Heterologous expression and functional characterization of *Drosophila suzukii* OR69a transcript variants unveiled response to kairomones and to a candidate pheromone

Alberto Maria Cattaneo (albertomaria.cattaneo@slu.se)
Swedish University of Agricultural Sciences: Sveriges lantbruksuniversitet https://orcid.org/0000-0002-7922-5215
Peter Witzgall
Swedish University of Agricultural Sciences: Sveriges lantbruksuniversitet
Charles A. Kwadha
Swedish University of Agricultural Sciences: Sveriges lantbruksuniversitet
Paul G. Becher
Swedish University of Agricultural Sciences: Sveriges lantbruksuniversitet
William B. Walker III
USDA-ARS Temperate Tree Fruit and Vegetable Research Unit

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Abstract

Background

Drosophila suzukii is among the main pests of berries, and to which control, primarily through application of insecticides is challenging and urges alternative strategies. Methods based on targeting chemical sensing and interfering with the behavior of this pest insect are promising, and are facilitated by the isolation and functional characterization of chemosensory receptors aimed to identify behaviorally active ligands. In our previous investigations, we functionally characterized subunits of the OR69a locus of *Drosophila melanogaster* as being capable to bind both pheromones and kairomones. OR69a is well conserved within the genus *Drosophila*, and represents a candidate target for a broad behavioral interference based on chemosensory communication towards the design of novel control strategies.

Results

By means of heterologous expression in empty ab3A neurons of *D. melanogaster* together with Single Sensillum Recording (SSR), we screened three OR69a-subunits we have identified in *D. suzukii* (DsuzOR69aA, DsuzOR69aB, DsuzOR69aC) with a panel of 48 ligands. We deorphanized these subunits to several compounds, including two possible kairomones (3-octanol and R-carvone) and (Z)-4-nonenal, a possible fly-emitted volatile compound, although with different pharmacological qualities. By coupling Gas Chromatography to Single Sensillum Recording (GC-SSR), we demonstrated these three ligands enhance ab3A-spiking at nanogram aliquots in a complementary fashion among the different OR69a variants, and we identified another possible kairomone, methyl salicylate, as the most active and specific ligand for the sole subunit DsuzOR69aB. In testing headspaces collected from *D. suzukii* female and from the fly-associated yeast *Hanseniaspora uvarum* by GC-SSR we did not observe activation from female headspaces but activation from headspaces collected from yeast. *In situ* hybridization analysis conducted on *D. suzukii* antennae suggested unique expression of OR69a-subunits into specific neurons as well as their possible co-expression within the same neurons.

Conclusions

The OR69a-subunits of *D. suzukii* constitute cation channels, which binding suggests kairomone specificity, even if effects may coexist for binding the possible fly-emitted volatile compound (Z)-4-nonenal in a complementary fashion. Among the possible kairomones, methyl salicylate is the most active ligand and it demonstrated to be specific to the sole DsuzOR69aB subunit, inspiring future investigation to validate potentials of this compound for *D. suzukii* pest control strategies.

Background

The spotted wing Drosophila, *Drosophila suzukii*, is one of the main pest insects threatening berry production [1] rapidly spread from its native Asia to Americas, Europe and Africa [2–4]. In the last decade, several ways for the control of this pest have been explored, from the use of microbiological strategies, to the selection of natural enemies, including predators and parasitoids [5–7]. Other methods to control this pest integrate agronomic strategies, among which, several target the increment of the reservoir of natural enemies [8].

Despite the various methods adopted up to now to control *D. suzukii*, farmers still rely on insecticide applications [9]. The infestation of ripening and ripe fruits is in conflict with insecticide use [2, 10–11], urging the identification of alternative strategies to limit this pest [12]. Among the first studies on *D. suzukii* behavior, it was demonstrated that chemosensory information influences reproduction of the insect [13]. While possible frontiers in mating disruption still seem to be far, other methods based on *D. suzukii* chemical sensing demonstrated promising effects in the development of *attract and kill* control [14–17].

With the aim to identify ligands active on insect chemical sensing, in reverse chemical ecology [18–19], molecular and pharmacological methods have already demonstrated potentials [20] and unveiled semiochemicals for integrated pest management [21]. Among these methods, heterologous expression *in vitro* and in *D. melanogaster* deorphanized pest chemoreceptors to ligands renowned for rather pheromone or kairomone activities [22–29].

Applying these methods to identify and functionally characterize receptors binding both types of semiochemicals may enhance pest control strategies targeting insect chemosensory communication. Among these receptors, we recently demonstrated in the closely related species *Drosophila melanogaster* that the OR69a-locus codes for both an OR-subunit responding only to food odorants (OR69aA) and for an OR-subunit (OR69aB) responding to both food odorants and to a novel sex pheromone: (Z)-4-undecenal. This pheromone is possibly emitted through autoxidation of *D. melanogaster* cuticular hydrocarbon (Z,Z)-7,11-heptacosadiene [30]. These two OR variants are co-expressed on the antennae in the same olfactory sensory neuron (OSN) type: ab9a [31–34] feeding into a circuit that mediates attraction of males and females, indicating OR69a as a promising target to interfere with behavior of other insects of the genus *Drosophila* wherein this receptor is conserved.

In this study, by means of single sensillum recording (SSR) in ab3A empty neuron system of *D. melanogaster* we identified, functionally expressed and characterized three OR69a subunits of *D. suzukii*: DsuzOR69aA, DsuzOR69aB and DsuzOR69aC. Upon deorphanization to several ligands, pharmacological studies based on ab3A-spiking suggested specificity to kairomones and to the possible fly-emitted volatile (Z)-4-nonenal. By optimizing gas chromatography coupled with single sensillum recording (GC-SSR), which has been previously adopted in our labs [35], we demonstrated complementary tuning of the three subunits to nanogram-aliquots of the selected ligands. Testing volatiles collected from the headspace of *D. suzukii* females and from the yeast *Hanseniaspora uvarum* by GC-SSR demonstrated absence of activation of any DsuzOR69a subunit by female headspace, but presence of active components

from yeast headspace. *In situ* hybridization on *D. suzukii* antennae unveiled both expression of the three subunits into specific neurons and, potentially, their co-expression within the same neurons, suggesting further that OR69a-tuning to ligands may happen in a complementary fashion.

Among the active ligands, we have identified methyl salicylate, known for its ecological significance to *D. suzukii*, as a compound emitted by its hosts [36] enhancing antennal [37] and neuronal [38] electrophysiological response. The demonstrated activation by this ligand of solely the DsuzOR69aB subunit and to the highest sensitivity opens future studies of the behavioral effects of methyl salicylate to validate its potential as a semiochemical for pest control strategies.

Results

Functional expression of DsuzOR69a subunits in ab3A neurons of D. melanogaster

Transcriptomic analysis and comparison with genomic data that will be part of a different project (Walker et al. in preparation) unveiled splicing of five DsuzOR69a transcripts expressed in *D. suzukii* antenna, here we named *DsuzOR69aA*, *DsuzOR69aB*, *DsuzOR69aC*, *DsuzOR69aD* and *DsuzOR69aE* (Fig. 1A, B; sequences available in the Additional file 1: Raw Data File 1).

Confirmation of the expression of OR69a-transgenes in the progenies generated from crossings of parental *w;Δhalo/CyO;pUAS-OR69a*A(aC) lines with *w;Δhalo;OR22a-Gal4* mutants and parental *w,pUAS-OR69aB;Δhalo/CyO;pOR22a-Gal4* lines with *w;Δhalo/CyO;+* mutants was demonstrated by recovering different rates of ab3A spiking, absent among empty neuron flies (*Δhalo* homozygous) without transgene OR expression. As further evidence of difference from the *Δhalo* homozygous, OR69a-progenies did not display ab3A-burst phenotypes described by Dobritsa et al. [39] (Fig. 1C). This phenotype is a prerogative of *Δhalo*, characterized by a basic, but limited, ab3A-spiking, which is absent in the newly designed version of the “empty neuron” heterologous expression system [40, Dr. Chih-Ying Su personal communication]. Interestingly, ab3A neurons expressing DsuzOR69aB subunits reported a relatively more frequent firing rate. When tested, every stimulus, including the solvent, elicited a generally strong and phasic spiking-effect. However, only few among the compounds of the panel generated a tonic response, such as a long-lasting rate of ab3A firing after the stimulus, and they demonstrated significant difference in ab3A spiking when compared with the solvent (Additional file 2: Raw Data File 2).

