli and macroglomerular complexes in other insects frequently receive sensory input from sex pheromone-sensitive OSNs (Christensen & Hildebrand, 1987; Boeckh & Tolbert, 1993; Hansson, 1997; Hildebrand *et al*., 1997; Hildebrand & Shepherd, 1997; Hansson & Anton, 2000; Galizia & Rössler, 2010). Thus, the substantially increased numbers of antennal OSNs in drones expressing OR11, OR18 or OR170 may not only indicate that these cells project their axons to macroglomeruli but also corroborate the concept that olfactory neurons equipped with male-biased receptor types could contribute to the detection of (sex) pheromone compounds.

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Disclosure

The authors declare that they have no conflict of interest.

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Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Fig. S1. Visualization of cells expressing OR18 in the antenna of drones

Fig. S2. Expression of OR170 in cells of the drone antenna

Fig. S3. Expression of Orco in the antenna of drones and workers

Fig. S4. Expression of OR18 in a subset of Orco-positive olfactory sensory neurons in the antenna of drones

Fig. S5. Defining the percentage of OR11-expressing neurons among the Orco-positive antennal olfactory sensory neurons in drones

Fig. S6. Determining the proportion of OR18-expressing olfactory sensory neurons in the antennae of drones

Fig. S7. Sensilla placodea in the antennae of drones