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Candidate pheromone receptors of codling moth *Cydia pomonella* respond to pheromones and kairomones

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Olfaction plays a dominant role in the mate-finding and host selection behaviours of the codling moth (*Cydia pomonella*), an important pest of apple, pear and walnut orchards worldwide. Antennal transcriptome analysis revealed a number of abundantly expressed genes related to the moth olfactory system, including those encoding the olfactory receptors (ORs) CpomOR1, CpomOR3 and CpomOR6a, which belong to the pheromone receptor (PR) lineage, and the co-receptor (CpomOrco). Using heterologous expression, in both *Drosophila* olfactory sensory neurones and in human embryonic kidney cells, together with electrophysiological recordings and calcium imaging, we characterize the basic physiological and pharmacological properties of these receptors and demonstrate that they form functional ionotropic receptor channels. Both the homomeric CpomOrco and heteromeric CpomOrco + OR complexes can be activated by the common Orco agonists VUAA1 and VUAA3, as well as inhibited by the common Orco antagonists amiloride derivatives. CpomOR3 responds to the plant volatile compound pear ester ethyl-(E,Z)-2,4-decadienoate, while CpomOR6a responds to the strong pheromone antagonist codlemone acetate (E,E)-8,10-dodecadien-1-yl acetate. These findings represent important breakthroughs in the deorphanization of codling moth pheromone receptors, as well as more broadly into insect ecology and evolution and, consequently, for the development of sustainable pest control strategies based on manipulating chemosensory communication.

Tortricid moths (Lepidoptera: Tortricidae) are among the most important pest insects as a result of their economic impact on agriculture. Within this family, the codling moth *Cydia pomonella* (L.) is now found in apple, pear and walnut orchards worldwide. They rely on odour and pheromone perception to find food, mates, and suitable substrates for oviposition¹,², and semiochemical-based technologies have been used as part of sustainable control strategies.

Insect pheromones are detected by olfactory sensory neurons (OSNs) that innervate specialized cuticular sensilla, primarily found on the antennal surface. Odorant detection is mediated by specific olfactory receptors (ORs) working together with an olfactory co-receptor (Orco) as heteromeric ligand-gated ion channels³–⁵. While many ORs are tuned to environmental odours such as plant volatiles, pheromone receptors (PRs), which constitute a monophyletic clade in the insect OR phylogeny, respond predominantly to sex pheromones⁶–⁹.

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Previous transcriptome-based studies\textsuperscript{10,11,} reported 58 putative codling moth ORs, of which 12 are grouped in the PR-clade. Deorphanization of these PRs may aid in attempts to increase the efficacy of pheromone-based mating disruption for this species. The main component of the codling moth pheromone is (E,E)-8,10-dodecadien-1-ol (codlemone). Volatile compounds emitted from host-plants, such as pear ester, ethyl (E,Z)-2,4-decadienoate, are known to enhance male attraction to codling moth pheromone\textsuperscript{8,12–14}. Disruption of mate-finding by air permeation with synthetic codlemone\textsuperscript{2,15} can accordingly be enhanced by adding pear ester\textsuperscript{12,16,17}. Semiochemical-based technologies enable sustainable control of the insect model codling moth and can be applied also to other insects\textsuperscript{18}.

In an attempt to identify the PRs responsible for detection of these ligands, we recently expressed CpomORs in Drosophila melanogaster OSNs by means of both the Or67d\textsuperscript{19,20} line for expression in at1 OSNs housed in trichoid sensilla\textsuperscript{19,19} and the H-\textit{halo Or22a-GAL4} line for expression in ab3A OSNs housed in basiconic sensilla\textsuperscript{20,21}. Single sensillum recordings (SSR) from OSNs expressing one of the candidate PRs, CpomOR3, revealed that it did not respond to any of the pheromonal compounds emitted by \textit{C. pomonella} females or closely related species within the genus \textit{Cydia}\textsuperscript{22,23}. Instead, it responds strongly to pear ester, which has a strong synergistic effect on male attraction to sex pheromone\textsuperscript{9,24,25}.

Here we used HEK293T-based heterologous expression together with standard calcium imaging and patch-clamp recordings, as well as \textit{in vivo} expression in \textit{D. melanogaster} OSNs, for reinvestigation of CpomOR3 function and \textit{de novo} characterization of two additional PR candidates, CpomOR1 and CpomOR6a, abundantly expressed in moth male OSNs and initially proposed to be possible receptors for codlemone\textsuperscript{25,26}. We found that all three receptors are functional when co-expressed in HEK293T cells with CpomOrco. While we were unable to detect a response of CpomOR1 to any of the native ligands tested, CpomOR3 responded to pear ester as well as the analogous methyl-(E,Z)-2,4-decadienoate and CpomOR6a responded to codlemone acetate ((E,E)-8,10-dodecadien-1-yl acetate) a strong pheromone antagonist of the codling moth\textsuperscript{26}. These findings represent important breakthroughs in understanding the mechanisms of codling moth attraction to biologically relevant odours and, consequently, for developing innovative pest control strategies based on disrupting olfactory communication.

**Results**

**Codling moth putative PRs and Orco.** The complete open reading frames (ORFs) for CpomOR1 (JN836674.1), CpomOR3 (KJ420588), and CpomOrco (JN836672) were sequenced in prior studies\textsuperscript{10,25}. For CpomOR6a, a 306bp sequence was identified in the codling moth antennal transcriptome\textsuperscript{20}. We performed 5\textsuperscript{′} RACE-PCR to complete the ORF of 1248 bp (updated to Genbank; accession number JN836671). Then, we compared the ORF sequence with the updated transcript variants found by Walker et al. (2016)\textsuperscript{15}, and determined that this sequence corresponds to CpomOR6a. Analysing the translated protein, seven transmembrane domains with an intracellular localization for the N-terminus were predicted for CpomOR6a. Distinct labelling of the plasma membranes of transfected cells indicated that the receptors were properly stabilized and fixed for immunolabeling with an anti-V5 antibody. Untransfected cells were used as a negative control. Distinct labelling of the plasma membranes of transfected cells indicated that the receptors were properly expressed in the system (Fig. 1). The putative PRs were also co-expressed with CpomOrco, resulting in a localization similar to that found when they were expressed alone (Fig. 1).

**Heterologous expression of codling moth putative PRs and Orco.** Prior to physiological tests of the codling moth receptors in the HEK293T heterologous expression system, we first established that the receptors were expressed at the cell membrane. Each of the receptors was cloned into the mammalian expression vector pcDNA5/TO with an N-terminal V5 epitope tag. Twelve to 16 h after transfection, the cells were permeabilized and fixed for immunolabeling with an anti-V5 antibody. Untransfected cells were used as a negative control. Distinct labelling of the plasma membranes of transfected cells indicated that the receptors were properly expressed in the system (Fig. 1). The putative PRs were also co-expressed with CpomOrco, resulting in a localization similar to that found when they were expressed alone (Fig. 1).