In contrast to DsuzOR69aA and DsuzOR69aC progenies, for which response to ethyl hexanoate was significant (Fig. 1D, Additional file 3: Fig. S1), DsuzOR69aB flies demonstrated non-significant ab3A-spiking to this ligand. To demonstrate that this effect was not related to the expression of the *D. melanogaster* wildtype OR22a subunit in the tested OR69a-flies, being that ethyl hexanoate is among the OR22a-activators [39, 41–42], responses to this ligand were compared to a negative control from a collection of white-eyed non-transformed insects (Best Gene, genotype *w;+;+* - see methods). Evidence of a phasic and delayed ab3A-effect for DsuzOR69aA and DsuzOR69aC was apparent, contrary to the tonic effect associated with ab3A activation of wildtype OR22a (Additional file 3: Fig. S1), suggesting transgenic expression of a different OR-subunit into the empty ab3A neurons.

SSR investigation on DsuzOR69a subunits

DsuzOR69a-subunits were heterologously expressed by generating ab3A-strains of *D. melanogaster* (Fig. 1C). Analyzing responses due to expression of different DsuzOR69a subunits, several compounds demonstrated significant ab3A-spiking when compared with the solvent (Fig. 1D, Table 1). Some of the compounds in particular reported activation for only one subunit, among which, ethyl-2-methylpent-3-enoate, decanal, (Z)-6-undecenal, limonene oxide, linalool oxide, L-limonene, valencene, S- and R-α-terpineol demonstrated significant difference in ab3A-spiking from the solvent only for DsuzOR69aA; methyl salicylate only for DsuzOR69aB; isoamyl acetate, ethyl lactate, (Z)-3-nonenal, (E,E)-2,4-decadienal and citral only for DsuzOR69aC.

On DsuzOR69aA, SSR analysis demonstrated strongest activation for the kairomonal monoterpene alcohols R-linalool, S-linalool, R-α-terpineol and S-α-terpineol, the aliphatic alcohol 3-octanol and other ligands in the panel tested, which elicited a significant effect when compared with the solvent. DsuzOR69aB demonstrated an overall high spiking frequency with a generally strong and phasic effect to multiple ligands. However, when compared with the solvent, significant spiking was associated only to the monoterpene ketones R-carvone and S-carvone, to the aliphatic alcohol 3-octanol, to the monounsaturated alcohol (E)-2-hexenol, to the unsaturated aldehyde (Z)-4-nonenal and to the monoterpenoid ester methyl salicylate, which induced the strongest responses together with R-carvone (Additional file 2: Raw Data File 2). Testing DsuzOR69aC, SSR analysis unveiled 3-octanol and (R)-carvone to be the most responsive ligands, while several among the other ligands induced a significant effect when compared with the solvent.

In parallel, within the whole panel we tested, some compounds demonstrated significant ab3A-spiking for all the tested Dsuz-subunits: 3-octanol, R/S-carvones and (Z)-4-nonenal. Based on this finding, compounds were used to test dose-response.

Dose-response characteristics of DsuzOR69a subunits

Testing dose response relationship by SSR, 3-octanol yielded the following pharmacological parameters: DsuzOR69aA, $EC50_{3-octanol}$ = 7.733 ± 1.909 µg, Hill coeff.$_{3-octanol}$ = 0.8866 ± 0.1736; DsuzOR69aB, $EC50_{3-octanol}$ = 7.495 ± 2.480 µg, Hill coeff.$_{3-octanol}$ = 1.032 ± 0.3818; DsuzOR69aC, $EC50_{3-octanol}$ = 6.681 ± 0.5721 µg, Hill coeff.$_{3-octanol}$ = 1.588 ± 0.2437 (Table 2).

After correction for differences in vapour pressure, our results suggested all DsuzOR69a subunits to be more sensitive to R-carvone. Indeed, based on EC50s, both normalization methods (normalization to the 3-octanol induced spiking or to the maximal effect after adjusting for vapour pressure) associate R-carvone to lower values than other ligands (DsuzOR69aA, $EC50_{R-carvone}$ = [1.529 ± 0.203 ; 1.555 ± 0.2932] µg; DsuzOR69aB, $EC50_{R-carvone}$ = [1.788 ± 0.4933 ; 2.223 ± 0.6643] µg; DsuzOR69aC, $EC50_{R-carvone}$ = [2.444 ± 0.8927 ; 2.471 ± 0.8861] µg; Table 2) with overall similar Hill-coefficients (DsuzOR69aA, Hill coeff.$_{R-carvone}$ = [1.327 ± 0.3112 ; 1.138 ± 0.1749]; DsuzOR69aB, Hill coeff.$_{R-carvone}$ = [1.341 ± 0.3775 ; 1.359 ± 0.409]; DsuzOR69aC, Hill coeff.$_{R-carvone}$ = [1.606 ± 0.6518; 1.624

± 0.6498] Table 2). In addition, for all DsuzOR69a subunits, saturation of the response to R-carvone was always reached at lower concentrations when compared to the other ligands (DsuzOR69aA: 25.0 µg; DsuzOR69aB: 25.0 µg; DsuzOR69aC: 10.0 µg; Table 2).

However, normalizing to 3-octanol induced ab3A-spiking we observed relatively low-efficacy of R-carvone on DsuzOR69aA ($Fmax_{R-carvone}$ = 0.4804 ± 0.01756) and on DsuzOR69aC ($Fmax_{R-carvone}$ = 0.5908 ± 0.05757) compared with the 3-octanol induced effect (DsuzOR69aA, $Fmax_{3-octanol}$ = 1.107 ± 0.082; DsuzOR69aC, $Fmax_{3-octanol}$ = 0.9862 ± 0.02832), which suggested possible partial agonism for R-carvone to these DsuzOR69a subunits (Table 2, Fig. 2A). This seems to be the case in particular for DsuzOR69aC, since doses above 50.0 µg of R-carvone associated for any replicate a visible reduction in ab3A-spiking (Additional file 2: Raw Data File 2). In contrast, when tested on DsuzOR69aB ($Fmax_{R-carvone}$ = 1.045 ± 0.05696) R-carvone displayed even higher efficacy than 3-octanol ($Fmax_{3-octanol}$ = 0.9795 ± 0.1075).

Testing dose response relationship by SSR for (Z)-4-nonenal yielded the following pharmacological parameters: DsuzOR69aA, $EC50s_{(Z)-4-nonenal}$ = [8.281 ± 2.775 ; 8.465 ± 3.601] µg; Hill $coeff._{(Z)-4-nonenal}$ = [1.211 ± 0.4912 ; 1.157 ± 0.5571]; DsuzOR69aB $EC50s_{(Z)-4-nonenal}$ = [2.901 ± 0.5136 ; 2.996 ± 0.7922] µg; Hill $coeff._{(Z)-4-nonenal}$ = [1.074 ± 0.1681 ; 1.198 ± 0.2865]; DsuzOR69aC, $EC50s_{(Z)-4-nonenal}$ = [9.993 ± 3.047 µg; 10.14 ± 3.085] µg; Hill $coeff._{(Z)-4-nonenal}$ = [1.312 ± 0.4962 ; 1.318 ± 0.5061]. For all DsuzOR69a subunits, (Z)-4-nonenal demonstrated high efficacy, similar to 3-octanol (DsuzOR69aA, $Fmax_{(Z)-4-nonenal}$ = [0.8592 ± 0.0966 ; 1.038 ± 0.1472]; DsuzOR69aB, $Fmax_{(Z)-4-nonenal}$ = [1.038 ± 0.03855 ; 1.088 ± 0.0583]; DsuzOR69aC, $Fmax_{(Z)-4-nonenal}$ = [0.9851 ± 0.1059 ; 0.9868 ± 0.1062]). Interestingly, for DsuzOR69aB, (Z)-4-nonenal reported very similar pharmacological parameters and a matching summary plot of the approximated Hill equation with R-carvone (Fig. 2A).

