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Supplemental Data

Odor Coding in the Maxillary Palp of the Malaria Vector Mosquito *Anopheles gambiae*

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Supplemental Experimental Procedures

Insects

An. gambiae sensu stricto, originated from Suakoko, Liberia, was reared as described [S1, S2] and 5- to 7-day-old females that had not been blood fed were used for experiments. For extracellular recordings, females with legs removed were mounted on a transparent Perspex block (1.1 × 1.1 × 1.5 cm) via a piece of transparent Scotch double-sided sticky tape. So that the mosquito could be immobilized, the wing, the antenna, the proboscis, and the junctions between palpal segments were pressed gently against the tape. The maxillary palp surface was viewed at 750× magnification through an Olympus IX50 inverted microscope. All recordings were made from capitate pegs on segments two to four of the maxillary palp.

Transmission Electron Microscopy

Heads with appendages were dissected from five-day old female mosquitoes and fixed with 4% glutaraldehyde for 4–5 days at 4°C. After washing in 2.5% potassium dichromate for 2 hr, tissues were then fixed with 1% osmium tetroxide for 4–5 days at 4°C. Tissues were later dehydrated in a graded alcohol series, embedded in Spurr's resin [S3] within BEEM capsules (Electron Microscopy Sciences), and sectioned at 50 nm with a Leica Ultracut UCT ultramicrotome. Sections were stained with 1% uranyl acetate and then lead citrate before being viewed in a Philips CM10 transmission electron microscope.

Mosquito Single-Sensillum Electrophysiology

Extracellular recordings of capitate pegs from female *An. gambiae* were made as described [S4] with an USB-IDAC analog-digital conversion interface with the software Autospike (Syntech). All odorants were of the highest purity grade commercially available except for the 90% aqueous solution of L(+)-lactic acid (pharmaceutical grade; > 95% L-isomer), 7-octenoic acid (>99%, a gift from M. Birkett), and Henkel 100 (a gift from T. Gerke). Odorants were dissolved and diluted in paraffin oil, except that ammonia was diluted in water and 7-octenoic acid was dissolved in tertyl-butyl methyl ether (TBME). In the latter case, TBME was allowed to evaporate for 15 min from samples before use. Hundred-fold dilutions of 92 compounds in paraffin oil were prepared gravimetrically on a Cahn C-33 μg balance (Cahn Instruments) and used to screen the response spectra of palpal ORNs.

Odorants were tested in random order; for the same odorant, lower concentrations were tested first to prevent possible adaptation to higher concentrations. Ten μl of diluted odorant was added onto a 1 × 1.5 cm piece of filter paper, which was placed into a Pasteur pipette. A 200 ms odorant stimulus was added to an air stream, which was set at 11.1 ml/s, with a stimulus controller (Syntech) so that the odorant-carrying air stream was bolstered up to a constant flow rate of 40 ml/s with charcoal-filtered and humidified air. For controls, paraffin oil, water, or TBME were used instead of diluted odorant.

For stimulations with CO_2, the maxillary-palp surface was initially exposed to a stream of synthetic air with monitored quality (21% oxygen and 79% nitrogen, Linde Gas) at a flow rate of 5 ml/s, as regulated by a flow meter and a C5-01/b stimulus controller (Syntech). Different concentrations (300, 600, 1200, 2400, and 4800 ppm) of CO_2 were produced by the mixing of 5% CO_2 (Linde Gas) with synthetic air in a 25-l Tedlar bag and later injected into the synthetic air stream at 5 ml/s via a 224-PCXR4 pump (SKC Gulf Coast). A second flow meter and stimulus controller were used to keep the total flow constant.

Reverse Transcriptase PCR

Five-day-old mosquitoes were cold anesthetized and dissected by hand on a chill table. For each sex, 140 maxillary palps were used for RNA preparation. Total RNA was prepared with RNeasy (QIAGEN) according to the manufacturer's instructions. One-third of each RNA preparation was used for oligo dT-primed cDNA synthesis with Superscript II Reverse Transcriptase (Invitrogen) for the generation of templates for subsequent PCR reactions. Negative control samples with no reverse transcriptase were included in each cDNA synthesis and subsequent PCR analysis.