**Physiological properties of the heterologously expressed codling moth putative PRs.** The codling moth receptors were co-transfected with a plasmid carrying a gene for nucleus-targeted Blue Fluorescent Protein (BFP) as a control for transfection efficiency (Fig. 2, a1). We began by testing the codling moth putative PRs coexpressed with Orco for functional expression using VUA11 (acetamide,N-(4-ethylphenyl)-2-[[4-ethyl-5-(3-pyridinyl)-4H-1,2,4-triazol-3-yl][thio]-) or VUA3 (acetamide,2-[[4-ethyl-5-(4-pyridinyl)-4H-1,2,4-triazol-3-yl][thio]-N-[4-(1-methylthethylphenyl][]) agonists that are known to activate both heteromeric and homomeric Orco-containing receptor channels from other insect species\textsuperscript{25}. HEK293T cells transfected with CpomOrco alone or CpomOrco in combination with either CpomOR6a, CpomOR3 or CpomOR1 generated calcium signals upon application of VUA1 or VUA3 (Fig. 2). Neither un-transfected nor transfected cells were sensitive to the maximum solvent concentration used (DMSO, 1%), suggesting that the agonist effects were not solvent dependent. Additionally, the overall sensitivity of the cells to the agonists correlated with BFP expression (Fig. 2, a1-a2). In the initial series of experiments, we examined whether the activation of ion channels underlies the generation of the agonist dependent calcium signals (Fig. 2, a3). Whole-cell voltage clamp mode was used to record from cells sensitive to VUAAs. As shown for one cell (Fig. 2, a4), at the holding potential −50 mV, VUA1 activated an inward current (black line) that kinetically preceded the calcium signal (red line) and thus may determine the agonist mediated calcium influx. Interestingly, the kinetics of the calcium responses mediated by the activity of either homomeric CpomOrco or heteromeric CpomOrco + OR complexes were different (Fig. 2, b). For example, cells transfected with CpomOrco + OR1 could be characterized by faster activation/deactivation kinetics (Fig. 2, b, c1). Similar results were observed for the two other heteromeric complexes: CpomOrco + Or6a and CpomOrco + Or3 (Fig. 2, c1).

The response amplitude of both individual cells and cell populations depended on the agonist concentration. The population (cumulative) responses were used to generate the agonist concentration dependences (Fig. 2, c1-c2). For quantitative analysis and comparison, the average peak amplitudes of the responses of different cells were
normalized to the responses elicited by application of a saturating concentration (1000 μM) of VUAA1 or VUAA3 (Fig. 2, c2). The comparison of the dose-response characteristics obtained for different OR complexes suggests that homomeric CpomOrco is less sensitive to these agonists than its heteromeric counterparts are. Furthermore, the parameters of the calcium responses mediated by the activity of heteromeric CpomOrco+OR complexes are characterized by faster activation/deactivation kinetics (Fig. 2, b, c1).

Amiloride derivatives (ADs) have been shown to block both heteromeric and homomeric currents during VUAA1 activation29,30. In our experiments with the putative Cpom PRs and Orco, the AD 5-(N-methyl-N-isobutyl) amiloride (MIA, 100 μM) applied extracellularly almost completely blocked the VUAA3 activated calcium signal (Fig. 3). Similar results were observed for both homomeric and all heteromeric complexes. In all cases, the effects were only partially reversible.

We next used whole-cell and outside-out patch-clamp recordings to characterize the basic electrophysiological properties of the CpomOrco+OR1 complex. All cells expressing CpomOrco+OR1 responded to VUAA3 (200 μM), generating an inward current that varied in amplitude from −49 to −1165 pA, with a mean amplitude of −431 ± 54 pA, n = 37. Un-transfected cells were not sensitive to VUAA3 (12 cells tested). The whole-cell currents gradually increased in a stimulus intensity dependent manner (Fig. 4, a1). In all cases, when cells were stimulated multiple times, the responses were characterized by constant amplitudes and stable kinetic parameters, indicative of the ionotropic nature of the receptors under the current experimental conditions. The results of subsequent experiments using outside-out patch-clamp recordings further support these observations (Fig. 4, a2). VUAA3...
Figure 2. Electrophysiology and pharmacology of CporORs expressed in HEK293T cells. (a) Activation of HEK293T cells expressing CporOrco by VUAA1. CporOrco was co-expressed with BFP (mostly localized in nuclei) for parallel expression control (a1); HEK293T cells were stimulated with VUAA1 (250 μM, a2). The agonist stimulation elicits Ca⁺⁺⁺ increase in the cells (a2, a3). Each image (a2) was acquired at the times indicated on panels and by red circles under calcium signal curves (intensity of fluorescent curves, a3). Note: the BFP negative cells (compare bright field image with BFP and a2) did not generate appreciable calcium signal. (a4) Activation of ion channels underlies the generation of the agonist dependent calcium signal. Whole-cell voltage clamp recording was obtained from the cell that generated calcium signal in response to VUAA1 (250 μM). VUAA1 activated inward current (black line, average of three responses, grey bars - SD) that kinetically preceded calcium signal (red line) and thus may underlie agonist dependent calcium influx. Holding potential was −50 mV. Time scale and diagram of stimulus application shown in a4 is common for a3 and a4. (b) The kinetics of calcium responses mediated by the activity of the homomeric Orco or Orco + OR complexes were different. Cells transfected with CporOrco + OR1 could be characterized by faster activation/deactivation kinetics (black circles and line). Calcium response traces represent average of normalized responses of many cells recorded from the same preparation. (c) VUAA1 stimulation elicits dose-dependent Ca⁺⁺⁺ increase in HEK293T cells expressing either homomeric Orco or Orco + OR complexes (plot series are labelled respectively). (c1) Data within each row were obtained from single preparation; right panels, the respective concentration dependencies. Note: the maximal agonist concentration used in these experiments (VUAA1 250 μM) is likely not a saturating concentration. (c2) Concentration dependences of VUAA1 and VUAA3. Data were obtained in the separate series of experiments. The response amplitudes were used to generate the agonist concentration dependences. The average peak amplitudes of the responses of different cells (n = 57–216) were normalized to the maximal responses usually elicited by application of a saturating concentration (1000 μM) of VUAA1 or VUAA3. Note: homomeric CporOrco (dark grey curves) is less sensitive to the agonists ([VUAA1]1/2 ~ 520 μM, [VUAA3]1/2 ~ 362 μM) than the heteromeric complexes (e.g. light grey curves, [VUAA1]1/2 ~ 150 μM, [VUAA3]1/2 ~ 140 μM).
(200 μM) applied repeatedly to the extracellular surface of membrane patch reversibly increased the membrane current noise likely associated with the activity of ion channels. As found for the whole-cell currents, the VUAA3 activated ion channel noise of membrane patches demonstrated little if any rundown (Fig. 4, a2). Note, the low single channel conductance and fast gating likely make the unitary currents undistinguishable.

Whole-cell recordings were also performed to estimate the selectivity of the Orco-based channel to a selection of inorganic monovalent cations (Fig. 4, b; see Methods for details). The permeability ratio sequence for the inorganic monovalent cations tested was $(P_{\text{X}^+}/P_{\text{Na}^+})$: $\text{Rb}^+(2\pm0.12) > \text{K}^+(1.37\pm0.03) \geq \text{Cs}^+(1.36\pm0.03) \sim \text{Na}^+ > \text{Li}^+(0.93\pm0.06)$.