GC-SSR

A subset of synthetic odorants reporting evident SSR-effects were chosen for further analysis by GC-SSR. Apart from 3-octanol, (Z)-4-nonenal, and R-carvone, already used for dose response (Fig. 2), we included methyl salicylate, given its specificity for DsuzOR69aB (Fig. 1). Before conducting any experiment, the GC-SSR equipment was assembled and its sensitivity was calibrated by testing decreasing ethyl hexanoate dosages down to 1.0 ng of recording from ab3A neurons of a chromosome 2 wild-type strain of *D. melanogaster* (Best Gene genotype w;+;+ - Additional File 4: Fig. S2). DsuzOR69aA subunit tested to synthetic ligands by GC-SSR demonstrated significant ab3A firing for 10.0 ng 3-octanol (two-tailed paired T-test [□=0.05]: p = 0.0062870; N = 6) and 10.0 ng R-carvone (p = 0.0034273; N = 5). DsuzOR69aB for 10.0 ng (Z)-4-nonenal (p = 0.0005518; N = 4), 5.0 ng methyl salicylate (p = 0.0147026; N = 4) and 10.0 ng R-carvone (p = 0.0004892; N = 5), while DsuzOR69aC for 10.0 ng 3-octanol (p = 0.0106037; N = 7) (Fig. 3A, Additional File 5: Raw Data File 3).

In accordance with the SSR analysis (Fig. 1D), GC-SSR confirmed activation to methyl salicylate for only DsuzOR69aB and not for DsuzOR69aA and DsuzOR69aC subunits. Injection of doses lower than 5.0 ng demonstrated evident firing of the ab3A neurons of DsuzOR69aB flies, capable of enhancing ab3A firing down to doses of 1.0 nanogram, contrary to the other compounds we have tested (Fig. 3B, C).

GC-SSR confirmed ab3A firing for R-carvone for DsuzOR69aA and DsuzOR69aB, but not for DsuzOR69aC, where although for some replicates it appeared to be active (Additional File 5: Raw Data File 3) it did not return a significant difference from the hexane when analysed by T-test. Significant effect of (Z)-4-nonenal was demonstrated for only DsuzOR69aB, while a significant effect of 3-octanol was demonstrated for DsuzOR69aA and DsuzOR69aC.

Analysing headspaces collected from 2- to 5-day-old *D. suzukii* females did not result in any effect on DsuzOR69a subunits (Fig. 4). Interestingly, parallel experiments conducted with headspaces of *Hanseniaspora uvarum* that were already available in our labs and on use for different projects [43, Klemann et al. in preparation] revealed activation of DsuzOR69a subunits in proximity of compounds released at different retention times (Fig. 5, Additional File 5: Raw Data File 3).

Fluorescence in-situ hybridization (FISH)

Quantification of the number and distribution of neuronal cells in *D. suzukii* antennae labelled with DsuzOR69a RNA probes indicated that there is no apparent difference between male and female [DsuzOR69aA - N_{male} = 11, N_{female} = 10; Mann-Whitney-U-test (MWU: □ = 0.05): p = 0.9681, U = 55; Two-sample T-Test (two-tailed, □ = 0.05): p = 0.690287; DsuzOR69aB - N_{male} = 11; N_{female} = 10; MWU: p = 0.09102, U = 30.5; T-Test: p = 0.107922; DsuzOR69aC - N_{male} = 13; N_{female} = 9; MWU: p = 0.89656, U = 56; T-Test: p = 0.528551] (Additional File 6: Raw Data File 4; Fig. 6). However, for DsuzOR69aB we observed a slight lower intensity in the staining of female neurons, for which counted number, in some cases, appeared to be lower. Distribution of neuronal cells labelled with DsuzOR69a RNA probes resulted in both cells stained with unique probes and cells stained with more than one probe (Fig. 6C).

Discussion

In this work, we isolated, heterologously expressed and functionally characterized three out of five OR-subunits translated from alternative mRNA transcript variants of the *D. suzukii* OR69a locus. Starting with an SSR-screening of candidate ligands based on orthologues of *D. melanogaster* [30], specific compounds were selected to study ligand-binding characteristics by performing dose-response experiments and to confirm their identity as ligands of these subunits by measuring their effects at the nanogram-level by an optimized GC-SSR. To our knowledge, this is among the first contributions where the use of the GC-SSR method helps the functional characterization of insect ORs expressed in empty neurons of *D. melanogaster* [44].

Based on the screening of synthetic ligands (Fig. 1D, Table 1), 3-octanol, (Z)-4-nonenal, R-carvone and S-carvone displayed significant activation of all the DsuzOR69a subunits we have tested. Interestingly, the unsaturated (Z)-4-nonenal was the sole aldehyde tested from the panel that we have identified to be active on all tested DsuzOR69as. To compare pharmacological features among these agonists, we performed a comparative dose-response analysis.

Based on the pharmacological parameters elaborated from normalized dose-response effects, DsuzOR69a subunits demonstrated that they are in general more sensitive to R-carvone than 3-octanol and (Z)-4-nonenal (Table 2). However, R-carvone seems to be only a partial agonist for DsuzOR69aA and DsuzOR69aC, given the reduced amplitude of the curve from the approximated Hill-equation when normalized to the 3-octanol effect and compared with 3-octanol and (Z)-4-nonenal (Fig. 2). Conversely, R-carvone demonstrated stronger agonism for DsuzOR69aB. Interestingly, for this subunit, R-carvone shared very similar pharmacological parameters with (Z)-4-nonenal (Table 2, Fig. 2), while (Z)-4-nonenal was always associated with among the highest Fmax, as a possible indication of a real DsuzOR69aB agonism [45].

To investigate further on the activation of DsuzOR69a subunits by these agonists, we coupled SSR with the gas-chromatographic equipment (see methods) to perform GC-SSR analysis testing compounds at nanogram-aliquots (Fig. 3) upon calibrating the equipment by adjusting glass tubing and airflow until effects were recordable at the lowest possible nanogram-doses (Additional File 4: Fig. S2). In GC-SSR experiments we included also methyl salicylate given the evidence from our SSR-screening demonstrating activation to this ligand by only the DsuzOR69aB subunit, where together with R-carvone, it is one of the two most active ligands (Fig. 1D, Additional file 2: Raw Data File 2).

Contrary to SSR (Fig. 1D, Fig. 2) no effects were recorded by testing 10.0 ng 3-octanol on DsuzOR69aB by GC-SSR (Fig. 3A). Not surprisingly, dose-response for DsuzOR69aB demonstrated the lowest efficacy for this compound when compared with R-carvone and (Z)-4-nonenal (Fig. 2A), as further evidence of its possible partial agonism [45–47]. While these results may indicate that doses of 10 ng were not sufficient to enhance a minimal activation of DsuzOR69aB for 3-octanol, they support that the GC-SSR method is more reliable than the SSR method in the deorphanization of ORs to their agonists. Indeed, upon puffing identical doses of different ligands for detection by SSR, spiking may be biased by their different volatility [48], by the loss of calibration during serial puffing, by the type of solvent, or by possible contaminants [49]. We expect these effects to be by-passed or at least reduced at minimum when compounds are released from the gas-chromatographer and directly provided to the antenna.

Instead, analysing DsuzOR69aA and DsuzOR69aC, 10.0 ng of 3-octanol were sufficient to enhance activation. Results from an SSR-comparison on OR69a subunits from several species of the genus *Drosophila*, which will be part of a different publication (Gonzalez et al. in preparation), demonstrated similar response spectrum to the ligands tested between the OR69aCs of *D. simulans* and *D. suzukii*, which included 3-octanol and R-carvone, that were also active on the OR69aAs of these Drosophilas. Possibly, the existence of an additional subunit such as OR69aC may increase the repertoire of ligands sensed by OR69aA and OR69aB. Indeed, although observed for the different class of IRs odorant receptors, different spectrums of ligand activation have been demonstrated among *Drosophila* neurons housing in ac2 and ac3 sensilla expressing different IR75-chemosensory subunits [50] that are translated from three splice-forms (IR75c, IR75b, IR75a) transcribed from the same locus (IR75cba, [51]). Future studies testing DsuzOR69aD and DsuzOR69aE subunits, as additional transcript variants from the DsuzOR69a-locus, will validate if this hypothesis holds true.

In analysing the effect to R-carvone by GC-SSR we observed activation for both DsuzOR69aA and DsuzOR69aB subunits. Taken with dose-response experiments, our results may suggest this ligand as real agonists for DsuzOR69aA and DsuzOR69aB. In analysing the effect to (Z)-4-nonenal by GC-SSR we observed activation for the sole DsuzOR69aB subunits. Pharmacological features and plotting of the approximated Hill equations for (Z)-4-nonenal and R-carvone were proximal to identity (Table 2, Fig. 2A), further suggesting (Z)-4-nonenal as a real agonist for DsuzOR69aB.