PCR was performed with a DNA Engine Dyad (MJ Research) under the following conditions: 94°C for 2 min; 40 cycles of 94°C for 10 s, 55°C for 30 s, a slow ramp at 0.4°C/s to 72°C, 72°C for 30 s; and 72°C for 4 min. Primer pairs that span introns were used in order to distinguish cDNA amplicons from those amplified from remaining genomic DNA. The complete set of *AgOR* primers can be accessed at www.cas.vanderbilt.edu/zwiebel/primers2.htm. PCR amplification products were run on a 1.5% agarose gel and verified by DNA sequencing.

In Situ Hybridization

Procedures for fluorescent double in situ hybridization were modified from previous work [S5–S7] as follows: Heads with maxillary

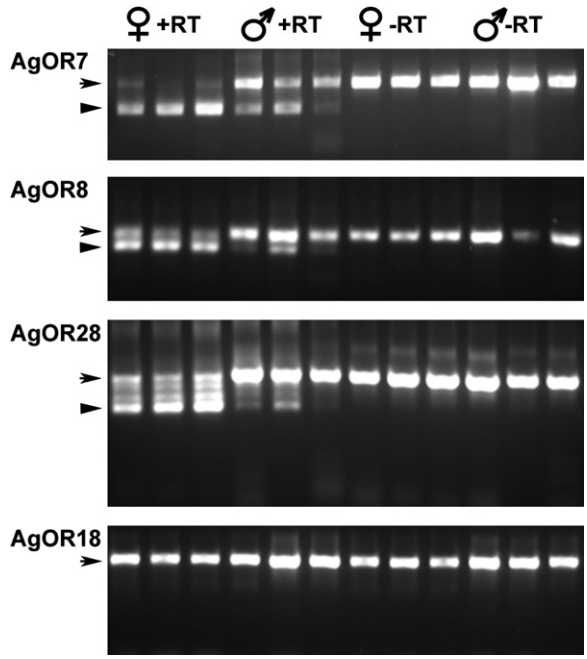

Figure S1. Expression of *AgOR* Gene in the Maxillary Palp as Determined by RT-PCR

Lanes are denoted as follows: Products from three independent female maxillary-palp RNA samples with RT, three independent male maxillary-palp RNAs with RT, triplicates of female maxillary-palp RNA with no RT, and triplicates of male maxillary palp RNA with no RT. Genomic DNA products are indicated by arrows, and cDNA products are indicated by arrowheads. *AgOR7*, *AgOR8*, and *AgOR28* are constantly detected in both male and female maxillary-palp RNA, whereas a different *AgOR*, *AgOR18*, is not detected.