The sequence is consistent with the selectivity sequences previously reported for Orco-based channels from other insects.\(^3\)\(^1\),\(^3\)\(^2\).

Deorphanization of codling moth putative PRs. As the physiological and pharmacological tests demonstrated that both the homomeric and heteromeric PR and Orco complexes were functionally expressed in the HEK293T cell system, we next used the system to look for natural ligands for the receptors. A library of potential codling moth pheromones and synergist compounds (Table 1), as well as additional compounds, such as plant volatiles and volatiles from fermentation and commercial substances (Supplementary Table S1) were screened against CpmOrco $+$ OR6a, CpmOrco $+$ OR3 and CpmOrco $+$ OR1. Treatment with VUAA3 (250 μM) was used as a positive control. In tests with CpmOrco $+$ OR6a, we observed clear activation in response to stimulation with (E,E)-8,10-dodecadien-1-yl acetate ((E,E)-codlemone acetate; Fig. 5, a1-a2). Subsequent experiments were used to calculate an EC50 of $51.84\pm13.21\,\mu\text{M}$ for codlemone acetate (Fig. 5, a2); however, the amplitude at saturating concentrations ($18.91\pm10.31$, dF) was $\sim27\%$ of the positive control amplitude ($69.71\pm27.29$, dF; Fig. 5, a1). Interestingly, compared to the positive control, we observed a long lasting codlemone acetate activation of transfected HEK293T cells, which led to a delayed recovery after stimulation (Fig. 5, a1).

Once we determined the response of CpmOR6a towards codlemone acetate using the HEK293T cell system, in parallel series of experiments we tested activation of CpmOR6a expressed in Drosophila at1 OSNs to a new panel of ligands, including codlemone, pear ester, codlemone acetate and structurally related compounds (Supplementary Dataset S2). As in the HEK293T cell system, the strongest response significantly different from the solvent was elicited by (E,E)-8,10-dodecadien-1-yl acetate ((E,E)-codlemone acetate) and (Z,Z)-8,10-dodecadien-1-yl acetate along with (E)-10-12-dodecadien-1-yl acetate (Fig. 5, b1 and Supplementary Fig. S5). Furthermore, dose response experiments with CpmOR6a towards (E,E)-codlemone acetate demonstrated that the threshold for detection was 1.0 microgram (Fig. 5, b2).
Previously we demonstrated that CpomOR3 expressed in ab3A and at1 OSNs is sensitive to pear ester 25, and here we confirmed this result using the HEK293T cell expression system: pear ester [(E,Z)-ED] elicited a response from cells expressing CpomOrco + OR3 (Fig. 6, a1-a2). Furthermore, we found that an analogous ester emitted by pear (methyl (E,Z)-2,4-decadienoate [(E,Z)-MD]) 33, also activates CpomOrco + OR3. EC50 estimations (EC50 HEK-(E,Z)-ED = 453.60 ± 119.6 μM; EC50HEK-(E,Z)-MD = 1082.08 ± 112.8 μM) and dose-response plots (Fig. 6, a2) suggest that CpomOrco + OR3 has a lower specificity for (E,Z)-MD than for (E,Z)-ED. As with CpomOrco + OR6a responses to (E,E)-codlemone acetate, we observed a slow recovery after CpomOrco + OR3 stimulation with (E,Z)-MD (Fig. 6, a1).

For the dose response of CpomOR3 when expressed in Drosophila ab3A OSNs (Fig. 6, b1-b2), a minimum dose of 100 ng loaded in the stimulus cartridge was required to elicit a response significantly different from the solvent for (E,Z)-ED and of 10 μg for (E,Z)-MD (Fig. 6, b2). Application of a different heterologous expression system confirmed CpomOR3 sensitivity to both pear ester and its analogous (E,Z)-MD, with further suggesting lower specificity for (E,Z)-MD than for (E,Z)-ED.

In contrast to CpomOR3 and CpomOR6a, we were unable to identify any ligands that activated CpomOrco + OR1. While calcium imaging experiments with VUA1 and VUA3 suggest that CpomOR1 is likely functionally expressed in the HEK293T system (Fig. 2, b, c), the receptor failed to respond to any of the ligands tested. In agreement with these results, CpomOR1 also failed to respond in Drosophila at1 OSNs to
| Compound | MW (g/mol) | Solubility (M) | LogP | Boiling point (°C at 760 mmHg) | CAS | Source | Reference |
|----------|------------|----------------|------|-------------------------------|-----|--------|-----------|
| (−)-β-caryophyllene | 204.35 | 3.40E-08 | 6.164 ± 0.248 | 268.4 ± 10.0 | 87-44-5 | Sigma | 1 |
| (E)-β-farnesene | 204.35 | 1.50E-08 | 6.139 ± 0.304 | 272.5 ± 20.0 | 18794-84-8 | Bedoukian | 1 and 25 |
| (E,E)-8,10-dodecadien-1-yl acetate (codlemone acetate) | 224.34 | 3.20E-08 | 5.611 ± 0.233 | 314.7 ± 11.0 | 53880-51-4D | Bedoukian Inc | 25 |
| (E,E)-8,10-dodecadienol (codlemone) | 182.30 | 3.40E-08 | 6.192 ± 0.203 | 270.7 ± 9.0 | 76600-88-9 | Fluka | 25 |
| (E)-β-farnesene | 204.35 | 1.00E-08 | 6.304 ± 0.316 | 279.6 ± 20.0 | 0.16-34-1 | Bedoukian | 1 |
| (Z)-3-hexenol | 100.16 | 0.14 | 1.697 ± 0.206 | 156.5 ± 0.0 | 982-96-1 | Aldrich | 1 |
| (Z)-3-hexenyl acetate | 142.20 | 0.025 | 2.400 ± 0.228 | 174.2 ± 19.0 | 3681-71-8 | Gift from Prof. Peter Witzgall | 1 |
| 1,8-p-menthadien-7-al (perillaldehyde) | 150.22 | 6.10E-03 | 2.798 ± 0.333 | 238.0 ± 29.0 | 2111-75-3 | Dr. Gigliola Borgono | 55 |
| 1-dodecanol | 186.33 | 5.90E-05 | 4.914 ± 0.177 | 258.0 ± 3.0 | 112-53-4 | Sigma Aldrich | 25 |
| 3-(4-methyl-1-oxopentyl)furan | 166.22 | 2.10E-03 | 2.851 ± 0.318 | 224.4 ± 13.0 | 553-84-4 | Gift from Dr. Gigliola Borgono | 55 |
| butyl hexanoate | 172.26 | 3.00E-03 | 3.842 ± 0.205 | 206.8 ± 8.0 | 626-82-4 | Bedoukian | 25 |
| ethyl-(E,Z)-2,4-decadienoate [(E,Z)-ED] | 196.29 | 1.00E-03 | 4.454 ± 0.229 | 264.7 ± 9.0 | 3025-30-7 | Aldrich | 1 and 25 |
| Linalool | 154.25 | 6.70E-03 | 2.795 ± 0.263 | 198.5 ± 0.0 | 70-70-6 | Firmenich | 1 |
| methyl salicilate | 152.15 | 0.021 | 2.523 ± 0.240 | 222.0 ± 0.0 | 119-36-8 | Fluka | 1 |
| methyl-(E,Z)-2,4-decadienoate [(E,Z)-MD] | 182.26 | 2.30E-03 | 3.944 ± 0.229 | 246.0 ± 9.0 | 4493-42-9 | Gift from Prof. Peter Witzgall | 33 |
| nonanal | 142.24 | 3.00E-03 | 3.461 ± 0.223 | 190.8 ± 3.0 | 124-19-6 | Aldrich | 1 |
| (E)-β-ocimene | 136.23 | 2.00E-05 | 4.418 ± 0.275 | 175.2 ± 10.0 | 3779-61-1 | Fluka | 1 |

Table 1. Library of potential codling moth pheromones and synergist compounds screened against C. pomonella ORs.