GC-SSR analysis demonstrated methyl salicylate to be active solely on the DsuzOR69aB subunit. Interestingly, the response to this ligand provides the highest spiking frequency among the other ligands tested and it is active up to doses lower than 1.0 ng (Fig. 3B, C). Methyl salicylate is the most active ligand we have found for DsuzOR69aB and, possibly, the main agonist for cation channels constituted with this transmembrane protein. Activation of DsuzOR69aB to methyl salicylate to 1.0 ng, but not lower aliquots, is in accordance of the optimization limit of our GC-SSR method, where doses of 1.0 ng represented the lowest detectable quantities by our assembled equipment (Fig. 3B, C and Additional File 4: Fig. S2). The ecological role of methyl salicylate is renowned among several plant mechanisms: from systemic acquired resistance to plant-plant interactions, as well as plant-insect interactions, including attraction of pollinators, repellency for phytophagous insects or attraction of their parasitoids [52]. Since long ago, methyl salicylate has been reported among the bouquet of several floral scents [53] and it can also be found in the headspace of fruits from strawberries [38], *Rubus* species and cherries [37], that are among the renowned hosts of *D. suzukii*. Previous investigations demonstrated significant antennal responses to methyl salicylate by GC-EAD when *D. suzukii* were tested with headspaces emitted by strawberries fruits and leaves, even from fruits when this volatile was emitted in small quantities [38]. A more recent investigation characterized methyl salicylate among the main volatiles emitted by intact berries of mistletoe (*Viscum album* subsp. *laxum*), representing a food source for winter and spring seasons for *D. suzukii* [36]. Interestingly, this study reports higher attraction and ovipositional behavior for mated *D. suzukii* when berries are artificially wounded and emitting significantly reduced levels of methyl salicylate. Furthermore, other studies reported methyl salicylate among the most toxic compounds for *D. suzukii*, demonstrating among the lowest LD50s when fumigant toxicity experiments were conducted on males and females [54]. All together, these data suggest methyl salicylate is deserving of investigations to verify its potential semiochemical properties towards the control of *D. suzukii*, in particular, to test repellency to this compound, taking evidences of the enhanced oviposition from its reduced emission from hosts [36]. To this hypothesis, activation of OR69aB in *D. suzukii* by a repellent would represent quite an evolutionary shift from *D. melanogaster*, for which the orthologue was demonstrated in our previous investigation to be tuned by the (Z)-4-undecenal pheromone reported to mediate attraction [30]. To validate this hypothesis, taking advantage of the ongoing development of CRISPR-cas9 technologies for *D. suzukii* [55] future behavioral experiments may compare sensing to methyl salicylate between wild type flies and CRISPR-knock out lines generated by inducing frame-shift editing within the start codon of the OR69aB-exon (Fig. 1A, Additional File 1: Raw Data File 1).

Our SSR-screening of DsuzOR69as is in accordance with the previous studies [30] and with evidence from an SSR-comparison of OR69a subunits from several species of the genus *Drosophila* (Gonzalez et al. in preparation). This study in preparation will demonstrate OR69aAs being mostly responsive to monoterpene alcohols and ethers, including linalool oxide and R-α-terpineol, representing the most shared ligands among the various orthologues. OR69aBs, instead, are mostly responsive to R- and S-carvones, that in our study we reported to be active on all the *D. suzukii* subunits we tested (Fig. 1D).

However, among our SSR analysis, we did not identify any significant activation for DsuzOR69as to (Z)-4-undecenal, which is instead active on the orthologues of *D. melanogaster*. Indeed, in Lebreton et al. [30] we demonstrated the existence of (Z)-4-undecenal in headspaces collected from *D. melanogaster*. The existence in *D. melanogaster* of (Z,Z)-7,11-heptacosadiene [(Z,Z)-7,11-HD] as the most abundant cuticular hydrocarbon [56], and experimental evidences of its autoxidation to the emission of aldehydes led us to hypothesize that (Z)-4-undecenal is the result of the (Z,Z)-7,11-HD autoxidation. OR69a subunits binding for both (Z)-4-undecenal and other odorants suggested a role of this receptor of *D. melanogaster* as a sensor for both pheromones and kairomones. In accordance, in the same investigation we reported chemical analysis on *D. melanogaster* and headspaces indicating small traces of (Z)-4-nonenal, associated with the presence of the (Z,Z)-5,9-HD isomer in limited quantities on the cuticular of these *Drosophila* [57–59]. Interestingly, (Z,Z)-5,9-HD is more abundant on the cuticle of the *D. melanogaster* subspecies Zimbabwe [59–61], and more recent investigations supported the autoxidation hypothesis demonstrating the emission of (Z)-4-nonenal from the Zimbabwe *D. melanogaster* [62].

Based on the most recent publications, there is no evidence in *D. suzukii* of the presence of cuticular hydrocarbons, which autoxidation may result in the emission of (Z)-4-nonenal [63–64], however, the specific effect of this ligand on DsuzOR69aB is compelling. In theory, if (Z)-4-nonenal would be emitted by *D. suzukii*, by the use of the GC-SSR method we optimized we would have recorded activation of DsuzOR69as upon testing respective headspaces collected from the female insects. However, volatile collection from 2- to 5-day-old *D. suzukii* females did not result in any effect on DsuzOR69as (Fig. 4). In any case, we cannot state whether (Z)-4-nonenal is emitted or not by *D. suzukii*. Indeed, apart from the possible absence of aldehyde ligands in the headspaces we have collected, final quantities of collected aldehydes may range below the detectable sensitivity of our equipment (< 1.0 ng, Additional File 4: Fig. S2, Fig. 3C), or may have difficulties to be released by our GC-equipment given their polar incompatibility with the HP-5 column we have utilized. These various scenario seems to be more reliable considering additional trials we performed by expressing OR69a orthologues of the *D. melanogaster* subspecies Zimbabwe (Additional File 7: Fig. S3). Testing headspaces from female insects, expecting to contain (Z)-4-nonenal [59–62], we did not record any effect for OR69aB. To shed more light on this aspect, additional experiments may be conducted by testing samples of Zimbabwe by GC-SSR using a different GC-column from HP-5 and adopting a different pheromone collection protocol [62]. On the other hand, activation of DsuzOR69aB to (Z)-4-nonenal, not expected to be emitted by *D. suzukii*, but identified in the emissions of *D. melanogaster* [30] and of its subspecies Zimbabwe [62], may suggest some sort of mechanisms of inter-specific communication among different insects of the genus *Drosophila*. For example, complex mechanisms exist at the base of inter-specific discrimination among insects within this genus [65] that are based on the different composition of cuticular hydrocarbons [66]. Although speculative, the detection of aldehyde pheromones by OR69a receptors upon their emission through autoxidation of cuticular hydrocarbons may be at the base of these inter-specific communication mechanisms. Not surprisingly, we have already demonstrated the existence of similar chemosensory systems in other insects where conserved pheromone receptors detect the main sex odors from other species [23] and the same kairomones from their hosts (Cattaneo, Witzgall and Walker in preparation). To verify possible emission of (Z)-4-nonenal from *D. suzukii* and its involvement in DsuzOR69a-based chemosensory communication, additional goals will be aimed at characterization of the rate of emission between males or females at different ages, virgin or mated, proposing (when possible) a similar approach used by Snellings et al. [63] Taking another possible scenario, (Z)-4-nonenal may be emitted by hosts of *D. suzukii* or may derive from different sources involved in the ecological relations with this insect, deserving future studies to validate alternative origins of the ligand. Indeed, a similar context exists for the *D. melanogaster* OR69a-ligand (Z)-4-undecenal identified in Clementine essential peel oil [67].

Apart from binding (Z)-4-nonenal, with an expected role as pheromone, evidences of the DsuzOR69as in binding kairomones may come from additional GC-SSR data demonstrating activation of the three DsuzOR69a subunits in proximity of GC-peaks of injected headspace collections from *H. uvarum* (Fig. 5, Additional File 8: Fig. S4). *H. uvarum* is one of the main yeast symbionts of berries, it is renowned for its ecological relations with *D. suzukii* and interferes with the behavior of the insect [16–17, 43, 68–70]. Although the compounds from *H. uvarum* headspace with activity on the DsuzOr69a-subunits were not identified, by invoking responses, it demonstrates involvements of DsuzOR69as in binding compounds emitted by this yeast, and it further remarks the ecological relations between this microorganism and the *D. suzukii* insect. In the frame of upcoming projects, GC-MS efforts will characterize the active ligands we have identified in *H. uvarum* headspace collections.