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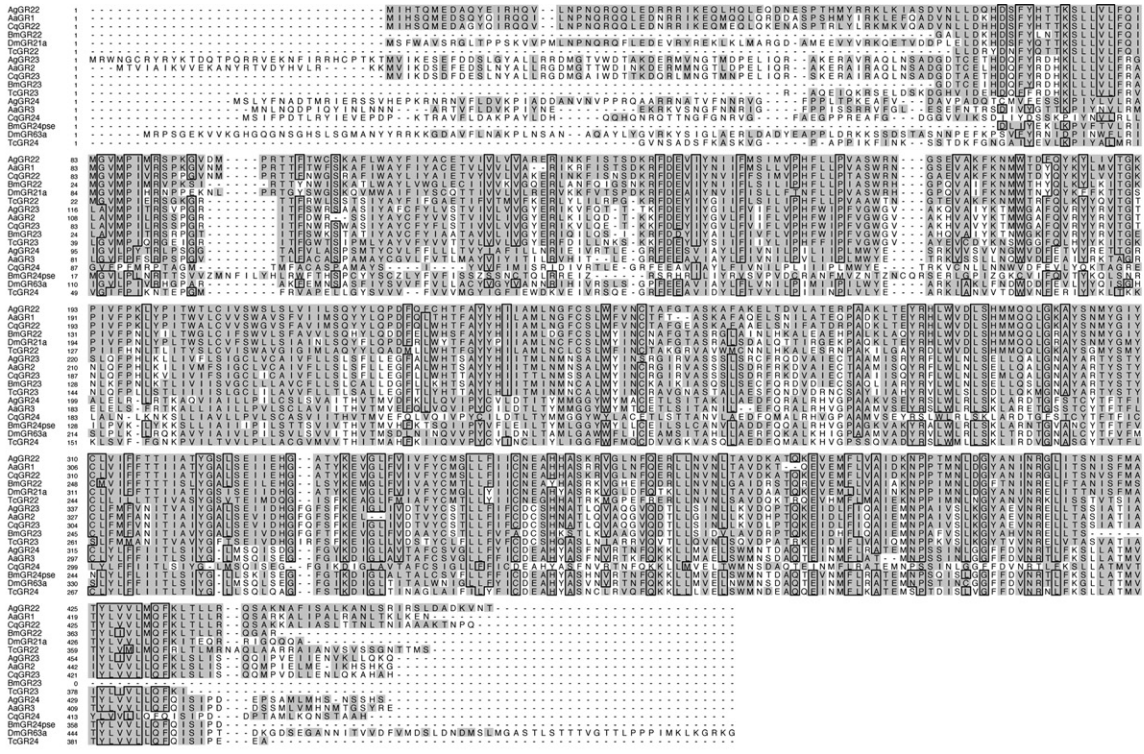
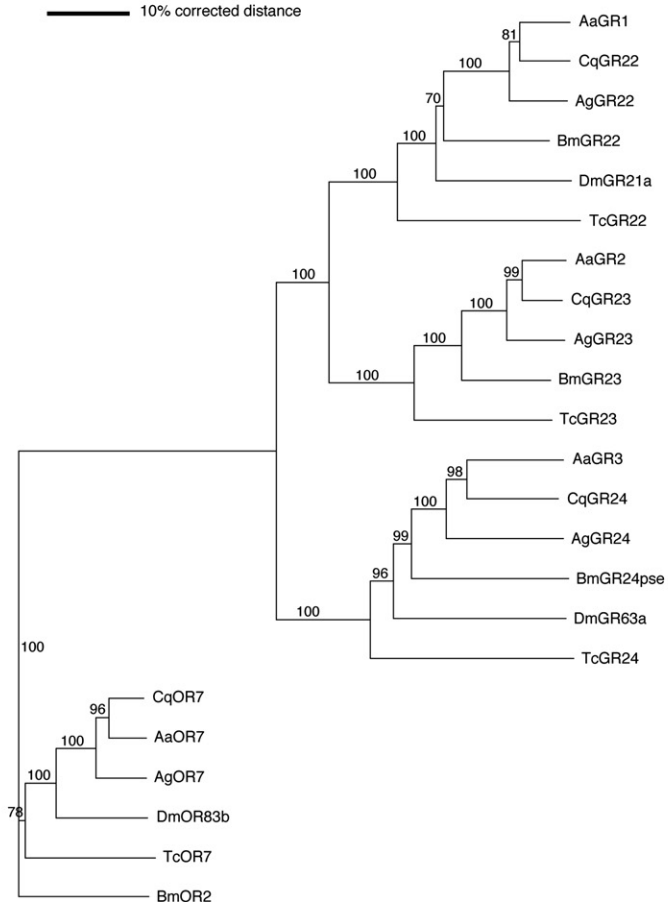
A