Pheromones and synergists (spikes/s = 0.61, n = 5; response minus basal activity, see methods section for detail) or their combinations with codlemone (spikes/s = 0.00, n = 5; Supplementary Dataset S3).

Discussion

Of the 58 ORs identified in C. pomonella11, 12 belong to the PR clade. Here we functionally expressed three of them, CpmOR1, CpmOR3 and CpmOR6a, in an HEK293T expression system with the goal of identifying their natural ligands. We found that in contrast to early studies that demonstrated that a channel forming subunit Orco (OR83b) is required as a chaperon to target the ligand-specific insect OR subunits to plasma membrane5, CpmORs are properly expressed and targeted when independently expressed in HEK293T cells (Fig. 1). Like all functionally characterized insect ORs, the codling moth receptors described here can be activated by a group of common synthetic agonists including the VUAA compounds28 that interact directly with Orco, indicating that they are indeed functional in HEK293T cells. Two criteria based on calcium responses to VUAAs can be used to functionally confirm the expression of the orphan ORs: (1) heteromeric OR complexes are more sensitive to the agonists as compared to homomeric Orcos (Fig. 2, c2) and (2) the parameters of the calcium responses mediated by the activity of heteromeric CpmOrco + OR complexes are characterized by faster activation/deactivation kinetics (Fig. 2, b, c1). These observations are consistent with data for heterologously expressed mosquito ORs28,34 and may suggest different functional modes of homomeric and heteromeric complexes. Further studies are necessary to understand the mechanisms underlying these differences.

One of the emerging characteristic pharmacological properties of insect OR-based channels is sensitivity to AOs29-30. Extracellular application of the AD MIA almost completely blocked the VUAA3-activated calcium signal. Similar results were observed for both homonomic (CpmOrco) and heteronomic CpmOrco + OR complexes. These results are consistent with the idea that all insect or, perhaps, even all arthropod chemosensory receptor channels (among ORs and IRs) can be characterized by somewhat common pharmacology29,30,35-37. Despite their apparent lack of selectivity, some AOs tested have demonstrated different antagonistic potencies against different OR complexes29, suggesting that broader screening of AD library may identify compounds with greater OR channel selective affinity.

Among the three CpmORs tested, CpmOR6a and CpmOR1 in particular were found to be robustly expressed in male moths10,11 and were thus initially proposed to be candidate receptors for codlemone. While we did not observe any response to codlemone with either (Supplementary Dataset S2, S3 and Supplementary Fig. S4), we did find that CpmOR6a interacts with codlemone acetate and its isomers (Fig. 5), pheromone components found in both C. pomonella and related species.22,38,39. The lower amplitude of the CpmOrco + OR6a responses to codlemone acetates may suggest that these compounds are partial agonists of the receptor. Another possible explanation is that only approximately 30% of receptors expressed represent heteromeric complexes sensitive to their cognate ligand/s with the majority of the receptors being homomeric (sensitive exclusively to common agonists, VUAAs). It remains necessary to determine whether a different ratio of OR/Orco expression would yield a higher ratio of heteromeric/homomeric OR complexes and thus different amplitudes in the response.
All four geometric isomers of codlemone acetate are reported to be pheromone compounds in tortricid species. While codlemone acetate is a minor pheromone component for *C. pomonella* that acts as a subtle behavioural synergist when blended at a low level with the main codling moth sex pheromone codlemone, it is a major pheromone component in other species and higher concentrations have a strong antagonistic effect on codling moth attraction. Species closely related to *C. pomonella*, like *C. nigricana*, *C. splendana*, *C. pyrivora*22, *C. latiferreana*40,41 and *Hedya nubiferana*38,39, use codlemone acetate as their main pheromone component. While speculative, a possible explanation of the existence of the codlemone acetate receptor in *C. pomonella* may be as a remnant of the former ancestor of the insect. However, conserving a receptor dedicated to detection other species may be important for reproductive isolation. Otherwise, since the pheromone is also emitted by moths within the same host range, their detection may facilitate host finding for *C. pomonella*. The arise of a receptor specialized for the detection of a main pheromone compound like codlemone, may likely represent a step towards allopatric speciation of the codling moth.

In *C. pomonella*, codlemone acetate isomers are detected by two types of OSNs located in sensilla trichodea on male antennae42, one of which responding primarily to the main geometric isomer of codlemone [(E,E)] with tenfold less sensitivity to other geometric isomers [(Z,E); (E,Z); (Z,Z)]. These OSNs are even less responsive to (E,E)-codlemone acetate and its geometric isomers [(Z,E); (E,Z); (Z,Z)]. The second type of OSNs detects all geometric isomers of codlemone acetate, with the (E,E)-isomer eliciting the strongest response, but is insensitive to all geometric isomers of codlemone. Our structure-activity SSR recordings (Fig. 5b) indicate that apart from (E,E)-codlemone acetate, *C.pomonella* is able to detect the isomers (E,Z)- and (Z,Z)-codlemone acetate, and possibly also (Z,E)-codlemone acetate, along with (E)-10:dodecadien-1-yl acetate (Supplementary Fig. S5), which correlates with the electrophysiological results obtained by Bäckman et al. (2000)42. Considering that this receptor does not respond towards codlemone, there must be another PR expressed by the type of OSNs that responds primarily to codlemone but also weakly to codlemone acetate. Walker et al. (2016)11 have proposed *CpomOR1* as
**Figure 6. Functional expression of C PomOR3.** (a) Functional expression of C PomOrco + OR3 in HEK293T cells. (a1) Comparison of C PomOrco + OR3 amplitudes of the calcium responses (mean of the maximum response ± SEM) to 500 μM pear ester (15.07 ± 9.48, df; left) and to 500 μM methyl ester (10.40 ± 5.91, df; right); n = 151. Black bar: stimulus. (a2) Normalized dose-response of pear ester [(E,Z)-ED, white] and methyl ester [(E,Z)-MD, grey]. (b) Functional expression of C PomOR3 in Drosophila ab3A OSNs. (b1) Spiking activity of OSNs in response to different doses of (E,Z)-ED (left) and (E,Z)-MD (right). Black bar: stimulus (500 ms). (b2) Mean ± SEM response of C PomOR3-expressing OSNs stimulated with different doses of (E,Z)-ED (white, n = 13) and (E,Z)-MD (grey, n = 13). Repeated measures ANOVA determined that different doses of the compound elicited significant differences (F(7, 91) = 42.17, p < 0.001). Post hoc tests using the Bonferroni correction revealed that C PomOR3 needed a minimum dose of 100 ng of (E,Z)-ED to elicit a response significantly different from the solvent (p = 0.026). On the other hand, for the dose response of (E,Z)-MD a repeated measures ANOVA determined that different doses of (E,Z)-MD also elicited significant differences in C PomOR3 (F(7, 84) = 41.68, p < 0.001). Post hoc tests using the Bonferroni correction revealed that OR3 needed a minimum dose of 10 μg of (E,Z)-MD to elicit a response significantly different from the solvent (p = 0.020).
Antennal RNA was quantified using Nanodrop (8000 UV-vis Spectrophotometer, Thermo Scientific, Wilmington, DE, USA). Antennae were flash-frozen using liquid nitrogen, and thereafter kept at −80 °C. For RNA extraction, pupae were obtained from a laboratory rearing (Andermatt Biocontrol, Grossdietwil, Switzerland), and adults were allowed to emerge in cages kept at 23 °C, ±5% RH and 16 h : 8 h light/dark cycle, and were fed 10% sugar solution. For RNA extractions, 2–3 day old females and males were used. Using sharp forceps, antennae were removed at the base of the pedicel and immediately flash-frozen using liquid nitrogen, and thereafter kept at −80 °C. RNA was extracted using the RNeasy kit (Qiagen, Hilden, Germany), which included a DNase digestion to eliminate genomic DNA contamination. 