In analyzing expression of DsuzOR69a subunits in antennal sensory neurons of *D. suzukii*, we did not notice a significant difference between males and females, neither in the neuronal number nor in their overall distribution (Fig. 6). Our fluorescent data seems to converge on two scenarios, demonstrating that single OR69a transcript variants may rather be identified in different neurons, as well as co-existent within the same neurons. The latter scenario seem to be coherent with expectations up to now reported for *D. melanogaster* suggesting co-existence of both OR69aA and OR69aB transcript variants within the same ab9a neurons [30–34]. However, the study of Couto et al. [32] demonstrated that the two OR69a subunits may be expressed through different promoters, which cannot exclude their possibility to be present among different sub-populations of neurons. For this reasons, co-expression of OR69a-subunits remains an open and current research question. To our knowledge, this represents the first *in situ* hybridization study conducted on *D. suzukii* neurons. Our results may represent the starting point for future projects performing deeper *in situ* hybridization by combining different probes all in once, to validate convergence of OR subunits within the same OSNs, to consider if their chimeric cation channel may deserve to be tested by SSR. Indeed, in Lebreton et al. [30] co-expression of OR69aA and OR69aB in the same empty neurons did not report neither qualitative nor quantitative differences in neuronal activity. However, the existence of five antennal OR69a variants in *D. suzukii* (Fig. 1A, B; Walker et al. in preparation), may suggest different functional dynamics deserving better investigation in future research.

Apart from the heterologous expression, the identification in the antennae of *D. suzukii* of neurons expressing DsuzOR69a-subunits may add to the recent electrophysiological studies conducted *in vivo* on this insect [71]: a more deepened GC-SSR analysis will investigate on these neurons the effects of the same active ligands we have identified in the course of our investigation.

Conclusions

For the first time, we have heterologously expressed and functionally characterized some chemosensory receptors of *D. suzukii*. By means of the empty neuron of *D. melanogaster*, we functionally characterized three OR69a subunits of *D. suzukii*. By SSR screening and GC-SSR analysis, we identified ligands

eliciting activation of DsuzOR69a-subunits to nanogram quantities, that based on our optimized method, suggested these compounds as real agonists of OR69a-based receptors. In terms of basic spike frequency, activation to single ligands, dose response characteristics and the overall effects to minimal dosages, DsuzOR69aB seems to be the most active OR69a subunit characterized up to now, although both DsuzOR69aA and DsuzOR69aC are functional and tuned to a wide panel of ligands, some of which are active at nanogram doses. Among the active ligands, methyl salicylate represents the strongest agonist, specific solely for the DsuzOR69aB. We suggest other ligands, like (Z)-4-nonenal, specific solely for DsuzOR69aB, R-carvone, common agonists for DsuzOR69aA and DsuzOR69aB, and 3-octanol as an agonist for DsuzOR69aA and DsuzOR69aC. Future projects may investigate the behavioral role of these ligands.

Although our results taken together may suggest the DsuzOR69a locus transcribing sensors for kairomones, activation of DsuzOR69aB to (Z)-4-nonenal warrants further efforts to validate its possible binding of pheromones, that more possibly, must be searched among the aldehydes emitted through autoxidation of *Drosophila´*s cuticular hydrocarbons. Future studies on the other two subunits translated from the DsuzOR69a-locus, DsuzOR69aD and DsuzOR69aE, will validate whether they may constitute receptors for kairomones or pheromones, to better understand the ecological role in *D. suzukii* of OR69a receptors.

Material And Methods

Insects

Drosophila melanogaster (Zimbabwe-S-29, Bloomington #60741, we previously used in Lebreton et al. [30]) and *D. suzukii* (Italian strain [13]) were maintained on a sugar-yeast-cornmeal diet at room temperature (25 ± 2°C) and a relative humidity of 50±5 % under 12:12 light: dark photoperiod. Newly emerging flies were collected after every 4 h from the onset of photophase. Virgin male and female flies were kept separately in fresh food vials.

Cloning and heterologous expression of DsuzOR69a(s) **in** Drosophila **empty neuron system**

OR69aA, OR69aB and OR69aC receptors were cloned from antennae of *D. suzukii*, upon their identification in antennal transcriptome that will be part of a different report (Walker et al. in preparation). Briefly, cDNA was generated from RNA extracts of antennae of 100 males and females using standard procedures. The complete ORFs encoding DsuzOR69aA, DsuzOR69aB and DsuzOR69aC were amplified by PCR combining forward-specific CDS-primers (DsuzOR69aA: 5'-ATGCAGTTGCACGACTATATGAGGTATA-3´; DsuzOR69aB: 5´-ATGCAGCTGGAGGACTTTATGTTCTATC-3´; DsuzOR69aC: 5´-ATGGAATTTCATGAGTATTTTGAGTATT-3´) with a common reverse primer (DsuzOR69aABC: 5´-TTATTTCAGGGAACGCACGCAGGTAAAC-3´, Additional File 1: Raw Data File 1), starting from antennal cDNA as a template, retro-transcribed by RT-for-PCR kit (Invitrogen, Life technologies, Grand Island, NY, USA). Purified PCR products were then cloned into the PCR8/GW/TOPO plasmid (Invitrogen). The integrity and the orientation of the insert was confirmed by Sanger sequencing 3730xl (Eurofins Genomics, Ebersberg - Germany). 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). Integrity and orientation of inserts was checked further by Sanger sequencing.

Transformant lines for *pUAS-DsuzOR6aA* and *pUAS-DsuzOR6aC* were generated by Best Gene (Chino Hills, CA, USA) injecting into Best Gene Strain #24749 with genotype M{3xP3-RFP.attP}ZH-86Fb and insertion locus on the 3rd chromosome. The transformant line *pUAS-DsuzOR69aB* was generated by injecting into the Best Gene Strain #32233 with genotype y1 w*P{CaryIP}su(Hw)attP8 and insertion locus on the X chromosome. To drive expression of DsuzOR69as in the A neuron of ab3 basiconic sensilla (ab3A OSNs), crossings were performed with balancer lines in accordance with procedures already published from our labs [72]. For *pUAS-DsuzOR69a*A- and aC-lines the final crossing was performed with *w;Δhalo/CyO;Or22a-Gal4* mutant line [39, 73]. For *pUAS-DsuzOR69aB*, after crossing with *w;Δhalo/CyO;Or22a-Gal4* generating *pOR22a-Gal4* insertion in homozygosis on the third chromosome, the final crossing was performed with *w;Δhalo/Cyo;+* lines. In any case, *Δhalo* homozygous were selected based on the straight wings phenotype. The final strains tested by SSR and GC-SSR were the following genotypes: **DsuzOR69aA** line, *w;Δhalo;pUAS-DsuzOR69a*A*/pOR22a-Gal4;* **DsuzOR69aB** line, *w, pUAS-DsuzOR69a*B*/w;Δhalo;pOR22a-Gal4/+;* **DsuzOR69aC** line, *w;Δhalo;pUAS-DsuzOR69a*C*/pOR22a-Gal4.* Insects were reared in our facilities at room temperature (19–22°C) under a 16:8-h light:dark photoperiod as described in Lebreton et al. [30].

Volatile collection from flies

Twenty virgin female *D. melanogaster* (4- to 6-day-old) and *D. suzukii* (2-, 4-, and 5-day-old) were exposed to baked standard glass rearing vial (24.5 x 95 mm, borosilicate glass; Fisher Scientific Sweden) for 24 h. Flies were removed and the vials were rinsed with hexane (200 µL) under ultrasonic water bath for 3 min. Collections were transferred to 1.5 mL GC-MS vials with insert and concentrated to about 5 µl under a fume hood.

Single sensillum recordings

DsuzOR69as expressed in the A neuron of ab3 basiconic sensilla 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 right antenna of each insect was gently pushed with a glass capillary against a piece of glass. This piece of glass and the pipette tip were fixed with dental wax on a microscope slide. Electrolytically 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 in ab3-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). To carry the odorant stimulus and prevent antennal dryness, a constant humidified flow of 2.5 L/min charcoal-filtered air was delivered through a glass tube and directed to the preparation. To confirm expression of *DsuzOR69a*-transgenes, basic spiking of ab3-neurons were compared with

parental flies *Δhalo*-homozygous (*w;Δhalo;Or22a-Gal4* mutants). A panel of 48 odorants (Table 1) was chosen based on a previously reported investigation of compounds emitted from fruit, yeast and insects tested on the *D. melanogaster* orthologues [30].