B



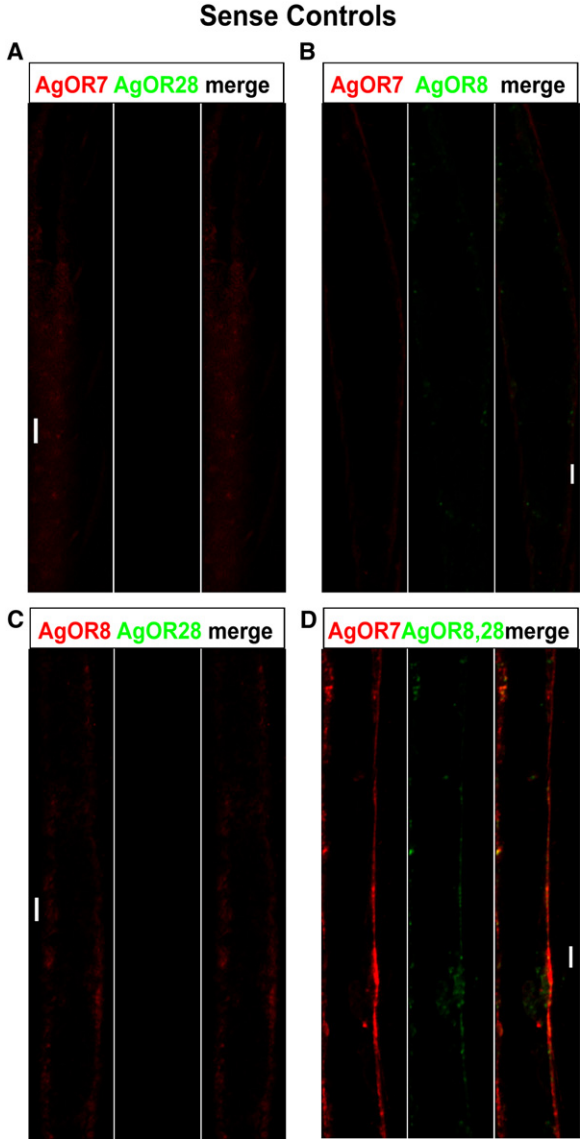


Figure S3. Sense Controls for the FISH Experiments

Sense probes for *AgOR7* and *AgOR28* (A), *AgOR7* and *AgOR8* (B), *AgOR8* and *AgOR28* (C), and *AgOR7* and *AgOR8*, *28* (D) do not reveal any specific labeling. The scale bar represents 10 μm.

palps attached were dissected from 5-day-old female mosquitoes and collected in 4% paraformaldehyde in phosphate-buffered saline (PBS) with 0.05% Tween-20 on ice. Twenty minutes after dissection was over, fixative was replaced with 4% paraformaldehyde in PBS without Tween-20, and fixation continued on ice for additional 5 hr. Tissues were dehydrated in an ethanol series, cleared with xylenes, and embedded in Paraplast plus (Kendall) before being sectioned at 8–10 μm on a Microme HM-340E microtome (Carl Zeiss). After 21 hr drying at 40°C, sections were dewaxed with Citri-Solv (Fisher) and rehydrated in an ethanol series to PBS. After fixation, acetylation, prehybridization, and the washes in between were performed as previously described [S5]. Templates for the in situ probes were 700–850 bp fragments PCR amplified from female maxillary palp cDNA, and these fragments were cloned into pCRII-TOPO (Invitrogen). Digoxigenin-labeled and fluorescein-labeled RNA probes were generated and spot tested according to the manufacturer's directions (Roche). Hybridization was performed in a Boekel InSlide-Out hybridization oven for 21 hr, and posthybridization washes followed. Fluorescein-labeled probes were visualized with anti-fluorescein-AP (Roche), and incubation with Fast Red substrates (Roche) followed. Digoxigenin-labeled probes were visualized with anti-DIG-POD (Roche), and incubation with fluorescein-labeled tyramides (Perkin Elmer) followed. No hybridization was observed with sense probes (Figures S3 and S4). Sections were mounted in Vectashield (Vector Laboratories) and visualized with an LSM510 confocal microscope (Carl Zeiss).

Receptor Expression in *Xenopus laevis* Oocytes and Two-Electrode Voltage-Clamp Electrophysiological Recording

Full-length coding sequences of *AgOR7*, *AgOR8*, and *AgOR28* were PCR amplified from female *An. gambiae* maxillary palp cDNA. *AgOR7* coding sequence was cloned into pT7TS (a gift from G. Lepperdinger with permission of D. Melton); coding sequences of *AgOR8* and *AgOR28* were first cloned into pENTR/D-TOPO (Invitrogen) and then subcloned into pSP64DV by means of the Gateway LR reaction. The pSP64DV vector was a Gateway-compatible destination vector converted from pSP64T-Oligo (a gift from A. George) with the Gateway Vector Conversion System (Invitrogen). cRNAs were synthesized from linearized vectors with mMESSAGE mMACHINE or mMESSAGE mMACHINE T7 Ultra (Ambion). Human $G\alpha_{15}$ RNA was transcribed from pSGEM-$G\alpha_{15}$ (a gift from H. Hatt) [S8].