The identification of a potential PR agonist is one of the first successful associations between an insect PR and a likely pheromone ligand using HEK293T-based heterologous expression. Among insect ORs, PRs have been especially difficult to functionally characterize, as illustrated even by our own experiments, where CpomOR1 does not produce any response to pheromones, synergists and their combination (Table 1 and Supplementary Dataset S3). These results highlight the importance of developing alternative testing methods. Overall, our findings suggest that the potential coding moth pheromone receptors exhibit biological and pharmacological properties surprisingly similar to insect ORs when expressed heterologously: CpomOrco-CpomOR complexes are capable of functioning as ionotropic receptor channels and can be characterized by relatively low conductance and fast gating parameters. CpomOrco-CpomOR complexes are activated by a group of common compounds, VUAs, and can be efficiently blocked by amiloride derivatives. Several ligands were identified: codlemone acetate, pear ester and the analogous methyl ester. These findings provide important insights into our understanding of the mechanisms of the moth chemoreceptor functioning and, consequently, for developing pest control strategies based on disruption of pest chemosensory communication.

**Methods**

**Insect dissection and RNA extraction.** *C. pomonella* pupae were obtained from a laboratory rearing (Andermatt Biocontrol, Grossdietwil, Switzerland), and adults were allowed to emerge in cages kept at 23 °C, ±5% RH and 16 h : 8 h light/dark cycle, and were fed 10% sugar solution. For dissections, 2–3 day old females and males were used. Using sharp forceps, antennae were removed at the base of the pedicel and immediately flash-frozen using liquid nitrogen, and thereafter kept at −80 °C. RNA was extracted using the RNeasy kit (Qiagen, Hilden, Germany), which included a DNase digestion to eliminate genomic DNA contamination. Antennal RNA was quantified using Nanodrop (8000 UV-vis Spectrophotometer, Thermo Scientific, Wilmington, DE, USA).

**Rapid amplification of cDNA ends (RACE) PCR.** While the full length sequences of *CpomOrco*, *CpomOr3*, and *CpomOR1* were previously reported, RACE PCR was performed to obtain the complete open reading frame of *CpomOR6a*. cDNA was reverse-transcribed from antennal RNA using the SMARTer kit (Clontech, Mountain View, CA, USA). Primer sequences were designed using existing contig data as reference, and thermodynamical features were checked by OligoEvaluator (Sigma Genosys, http://www.oligoevaluator.com/). Putative oligomerization was checked by OligoAnalyzer 3.1 (Integrated DNA Technologies, http://eu.idtdna.com/calc/analyzer), and melting temperatures were estimated using the salt-adjusted algorithm of the OligoCalc website (http://www.basic.northwestern.edu/biotools/OligoCalc.html). For primers, the goal was a GC% 40–60, Tm < 70 °C, and to create a product with at least 150 bases of overlap with existing contig data.

### Table 2. Primer sequences and melting temperatures.

| OR6a 5'-RACE Primer | Sequence                  | Tm (°C) |
|----------------------|--------------------------|---------|
| 5'-OR6a              | CCCATGTTACTGCATATCCTGAC  | 65.42   |

**CDS-primers**

|                        | Sequence             | Tm (°C) |
|------------------------|----------------------|---------|
| Fw_Orco                | ATGATGGGTAAGTGAATATCA| 57.60   |
| Rv_Orco                | TTACTTCAGTTGTACTAAACATGTA| 61.70   |
| Fw_OR6a                | ATGCAGACAAAGGCACACAGG| 61.00   |
| Rv/OR6a                | TTAGTCTGCGAATGTGGCTAG| 61.00   |
| Fw/OR3                 | ATGTCTCTCTGGACAAATGAAAGC| 60.80   |
| Rv/OR3                 | TTAGTCTATTCTTCTGAGT| 58.30   |
| Fw/OR1                 | ATGCATCCCGAAACGGCTTG| 62.00   |
| Rv/OR1                 | TTACCCCTCAGCAGGAAG| 60.50   |
designed sequence of the 5′_OR6a primer, which successfully extended the CDS of CpomOR6a, is reported in Table 2. SMARTer RACE PCR was performed using an adjusted version of the supplied protocol. Supplied thermostable DNA polymerase was used with a temperature program of 95°C for 2 min, followed by 30 cycles of 95°C for 1 min, 65.42°C for 90 s 68°C for 2 min, and a final elongation of 68°C for 7 min. The 5′_OR6a primer was combined together with Universal primer A mixed supplied in the kit, with 2% DMSO per reaction volume added. PCR products were analysed by electrophoresis on 1.5% agarose gel. Bands were visualized after staining with ethidium bromide using a Gel Doc XR (Bio-Rad, Hercules, CA, USA). Relevant bands were excised and purified by the Gel extraction kit (Qiagen). Quantification was performed using a Nanodrop 3300 Fluorospectrometer (Thermo Scientific) using the PicoGreen® dsDNA reagent kit (Molecular Probes, Life Technologies).

Samples were sequenced (Sanger sequencer, 3730x) Applied Biosystems, Life Technologies) using gene specific primers. The 5′_ sequenced region was assembled with existing contig data and the candidate CDS was identified using the online tool ORF Finder (http://www.ncbi.nlm.nih.gov/gorf/orffig.cgi).

For functional expression, total RNA extracted from male and female antennae were submitted to full-length cDNAs synthesis using RT-for-PCR kit (Clontech), and the full length CDS was amplified (primers Fw_OR6a and Rw.OR6a in Table 2) and sequenced to confirm that the assembly was correct. The nucleotide sequence was converted to amino acids using the ExPaSy translate tool (http://web.expasy.org/translate/), after which transmembrane domains were predicted using TMHMM v2.0 (http://www.cbs.dtu.dk/services/TMHMM/) and TOPCONS (http://topcons.cbr.su.se/). The Topology of the transmembrane protein was visualized using TOPO 2.0 (http://www.sacs.ucsf.edu/cgi-bin/open-topo2.py). The completed sequence of OR6a has been updated in Genbank, JN836671.