Based on the database of odorant responses (http://neuro.uni-konstanz.de/DoOR/content/DoOR.php – [41-42], the panel included also 2-heptanone (CAS 110-43-0) and 3-octanol (CAS: 589-98-0) as positive controls to validate recordings from ab3 sensilla by testing activation of *D. melanogaster* ab3B. To discriminate ab3 from ab2 sensilla, the ab2A activator ethyl acetate (CAS: 141-78-6) was included as a negative control. To test absence in the ab3A neuron of the wild-type expression of OR22-subunits, ethyl hexanoate (CAS 123-66-0) was included as an additional negative control.

To screen the panel, odorants were diluted in hexane (Sigma Aldrich, St. Louis, MO-USA) at 1.0 μg/μL. Stimuli were prepared applying 10.0 μL of each dilution on grade 1 - 20 mm circles filter paper (GE Healthcare Life Science, Little Chalfont, United Kingdom), previously inserted into glass Pasteur pipettes (VWR, Milan, Italy), for a total amount of 10.0 μg of compound per stimulus. Puffing provided additional 2.5 mL air through the pipette for 0.5 seconds, by inserting the pipette within a side hole of the glass tube directing the humidified air-flow to the antennae. To characterize the intensity of the response, spike frequency was calculated as in Lebreton et al. [30] by subtracting ab3A spikes counted for 0.5 seconds before the stimulus from the number of spikes counted for 0.5 seconds after the stimulus, with the aim to calculate spike-frequency in terms of Δspikes/0.5sec. Given the impossibility to distinguish ab3A from ab3B spiking on *DsuzOR69aB*-transfected insects for most of the compounds tested, spike counting was performed subtracting the total number of spikes after stimulus to the total number of ab3A and ab3B spikes before stimulus. This spike counting approach was decided upon discussion with renowned experts in the use of the SSR-method (Dr. Mattias Larsson and Dr. Teun Dekker, Department of Plant Protection Biology, Swedish University of Agriculture Science; Dr. Francisco Gonzalez, Chemtica Internacional, S.A., Costa Rica). This approach is a reasonable when having difficulties in reliably distinguishing spikes from individual neurons [50, 74]. Responses to compounds of the panel were compared for 9 to 10 replicates, using a single insect as a replicate. To validate significant differences in spike counting, spike frequency of each compound were compared with respective spike frequencies enhanced by the solvent (hexane) by Mann-Whitney U-test ($p<0.01$; two tails) as done in our previous studies [23]. For box-plot analysis (Figure 1 D), Δspikes/0.5sec of each recording was normalized to the spike average of its specific insect replicate.

SSR method was adopted to perform dose-response experiments, selecting specific compounds of the panel giving significant ab3A-spiking for all the tested DsuzOR69a subunits: 3-octanol, R-carvone and (Z)-4-nonenal (Figure 1 D). The choice to use the more active R-carvone for dose-response analysis was determined by the comparison of the overall effects for R- and S- carvones on the three subunits, that were always more responsive to R-carvone (Additional file 2: Raw Data File 2). Compounds were diluted in hexane within a range between 0.1 μg/μL and 10 μg/μL, in order to test aliquots between 1 μg and 150 μg by applying at most 15 μL of the dilution on the filter paper. For each dose, ab3A spiking for 0.5 sec was doubled to calculate spike frequency in Δspikes/second. Spike frequencies were normalized to the effect from their respective saturating doses after correction for differences in vapour pressure [48]. To this normalization, 3-octanol molecular weight and vapor pressure parameters were chosen for adjustment (Table 1), as 3-octanol is the most volatile among the three ligands, adopting approaches we previously described [23, 26-27]. By an additional method, ab3A-spiking were normalized to the effect from saturating doses of 3-octanol, following similar protocols adopted for normalization of the effect to the effect enhanced by common ligands [23]. Normalized data were analysed by Sigma Plot 13.0 (Systat Software Inc., San Jose, CA, USA). Responses to selected compounds were compared for 3 to 5 replicates, considering a replicate as a single insect. Saturating doses were chosen comparing doses enhancing highest averages in ab3A spiking and taking the minimal dose among them as the saturating (Additional file 2: Raw Data File 2).

GC-SSR

GC-SSR was performed interfacing GC-equipment available in our labs with a SSR rig. Samples were injected on a 7890 GC-systems (Agilent technologies Inc., Santa Clara, CA, USA) provided with a 30 m x 0,32 mm fused silica capillary column (Agilent Technologies Inc.), coated with HP-5, df = 0,25 μm, programmed from 30°C (hold 3 min) at 8°C/min to 250°C (hold 5 min) (software: GC-SSR-1 - Agilent.OpenLab, Agilent Technologies). The split of out-let from GC column was a 1:1 ratio between the flame ionization detector and the mounted antenna, according to instrument settings. A humidified flow of 3.5-4.0 L/min charcoal-filtered air was directed into a 90-degree-angled glass tube provided with a hole on the angle where part of the column exiting from the transfer line accessed. To optimized the method, we adjusted glass-tubing length to 17 cm and tested ab3 sensilla of white-eyed non-transformed insects expressing OR22a (Best Gene, genotype *w;+;+*) until we demonstrated effects by testing doses of ethyl hexanoate proximal to 1.0 ng (Additional File 4: Fig. S2), which was considered as the sensitivity limit of the method. Recording was set for up to 35 min upon preliminary observation of retention times for the injected compounds.

By GC-SSR we tested insects expressing DsuzOR69a subunits for 0.1-10.0 ng aliquots of synthetic ligands (depending on the experiment) reporting evident SSR-effects (3-octanol, (Z)-4-nonenal, methyl salicylate, R-carvone). Synthetic ligands were diluted in hexane between 0.0001 and 0.010 μg/μL depending on experimental conditions and 2.0 μL were injected into the gas-chromatographer. Parallel experiments were conducted testing *D. suzukii* virgin female volatile collection from 2-, 4- and 5-day-old live insects and headspace collections from *H. uvarum* already available in our labs. To test headspace collections by GC-SSR, aliquots of 4.0 μL were injected into the gas-chromatograph.

To perform statistical analysis, spikes were counted within 5 seconds from the emission of their respective GC-peaks. For headspace collections, spikes were counted from the beginning of the ab3A-effect. Numbers were subtracted to spikes from 5 seconds anticipating the effect and divided by 5 to calculate Δspikes/second.

Fluorescence in-situ hybridization (FISH)

FISH was performed by using single own-synthesized DIG- and FLUO-probes starting from linearized pCR8-TOPO-vectors containing DsuzOR-coding sequences. In brief: 1.5 μg of pCR8-TOPO DNA containing DsuzOR69aA/B/C, -DsuzOrco (positive control) and -DsuzIR60b (negative control) were linearized with HpaI (DsuzOR69as) or BbsI (DsuzOrco, DszuIR60b) following recommended protocols (New England Biolabs, Ipswich, MA, USA) to be purified in RNAse-free water and checked on agarose gel electrophoresis to verify the linearization of the plasmids. One third of the purified volume (~0.5 μg) was amplified with

T7-RNA polymerase (Promega, Madison, WI, USA) integrating DIG- or FLUO-labelled ribonucleotides (BMB Cat. #1 277 073, Roche, Basel, Switzerland) following recommended protocols (https://www.rockefeller.edu/research/uploads/www.rockefeller.edu/sites/8/2018/10/FISHProtocolKSVRevised.pdf). *D. suzukii* antennae were collected from male and female adult insects of our rearing facility (FORMAS Swedish Research Council - project numbers 2011-390 and 2015-1221). RNA FISH on whole mount antennal were done essentially as described [75] staining with a single probe for each experiment. Imaging was performed on a Zeiss confocal microscope LSM710 using a 40´ immersion objective; settings were adjusted based on single antennae: DIG-labelled probes staining specific neurons were visualized setting Cy5-laser between 4-10% and calibrating gain in a range of 700-900. Staining was compared with male and female FISH-negative control probes prepared using IR60b, an Ionotropic Receptor which expression, based on our transcriptomic analysis, was demonstrated absent in the antennae of both males and females and that will be part of a different report (Walker et al. in preparation). A FLUO-labelled RNA-probe for DsuzOrco was used to stain antennae as a positive control and visualized using 488-laser at 4%, gain 700-900. Neuronal counting was performed using the cell-counter tool of Image J (Fiji, https://imagej.nih.gov/ij/). To identify differences between males and females, neuron numbers were compared with Mann-Whitney U-test and Two-samples test (= 0.05). After counting, region of interest (ROI) associated with neurons from different samples of DsuzOR69a-stained antennae were compared with ImageJ. In all cases, positions of neurons respected the same distribution along the antenna and partially overlapped among the different staining for DsuzOR69a-transcripts; a stylized antenna (Figure 6C) was generated using PowerPoint 2016.