Mature healthy oocytes (stage V–VII) were treated with 2 mg/ml collagenase S-1 in washing buffer (96 mM NaCl, 2 mM KCl, 5 mM $MgCl_2$, and 5 mM HEPES [pH 7.6]) for 1–2 hr at room temperature. Oocytes were later microinjected with 27.6 ng *AgOR8* or *AgOR28* cRNA, 27.6 ng *AgOR7* cRNA, and 0.276 ng $G\alpha_{15}$ cRNA. After injection, oocytes were incubated for 3–5 days at 18°C in 1XRinger's solution (96 mM NaCl, 2 mM KCl, 5 mM $MgCl_2$, 0.8 mM $CaCl_2$, and 5 mM HEPES [pH 7.6]) supplemented with 5% dialyzed horse serum, 50 μg/ml tetracycline, 100 μg/ml streptomycin, and 550 μg/ml sodium pyruvate.

Whole-cell currents were recorded from the injected *Xenopus* oocytes with a two-electrode voltage clamp. Odorants were dissolved in dimethyl sulfoxide (DMSO) at a 1:10 ratio so that stock solutions could be made. Prior to recording, stock solutions were diluted in 1XRinger's solution to the indicated concentrations before being applied to *Xenopus* oocytes in an RC-3Z oocyte recording chamber (Warner Instruments). Odorant-induced currents were recorded with an OC-725C oocyte clamp (Warner Instruments) at a holding potential of −80mV. Data acquisition and analysis were carried out with Digidata 1322A and pCLAMP software (Axon Instruments).

Identification of Putative Insect CO_2 Receptor Sequences

The public DNA database of all available insect genome sequences at National Center for Biotechnology Information and the *Culex pipiens quinquefasciatus* (Johannesburg strain) whole genome

Figure S2. The Putative Insect CO_2 Receptors Are Highly Conserved

(A) Deduced amino acid sequences were aligned with the ClustalX algorithm [S10] and viewed in SeqVu (The Garvan Institute of Medical Research). For all alignments, similarity shading is based upon an 85% Goldman-Engelman-Steitz scale, and identity boxing is based on a 65% scale [S14] with SeqVu.

(B) Phylogenetic relationship of putative insect CO_2 receptors. The corrected distance tree was generated with PAUP* v4 with distances corrected in TreePuzzle v5 with a maximum-likelihood model and the BLOSUM62 amino acid matrix [S12, S15–S17]. The tree is rooted with the group of *DmOR83b* orthologs. Bootstrap support from 1000 replications of neighbor joining with uncorrected distances is shown on the relevant branch points. Although these putative insect CO_2 receptors are closely related to each other, they form three distinct phylogenetic clusters with *AgGR22*, *AgGR23*, and *AgGR24*, respectively. And although the three mosquito species, *An. gambiae*, *Ae. aegypti*, and *Culex pipiens quinquefasciatus*, the silkworm *Bombyx mori*, and the red flour beetle *Tribolium castaneum* all seem to have three homologous CO_2 receptor genes, *Drosophila*, on the other hand, does not have a close homolog of *AgGR23*.