**Cloning of olfactory receptors for heterologous expression in HEK293T cells.** In order to produce ampicils suitable for cloning into pDONR221 (Invitrogen Life technologies, Grand Island, NY, USA), we inserted attB regions suitable for BP-clonase-recombination upstream of the CDS primer sequences (attB1 forward region: 5′-GGGGCAAGTTGTACAAAAAAGCAGGTTAACAAAA-3′; attB2 reverse region: 5′-GGGGACACCTTTGATACAAAGAAGCTGGGT-3′; Gateway Technology, Invitrogen). CDS primers (Table 2) were designed to amplify full-length CDS pCpomOR sequences (Genbank database accession numbers, CpomOrco: JN836672; CpomOR6a: JN836671; CpomOR3: KJ420588; CpomOR1: JN836674.1). For forward primers, a NotI restriction site (5′-GGCCGCGC-3′) followed by the HEK-cell optimized 5′-CACC-3′ Kozak sequence (Dr. Jacob Corcoran, personal communication) and the gene-specific forward sequence, were located downstream of the attB1 sequence. For reverse primers, an Apal restriction site (5′-GGCCGCC-3′) followed by the reversed-stop codon (5′-TTA-3′) and the gene-specific reverse sequence, were located downstream of the attB2 sequence. To create V5-N-terminal variants suitable for immunohistochemical experiments, 42 nucleotides (5′-GGCAAGCTTATCCCTAATCCTCTGCTGGGCCTGGACAGCACC-3′) coding for 14 additional amino acids of a V5-epitope tag (Nt-GKPIPNPLGLDST-Ct) were added to the forward primer between the start codon and the rest of the gene-specific forward sequence.

Amplification was performed with Advantage 2 polymerase (Clontech) using a temperature program of 94°C for 5 min followed by 35 cycles of 94°C for 1 min, 59°C for 2 min with a final elongation step of 68°C for 7 min. A 4.0 μL PCR volume was mixed with 1.0 μL of BP-clonase (Gateway Technology, Invitrogen) and 150 ng of pDONR221 (Invitrogen), and was incubated for 4 h at 25°C. Of this reaction volume, 2.0 μL was used to transform TOP10 competent cells (Invitrogen). After transformation, 50 μL of the reaction was plated on 50 μg/mL Kanamycin selective media and incubated overnight at 37°C.

Colonies were grown, and diluted in 50 μL selective LB media with 50 μg/mL Kanamycin, to be grown for 2 hours at 37°C and 225 rpm. Colony PCR was performed to confirm inserts, using 1.0 μL culture from single colony-volumes with the M13FW universal primer and the relevant reverse OR-primer. Amplifications were performed using the GoTaq Green Master Mix (Promega, Fitchburg, WI, USA) with a temperature program of 95°C for 15 min, followed by 35 cycles of 95°C for 45 s, 55°C for 1 min, 72°C for 2 min, and a final elongation of 72°C for 7 min. Colony PCR samples were analysed as described above. Cultures producing relevant bands in colony PCR were grown at 37°C and 225 rpm overnight in 5.0 mL selective LB media with 50 μg/mL Kanamycin. The pDONR221 plasmids containing pCpomOR ORFs were purified using a miniprep kit (Qiagen). Plasmid quantification was performed using Nanodrop (8000 UV-vis Spectrophotometer), and samples were sequenced (Sanger sequencer, 3730xl) using M13 universal primers.

A 2.0 μL aliquot of each pDONR221/CpomOR and pDONR221/V5-CpomOR DNA was digested overnight at the limit of star activity, in a reaction volume with 0.5X FastDigest NotI and Apal added (Thermo Scientific), following the recommended protocol. Reaction volumes were run on 1.5% agarose gel and visualized after staining with ethidium bromide and digested bands were purificed by Gel extraction kit (Qiagen). Quantification was performed as described above. From the purified bands, 50 ng of the reaction was combined with 50 ng pcDNAs/TO (Invitrogen Life technologies) previously digested and purified, 1.0 μL T4 DNA ligase and 1X of the supplied reaction buffer (Thermo Scientific), which was incubated 2 h at room temperature for ligation. Of this reaction volume, 2.0 μL was used to transform TOP10 competent cells. Colony PCR was performed to screen positive colonies, and colonies selected for correct inserts were amplified, vectors were extracted and purified by miniprep, and sequenced (Sanger sequencer, 3730xl). In order to perform heterologous expression, pcDNAs/TO/CpomORs and pcDNAs/TO/V5-CpomORs were scaled up using GeneJet Plasmid Midiprep Kit (Qiagen).

**Heterologous expression in HEK293T cells and transient transfection.** HEK293T cells were grown in HEK cell media [Dulbecco’s modified Eagle's medium containing 10% fetal bovine serum (MP Biomedicals, Solon, OH, USA), 2.0 mM L-glutamine, and 100 μg/mL Penicillin/Streptomycin (Invitrogen)] at 37°C and 5% CO₂. To test transient expression of CpomORs for calcium imaging or patch-clamp recording experiments, 35-mm petri dishes containing semi-confluent HEK293T cells were transiently transfected. To transfect cells
with CpomOrco, we used 1.0 μg of pcDNA5/TO/CpomOrco DNA. In order to promote expression of the ORs, we used double aliquots for pcDNA5/TO/CpomOR DNAs (2.0 μg), combined with 1.0 μg of pcDNA5/TO/CpomOrco for co-transfections (CpomOrco + ORs). To report expression for calcium imaging experiments, 1.0 μg of a separate plasmid DNA (pEFP2-Nuc, a gift from Robert Campbell (Addgene plasmid # 148933)) carrying the coding sequence for a blue fluorescent protein (BFP) was used. In patch-clamp recordings, 1.0 μg of a separate plasmid DNA (pXOOM, Clontech) carrying the coding sequence for a green fluorescent protein (GFP) was used to report expression. In order to report candidate OR-expressing cells, expression of both fluorescent reporter genes was under the regulation of the same promoter for CpomOR genes (CMV). Transfection DNAs were dissolved in 100 μL sterile DMEM, mixed with 3.0 μL Calfectin (SignaGen, Rockville, MD, USA) following the recommended protocol. To estimate transfection efficiency, a parallel transfection was conducted using the positive control vector pcDNA5/TO/LACZ (Invitrogen) and staining with 0.1% XGal53. The predominant majority of LACZ transfected cells demonstrated a strong staining indicating a high transfection efficiency.

Transfections were conducted overnight. HEK cell media was replaced with 1.0 mL fresh media to incubate cells at 37 °C for up to 6 h, at which point part of the cell culture was spread in the middle of a 35-mm plate or small clusters. After 12 h of incubation at 37 °C and 5% CO2, cells were rinsed at the sides with 2.0 mL fresh HEK media. Cells were allowed to recover for at least 1 h prior to calcium imaging.