Abbreviations

OR69a: Odorant Receptor 69a; ab3: antennal basiconic sensilla 3; ab3A/ab3B: neurons A and B contained into the antennal basiconic sensilla 3; SSR: Single Sensillum Recording; GC-SSR: Single Sensillum Recording coupled with Gas-Chromatography; FISH: Fluorescent *in situ* hybridization; EC50: concentration/dose eliciting the half-maximal effect; (Z,Z)-7,11-HD: (Z,Z)-7,11-heptacosadiene.

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and materials

All data and material generated during this study are included within the article as additional files (supplementary figures and raw data files). More data can be provided contacting the corresponding author at albertomaria.cattaneo@slu.se.

Competing interests

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Authors' contributions

A.M.C. conceived and designed the experiments, generated transgenic fly-lines and performed SSR, dose-response, GC-SSR, data and statistical analysis and FISH studies. W.B.W.III. performed genomic, transcriptomic and phylogenetic analysis that led to the identification and characterization of DsuzOR69a transcript variants that he isolated and cloned to generate transgenic *Drosophila*. P.W. designed the bouquet of candidate ligands that have been tested on OR69a subunits, prepared diluted aliquots tested by SSR and provided headspace collections from *H. uvarum*. C.K. and P.G.B. generated headspace samples collected from *D. suzukii* and *D. melanogaster* flies. W.B.W.III and A.M.C. supervised the research. P.W. provided financial support. A.M.C. wrote the manuscript.

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Tables

Table 1 Panel of candidate ligands tested on DsuzOR69a subunits. Compound classes, CAS numbers and physiochemical properties are reported, including averages and standard deviation of the response to each ligand, based on number of replicates: DsuzOR69aA, N = 9; DsuzOR69aB, N = 9; DsuzOR69aC, N = 10. Asterisks denote significance (Mann Whitney U-test, p<0.01, two tails).

Compound class	Compound	CAS	Molecular weight (g/mol)	Vapor pressure (mmHg @ 25°C)	DsuzOR69aA			DsuzOR69aB			Dsu
					Average	st.dev.	Signficance	Average	st.dev.	Signficance	Ave
Fatty acid ester	**Ethyl-3-hydroxy butyrrate**	5405-41-4	132,1592	0,3620	0,78	9,95		103,00	21,54		15,
Fatty acid ester	**Ethyl acetate**	141-78-6	88,1062	111,7160	2,22	10,20		64,67	32,81		6,9
Fatty acid ester	**Isoamyl acetate**	123-92-2	130,1870	5,6000	11,33	11,41		98,56	22,59		17,
Fatty acid ester	**Ethyl-(E)-2-methylpent-3 enoate**	1617-23-8	142,1980	1,6000	8,11	7,32	*	82,11	42,32		12,
Fatty acid ester	**Ethyl hexanoate**	123-66-0	144,2139	1,6650	14,33	9,09	*	71,11	37,02		29,
Fatty acid ester	**Ethyl-3-hydroxyhexanoate**	2305-25-1	160,2131	0,0060	23,44	7,14	*	79,00	42,51		26,
Fatty acid ester	**(Z)-3-hexenyl-acetate**	3681-71-8	142,1980	1,2190	22,89	9,09	*	99,11	19,15		30,
Fatty acid ester	**Ethyl lactate**	97-64-3	118,1323	1,1630	7,89	12,64		70,67	34,28		27,
Aromatic ester	**Methyl salicylate**	119-36-8	152,1494	0,0343	0,56	5,87		132,00	29,22	*	9,7
Unsaturated aldehyde	**Decanal**	112-31-2	156,2686	0,2070	9,33	5,44	*	78,78	23,83		14,
Unsaturated aldehyde	**Undecanal**	112-44-7	170,2955	0,0830	5,22	12,43		65,56	39,52		9,3
Monoinsaturated aldehyde	**(Z)-3-nonenal**	31823-43-5	140,2257	0,3960	6,22	8,50		88,89	40,37		16,
Monoinsaturated aldehyde	**(Z)-4-nonenal**	2277-15-8	140,2257	0,3980	12,89	8,62	*	123,44	29,40	*	24,
Monoinsaturated aldehyde	**(E)-4-decenal**	65405-70-1	154,2527	0,1190	18,00	4,81	*	95,00	34,83		19,
Polyinsaturated aldehyde	**(E,E)-2,4-decadienal**	25152-84-5	152,2367	0,0300	2,22	8,31		90,00	44,38		22,
Monoinsaturated aldehyde	**(E)-4-undecenal**	68820-35-9	168,2796	0,0450	1,56	9,11		71,56	39,62		10,
Monoinsaturated aldehyde	**(Z)-4-undecenal**	68820-32-6	168,2796	0,0450	3,78	6,05		64,00	19,68		10,
Monoinsaturated aldehyde	**(Z)-6-undecenal**	60671-73-0	168,2796	0,0454	8,78	6,91	*	80,22	39,12		12,
Terpenoid aldehyde	**Citral**	5392-40-5	152,2367	0,2000	0,89	4,41		85,67	35,84		17,
Monoterpenoid aldehyde	**β-ciclocitrale**	432-25-7	152,2370	0,1760	0,44	4,40		98,25	34,25		16,
Monoterpenoid ester	**Geranyl acetate**	105-87-3	196,2898	0,0260	2,11	3,41		30,78	13,49		2,4
Monoterpenoid ether	**1,8-cineol**	470-82-6	154,2527	1,9000	4,89	5,86		83,33	43,08		8,5
Monoterpenoid ether	**Limonene oxide**	203719-54-4	152,2367	0,5150	17,56	4,83	*	93,11	36,22		9,2
Monoterpene ether	**Linalool oxide**	60047-17-8	170,2519	0,0210	36,00	18,84	*	62,44	29,39		1,6
Monoterpene	**α-pinene**	80-56-8	136,2375	4,7500	7,22	11,66		94,11	38,08		9,4
Monoterpene	**β-pinene**	18172-67-3	136,2375	2,3990	3,67	11,08		74,00	46,09		10,
Sesquiterpene	**Valencene**	4630-07-3	204,3563	0,0110	7,00	6,85	*	70,11	50,34		9,6
Monoterpene	**β-myrcene**	123-35-3	136,2375	2,2900	1,78	4,78		60,67	38,84		6,0

Monoterpene	**D-limonene**	5989-27-5	136,2375	0,1980	0,56	6,87		78,22	43,73		10,
Monoterpene	**L-limonene**	5989-54-8	136,2375	1,5410	8,22	5,85	*	85,33	40,97		8,9
Terpene alcohol	**R-linalool**	126-91-0	154,2527	0,0910	73,67	35,69	*	66,56	34,13		18,
Terpene alcohol	**S-linalool**	78-70-6	154,2527	0,0160	115,56	39,84	*	67,78	29,02		15,
Monoterpene alcohol	**R-α-terpineol**	7785-53-7	154,2527	0,0280	99,67	37,30	*	101,11	32,06		11,
Monoterpene alcohol	**S-α-terpineol**	7785-53-7	154,2527	0,0280	51,33	18,91	*	73,44	28,06		9,2
Monoterpenoid alcohol	**Citronellol**	106-22-9	156,2686	0,0200	4,67	6,77		59,44	23,86		9,7
Monoterpenoid alcohol	**Geraniolo**	106-24-1	154,2527	0,0210	4,89	5,65		65,67	16,64		3,6
Monoinsaturated alcohol	**(Z)-3-hexenol**	928-96-1	100,1608	1,0390	14,33	11,18	*	107,78	25,00		18,
Monoinsaturated alcohol	**(E)-2-hexenol**	928-95-0	100,1608	0,8730	6,11	11,29		122,22	23,86	*	28,
Aromatic alcohol	**4-methylphenol**	106-44-5	108,1400	0,1100	-0,67	2,98		110,89	24,67		1,3
Aliphatic alcohol	**3-octanol**	589-98-0	130,2307	0,5120	62,33	38,33	*	109,89	20,84	*	67,
Aliphatic alcohol	**Decanol**	112-30-1	158,2845	0,0085	2,33	6,99		61,56	20,68		10,
Polyinsaturated aliphatic alcohol	**Farnesol**	4602-84-0	222,3714	0,0004	1,00	7,89		61,56	45,13		4,8
Monoterpenoid chetone	**R-carvone**	99-49-0	150,2208	0,1600	21,67	8,79	*	134,44	24,31	*	42,
Monoterpenoid chetone	**S-carvone**	99-49-0	150,2208	0,1600	9,33	6,34	*	117,78	14,42	*	18,
Aliphatic Chetone	**2-heptanone**	110-43-0	114,1878	4,7320	23,11	17,39	*	85,44	29,78		16,
Lactone	**δ-decalactone**	780-86-2	170,2519	0,0020	2,44	7,14		78,11	38,80		8,9
Lactone	**γ-δ-dodecalactone**	713-95-1	198,3057	0,0020	1,00	6,94		77,78	47,15		9,1
Alkene	**(E,E)-α-farnesene**	502-61-4	204,3563	0,0100	0,89	6,51		68,44	34,51		2,8
Solvent	**Hexane**	110-54-3	86,1776	151,0000	-1,22	5,09	-	68,44	33,66	-	6,0