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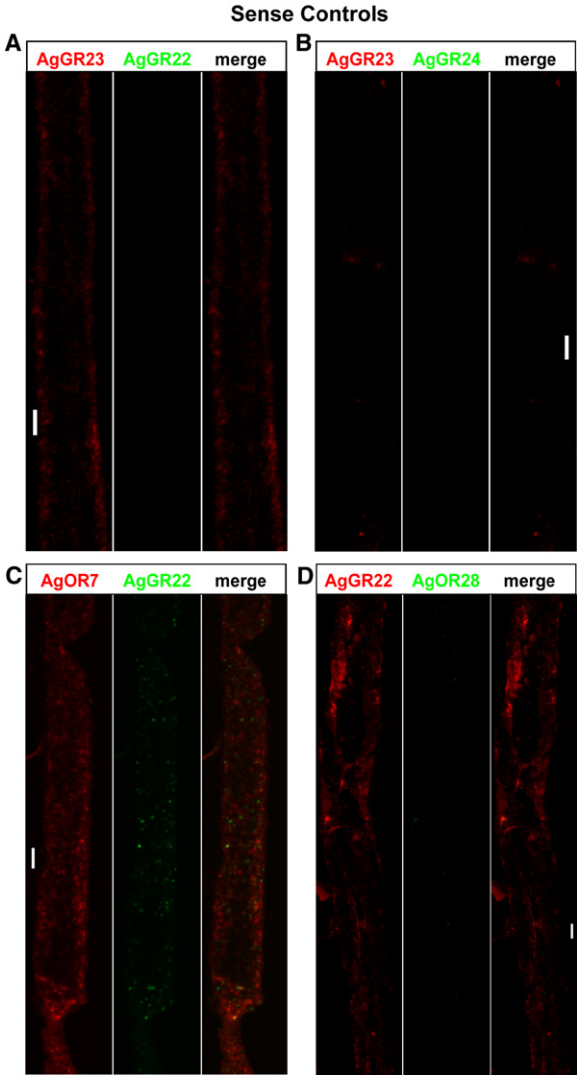

Figure S4. Sense Controls for the FISH Experiments

Sense probes for *AgGR23* and *AgGR22* (A), *AgGR23* and *AgGR24* (B), *AgOR7* and *AgGR22* (C), and *AgGR22* and *AgOR28* (D) do not reveal any specific labeling. The scale bar represents 10 μm.

sequencing database at the Broad Institute (http://www.broad.mit.edu/annotation/genome/culex_pipiens) were queried with sequences of *AgGR22*, *AgGR23*, and *AgGR24* or *AaGR1*, *AaGR2*, and *AaGR3* (L.B. Kent and H.M. Robertson, personal communication) by tBLASTn [S9]. Putative insect CO_2 receptor sequences were manually annotated with Sequencher v4.5 (Gene Codes). Specifically, the first 247 amino acid sequences of the *Bombyx mori BmGR23* were extracted from contig 17455, and the remaining 108 amino acid sequences were from contig 8952. In addition, the *Bombyx BmGR24* is likely to be a pseudogene and therefore renamed *BmGR24pse* because there are several in-frame stop codons in its N-terminal portion, although it remains possible that there is an intact version of the exon that has not yet been identified in the genome project.

Phylogenetic Analysis

Deduced amino acid sequences of putative insect CO_2 receptors were aligned with ClustalX [S10], and phylogenetic analysis was performed with distance methods in PAUP* v4.0b10 [S11]. The details of corrected distance phylogenetic methods can be found in the Figure S2 legend and [S12]. Bootstrap analysis was used to assess statistical support for relationships via uncorrected neighbor-joining analysis of 1000 pseudoreplicated data sets.

Drosophila Transgenes and Electrophysiology

All constructs were injected into w^{1118} flies. cDNAs of *AgGR22* and *AgGR24* were amplified with the following primers: 5′-TGCGGCCGCATGATTCACACACAGATGGAA-3′ and 5′-GGGTACCTTAGGTGTTCACTTTGTCTG-3′ for *AgGR22*, and 5′-TGCGGCCGCATGAGTCTCTACTTCAACGCG-3′ and 5′-GGGTACCCTAAGAATGAGACGAATTAC-3′ for *AgGR24*.

The predicted protein sequences encoded by these cDNAs correspond to the amino acid sequences shown in Figure S2. For the *AgGR23* genomic clone, 5′-TGCGGCCGCACGCTGGAACGGTTGTCGCTA-3′ and 5′-GGGTACCTTACTGTTTCTGTAGCAGCT-3′ were used.

Electrophysiology was performed as previously described [S13].

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