**Immunohistochemistry.** To study membrane localization of ORs in HEK293T, cells were transfected with pcDNAs/TO/V5-CpomOrco or co-transfected with pcDNA5/TO/CpomOrco combined with pcDNA5/TO/V5-CpomOrco. To compare heterologous expression of ORs alone, further transfections were prepared for pcDNA5/TO/V5-CpomOrco without CpomOrco DNA. V5-CpomOrco was used as a positive control. Non-transfected HEK cells were used as a negative control. After growth, HEK cells were rinsed once with Ca2+ free HEK media. Cells were incubated for 1 h at room temperature in 0.5–1.0 mL HEK cell Ringer (mM: 140 NaCl, 2.0 CaCl2, 10 HEPES, pH 7.5), and placed on the stage of an inverted microscope (Olympus IX-71, Olympus Corp., Tokyo, Japan) equipped with a cooled CCD camera (ORCA R2, Hamamatsu, Hamamatsu City, Japan). Calcium imaging was performed using Imaging Workbench 6 software (INDEC BioSystems, Santa Clara, CA, USA). Each cell was assigned a region of interest (ROI) and changes in fluorescence intensity within each ROI were measured and expressed as the fractional change in fluorescence intensity (dF/F0) or in some cases as dF/F0 with F0 as the baseline fluorescence level before agonist application. Stored time series image stacks were analysed off-line using Imaging Workbench 6, Clampfit 10.5 (Molecular Devices), SigmaPlot 11 (Systat Software Inc., San Jose, CA, USA) or exported as TIFF files into ImageJ 1.42 (available from public domain at http://rsbweb.nih.gov/ij/index.html). Values were adjusted by the company-provided software (Leica Microsystems LAS AF TCS MP5). Images were analysed using the same software and elaborated using ImageJ 1.42 (available from public domain at http://rsbweb.nih.gov/ij/index.html).

**Confocal microscopy.** Samples were analysed with Leica TCS-SP5 Confocal Laser Scanning Microscope (Leica Microsystems, Wetzlar, Germany) or exported as TIFF files into ImageJ 1.42 (available from public domain at http://rsbweb.nih.gov/ij/index.html). Hardware was set to have all lasers active (405 Diode, UV; Argon, Visible; DPSS 561, Visible; and HeNe 633, Visible) with Argon, Visible at 29%. In order to distinguish nuclei fluorescence from antibody-labelled plasma membrane extrusions, DAPI was excluded using pre-set DAPI parameters, calibrated Laser Line UV (405) at 27% and all other Laser Line at 0%. Emission PMT was calibrated between 417 and 496 nm, Gain: 693 nm, Offset: 0. Transmission: 504, Offset: 0. To detect DyLight Antibody with excitation/emission range 493/518 nm, pre-set FITC parameters were adopted, using Laser Line Visible (488) at 76% and all other Laser Line at 0%. Emission PMT was calibrated between 500 and 560 nm, Gain: 808 nm, Offset: 0, Transmission: inactive. All parameters were adjusted by the company-provided software (Leica Microsystems LAS AF TCS MP5). Images were analysed using the same software and elaborated using ImageJ 1.42 (available from public domain at http://rsbweb.nih.gov/ij/index.html).

**Dose/response characteristics of VUAA-compounds.** Non-specific agonists Acetamide,N-(4-ethylphenyl)-2-[(4-ethyl-5-(3-pyridinyl)-4H-1,2,4-triazol-3-yl)thio]- (VUAA1), CAS 525882-84-7 (Glixx Laboratories, Southborough, MA, USA) and Acetamide,2-[(4-ethyl-5-(4-pyridinyl)-4H-1,2,4-triazol-3-yl]
thio]-N-[4-(1-methylethyl)phenyl]- (VUAA3), CAS 585550-72-7 (Molport, Riga, Latvia), were dissolved in Dimethyl Sulfoxide (DMSO, Sigma Aldrich) and stored as a stock solutions (100 mM) at −20°C. The final working concentrations of VUAA1 and VUAA3 were usually prepared right before the experiments. Amplitudes of the calcium responses were used to generate dose-response characteristics. The values were normalized to the response amplitude recorded at 1000 μM VUAA1/VUAA3. The amiloride derivative 5-(N-methyl-N-isobutyl) amiloride (MIA), CAS 2609-46-3 (Sigma Aldrich) was prepared as DMSO stock solution (100 mM) and used to block VUAA3 activated calcium signals at the working concentration 100 μM.

Screening of ligand candidates. HEK293T cells expressing CpomOrco + OR6a, CpomOrco + OR3 and CpomOrco + OR1 were stimulated with an array of compounds such as: potential insect pheromone compounds and plant volatile synergists active on the olfactory system and behaviour of the codling moth1,25, non-host plant volatiles, volatiles from fermentation and commercial substances (Supplementary Table S1).

Physical parameters were obtained from SciFinder (SciFinder, 2015; Chemical Abstracts Service: Columbus, OH, 2015; accessed Jan-Nov 2015) and Chemspider (http://www.chemspider.com/). Compounds were diluted in either DMSO or ethanol (Sigma Aldrich) depending on their solubility properties. Each compound was tested at 100 μM (10 s stimulus duration). VUAA1 100 μM was used as a positive control in all cases (stimulus duration 5.0 s). Responses to codlemone acetate (for CpomOR6a) and pear and methyl esters (for CpomOR3) were normalized to the 250 μM VUAA3 evoked calcium signal response amplitude. Following modification, the Hill equation, \( F = \frac{[A]^h}{\left([A]_0^h + [A]^h\right)} \), was used to fit the concentration dependence data for agonist/ligand dependent OR channel activation, where \( F \) are the normalized fluorescent intensity values, \([A]\) is the agonist/ligand concentration, \([A]_{0.5}\) is the half-effective concentration (EC50), and \( h \) is the cooperativity coefficient.

Electrophysiology and data analysis. GFP positive HEK293T cells were visualized using either an Axiovert 100 inverted microscope (Carl Zeiss, Inc., München, Germany) equipped with a mercury vapour compressed-arc lamp (HBO100) coupled to widefield fluorescence filter set (1114-459, Carl Zeiss, Inc.) or Olympus IX-71 inverted microscope described above (Olympus Corp.). The OR channel activity was investigated using whole-cell and outside-out patch-clamp recordings. The currents were measured with a 200B patch-clamp amplifier (Molecular Devices) and a digital interface (Digidata 1320 A, Molecular Devices), low pass filtered at 5.0 kHz, sampled at 1–2 kHz. Analysis of the data was carried out using pCLAMP 9.2/10.5 software (Molecular Devices) and SigmaPlot 10 (Systat Software Inc). OR channel related currents were investigated at a holding potential of −50/50 mV unless otherwise specified. The polarity of the currents/voltages was presented relative to intracellular membrane surface. Appropriate corrections for liquid junction potentials were made when necessary. Patch pipettes were fabricated from borosilicate capillary glass (BF150-86-10, Sutter Instrument, CA, USA) using a Flaming-Brown micropipette puller (P-87, Sutter Instrument). Only patches with initial cell-attached seal resistance estimated higher than 1.0 GΩms were used in the experiments. Intracellular (pipette) solution for whole-cell experiments was NaCl 140 mM, EGTA 0.5 mM, Heps 10 mM, pH 7.4 (adjusted with Tris-base, standard Na+ 140 mM), while bath solution was usually NaCl 140 mM, CaCl2 1.0 mM, MgCl2 0–1.0 mM, KCl 50 mM, Heps 10 mM, pH 7.4 (adjusted with Tris-base or NaOH). The OR channel current noise was recorded using outside-out patches excised from HEK293T cells and evoked by stimulation with 250 μM VUAA3. The electrode solution was KCl 140 mM, EGTA 2.0 mM, Heps 10 mM, pH 7.4, while bath solution was NaCl 140 mM, EGTA 2.0 mM, Heps 10 mM, pH 7.4. Whole-cell recordings were used to estimate the selectivity of the OR channel to monovalent cations. The intracellular solution was standard Na+ 140 mM solution. VUAA3 (200 μM) was added to all extracellular test solutions. Cells were first exposed to standard NaCl 140 mM solution. Then, the same cell was exposed to a solution (LiCl 140 mM; KCl 140 mM; CsCl 140 mM; RbCl 140 mM) in which the Na+ ions were replaced by one of the following cations: Li+, K+, Cs+, Rb+. The whole-cell current-voltage characteristics were generated using series of 15 ms step at -100 (−110) mV followed by a 150 ms voltage ramp (linear change in voltage ~0.67 mV/ms) from -100 (−110) mV to +90–100 mV were applied from a holding potential of -50 (−60) mV. The interval between sweep starts was 1.0 s. Series of current traces (50–100 whole cell-I V curves) were then averaged and a potential at which current voltage characteristic of VUAA3 activated integral current intercepts current voltage characteristic of basal current was used as a reversal potential (VR) of the OR channel current in a given ion conditions. To determine the reversal potential shift (ΔVR), the VR of the currents obtained in symmetrical Na+ conditions (VRNa+) was subtracted from the VR obtained, then Na+ was replaced by respective cations (VRX). The ΔVRX means were then used to estimate the permeability ratios (PX+/PNa+) using the Goldman-Hodgkin-Katz potential equation (\( E_{X-X} = \frac{RT}{F} \log \frac{P_{Na}^{+}[Na^{+}]}{P_{X}^{+}[X]} \))56.

Heterologous expression of CpomORs in D. melanogaster OSNs. To perform heterologous expression in D. melanogaster OSNs, CpomORs were amplified using primers in Table 2 and purified PCR products were cloned into the PCR8/GW/TOPO plasmid (Invitrogen) following the recommended protocol. Cassettes with inserts were then transferred from their PCR8/GW/TOPO plasmids to the destination vector (pUASg-HA. attB, constructed by E. Furger and J. Bischof, kindly provided by the Basler group, Zürich), using the Gateway LR Clonase II kit (Invitrogen). The integrity and orientation of inserts was confirmed by sequencing (Sanger sequencer, 3730xl). Transformant UAS-CpomOR3 and UAS-CpomOR1 lines were generated by Best Gene (Chino Hills, CA, USA), using the PhiC31 integrase system, while the transformant UAS-CpomOR6a line was generated in our labs. Briefly, recombinant pUASg-HA. attB-CpomOR plasmids were injected into embryos of a D. melanogaster line containing an attP insertion site within the third chromosome (genotype y1 M[vas-int.Dm]ZH-2A w*; M(3xP3-RFPattP)ZH-86(Fb), leading to non-random integration. To drive expression of CpomORs in at1 OSNs in place of the endogenous receptor Or67d, transformant UAS-CpomOR lines were crossed to the knock-in
**Single sensillum recordings.** CpomOR1 and CpomOR6a expressed in at1 OSNs, and CpomOR3 expressed in ab3A OSNs, were tested through single sensillum recordings (SSR). Three to 8-day-old flies were immobilized in 100 μL pipette tips with only the top half of the head protruding. The left antenna of each insect was gently pushed with a glass capillary against a double-sided adhesive tape placed on a piece of glass. This piece of glass and the pipette tip were fixed with dental wax on a microscope slide. Electrotyically sharpened tungsten electrodes (Harvard Apparatus Ltd, Edenbridge, United Kingdom) were used to penetrate the insect’s body. The reference electrode was manually inserted in the right eye of the fly, while the recording electrode was manoeuvred with a DC-3K micromanipulator equipped with a PM-10 piezo translator (Märzhäuser Wetzler GmbH, Wetzler, Germany) and inserted at the base of the determined sensilla. Signals coming from the olfactory sensory neurons were amplified 10 times with a probe (INR-02, Syntech, Hilversum, the Netherlands), digitally converted through an IDAC-4-USB (Syntech) interface, and visualized and analysed with the software Autospike v. 3.4 (Syntech). A constant flow of 0.65 m/s of humidified air (charcoal-filtered) was delivered through a glass tube to the antenna. The panel of odorants was given to the insect by inserting pipettes containing a piece of filter paper with the correspondent stimulus in a lateral hole of the glass tube and puffing a flow of 2.5 mL of air during 0.5 s through the pipette. For CpomOR1 and CpomOR6a the panel of odorants was prepared by applying 10 μL of a solution of 1.0 μg/μL of the compounds in Supplementary Dataset S2 and Supplementary Dataset S3, for a total amount of 10 μg per stimulus. In the case of CpomOR3, a similar dilution process was used for the dose response experiments of ethyl-(E,Z)-2,4-decadienoate and methyl-(E,Z)-2,4-decadienoate, as well for dose response of experiments of codlemon acetate in CpomOR6a. Compounds were diluted from concentrations ranging from 0.01 ng/μL to 10 μg/μL in decadic steps, allowing reaching concentrations from 100 pg to 100 μg per stimulus when 10 μL of the dilution was applied in the piece of filter paper. In all cases, to characterize the intensity of the response, spike frequency was calculated by subtracting the spikes recorded 0.5 s after the stimulus from the number of spikes recorded 0.5 s after the stimulus and multiplied by 2 to get the response in spikes/s. The number of spikes was corrected accounting for differences in vapour pressure34. Dose response experiments between pear ester and its analogue were compared with two-way ANOVA with repeated measures, followed by LSD post-hoc test. Dose response experiments of codlemon acetate in CpomOR6a were analysed with one-way ANOVA with repeated measures followed by LSD post-hoc test.

**Source, identity and purity of tested compounds statement.** The authors declare that all compounds used in this study were pure according with respective supplier’s standards or sampled from pure stocks used in previously published methods.

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Author Contributions
A.M.C., Y.V.B. and F.G. conceived and designed the experiments. A.M.C. and E.A.C. optimized HEK293T cells transfection and immunohistochemistry protocols. A.M.C. and Y.V.B. performed Calcium Imaging on HEK293T cells. Y.V.B. performed patch-clamp experiments on HEK293T cells. F.G. performed SSR experiments on Drosophila ab3A and at1 OSNs. A.M.C. extended full-length CDS of CpomOR6a and CpomOR3. A.M.C. and J.M.B. cloned full-length coding sequences of OR genes into pcDNA5/TO and pUASg-HA.attB respectively. U.S. cloned CpomOR3 and V5-CpomOR3 into pcDNA5/TO. E.J-J., N.M., J.M.B. and W.B.W. conducted gene annotations and bioinformatics analyses. W.B.W. generated flies expressing CpomOR6a. P.W., G.A. and Y.V.B. supervised the research. A.M.C., Y.V.B. and E.A.C. wrote the manuscript.

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