Table 2 Dose-response characteristics parameters for DsuzOR69a subunits. Comparison of pharmacological parameters for 3-octanol, R-carvone and (Z)-4-nonenal ligands among DsuzOR69a subunits, characterizing hill-3 parameters plots of dose-responses [potency (EC50), Hill coefficient, maximal normalized Δspikes/second-frequencies (Fmax)]. Two normalization methods were adopted: adjustment to 3-octanol vapour pressure and normalization to ab3A spiking from saturating doses; normalization to ab3A spiking from saturating doses of 3-octanol (Figure 2, Additional file 2: Raw Data File 2).

Subunit	Normalization	Parameters	3-octanol	R-carvone	(Z)-4-nonenal
DsuzOR69aA	Adjusted to vapor pressure	EC50 (µg)	7.733 ± 1.909	1.529 ± 0.203	8.465 ± 3.601
		Hill coefficient	0.8866 ± 0.1736	1.138 ± 0.1749	1.157 ± 0.5571
		Fmax	1.107 ± 0.082	0.9807 ± 0.02537	1.038 ± 0.1472
	Saturation to 3-octanol	EC50 (µg)	7.733 ± 1.909	1.555 ± 0.2932	8.281 ± 2.775
		Hill coefficient.	0.8866 ± 0.1736	1.327 ± 0.3112	1.211 ± 0.4912
		Fmax	1.107 ± 0.082	0.4804 ± 0.01756	0.8592 ± 0.0966
Saturation (µg)			50,0	25,0	50,0
DsuzOR69aB	Adjusted to vapor pressure	EC50 (µg)	7.495 ± 2.480	1.788 ± 0.4933	2.901 ± 0.5136
		Hill coefficient	1.032 ± 0.3818	1.359 ± 0.409	1.074 ± 0.1681
		Fmax	0.9795 ± 0.1075	0.9319 ± 0.04635	1.038 ± 0.03855
	Saturation to 3-octanol	EC50 (µg)	7.495 ± 2.480	2.223 ± 0.6643	2.996 ± 0.7922
		Hill coefficient	1.032 ± 0.3818	1.341 ± 0.3775	1.198 ± 0.2865
		Fmax	0.9795 ± 0.1075	1.045 ± 0.05696	1.088 ± 0.0583
Saturation (µg)			100,0	25,0	25,0
DsuzOR69aC	Adjusted to vapor pressure	EC50 (µg)	6.681 ± 0.5721	2.444 ± 0.8927	10.14 ± 3.085
		Hill coefficient	1.588 ± 0.2437	1.606 ± 0.6518	1.312 ± 0.4962
		Fmax	0.9862 ± 0.02832	0,.9591 ± 0.09517	0.9851 ± 0.1059
	Saturation to 3-octanol	EC50 (µg)	6.681 ± 0.5721	2.471 ± 0.8861	9.993 ± 3.047
		Hill coefficient	1.588 ± 0.2437	1.624 ± 0.6498	1.318 ± 0.5061
		Fmax	0.9862 ± 0.02832	0.5908 ± 0.05757	0.9868 ± 0.1062
Saturation (µg)			100.0	10.0	100.0
Adjustment to vapor pressure coefficients				3.691192323	1.385163792

Figures

Figure 1

Functional expression of OR69a subunits and identification of candidate ligands. A. Intron/exon comparison among the DsuzOR69a locus (above) and transcript variants (below) (Additional File 1: Raw Data File 1) generated using the online Exon-Intron Graphic Maker version 4 (http://wormweb.org/exonintron) as we previously reported for other insect transmembrane proteins [76]. Grey rectangles: 5'- and 3'-UTRs; white rectangles: unspliced region (depending on the transcript variant); coloured rectangles: transcript variant-specific exons; black rectangles: common exons; lines: introns; scale bar: 100 bp. **B.** Extracted part of the maximum likelihood phylogenetic tree of DsuzORs (Walker et al. in preparation) representing OR69a sequences of *D. suzukii* and *D. melanogaster*, rooted to Orco. Node support was assessed with 600 bootstrap replicates. **C.** Comparison of basic firing rates of ab3A and ab3B neurons recorded from ab3 sensilla for pUAS-lines (above) and *w;Δhalo;Or22a-Gal4* mutant flies (below). Red bar: 2-heptanone test-stimulus on *w;Δhalo;Or22a-Gal4* mutant flies reporting ab3A-burst phenotype [39]. **D.** Box plot of normalized ab3A spiking from transgenic *D. melanogaster* expressing OR69a subunits, tested with the compound library reported in Table 1. Asterisks indicate compounds enhancing significant difference in spike frequency (Δspikes/0.5sec) when compared with the solvent (Mann Whitney U test: $p<0.01$; two tails, N = [8 - 10]). Colours of asterisks is based on OR69a-subunits: blue, DsuzOR69aA, green: DsuzOR69aB, red, DsuzOR69aC.

Figure 2

Dose-response characteristics of DsuzOR69a-subunits to selected ligands. Effects of 3-octanol, R-carvone and (Z)-4-nonenal on DsuzOR69a-subunits. The ab3A neurons expressing OR69as generated spiking in response to application of the compounds. The effects were concentration dependent and reversible. **A.** Left: graphs of concentration dependences of the compounds expressed as a function of normalized spike frequency [(Δspikes/second)/spikes] versus µg-

doses. Responses were individually adjusted to 3-octanol vapour pressure and normalized to their saturating concentrations (Table 2, Additional file 2: Raw Data File 2). Error bars represent standard error of mean. Data were fit with the Hill equation (solid lines). Right: summary plots based on normalization upon adjustment to vapour pressure and normalization to saturating doses of 3-octanol; different colours depicts different compounds (3-octanol, blue; R-carvone, yellow; (Z)-4-nonenal, Magenta haze). **B.** spike trains of ab3A generated by 10.0 µg dose of the specific stimuli; black bar: stimulus.

Figure 3

GC-SSR of DsuzOR69a-subunits tested to synthetic ligands. A. Spike frequency plot (SSR = 80 Hz, Bin-width = 8.0 s, Filter = 2 Taps; above) associated with ab3A firing for transgenic *D. melanogaster* expressing DsuzOR69aA (N = [4-6]), -aB (N = [5-7]) and aC (N = [5-7]) subunits, tested to a blend of synthetic ligands at 10.0 ng, except for methyl salicylate, 5.0 ng (GC 5.0 mV; Time: sec; below) released at their respective retention times in the following order: 3-octanol, (Z)-4-nonenal, methyl salicylate and R-carvone respectively. Below: box-plot generated upon analysis of spike counting for the effects elicited by the synthetic ligands: blue, DsuzOR69aA; green, DsuzOR69aB; red, DsuzOR69aC; asterisks denote significant effects (Additional File 5: Raw Data File 3). **B.** Spike frequency associated with ab3A firing for a transgenic *D. melanogaster* fly expressing DsuzOR69aB, tested to a blend of the synthetic ligands mentioned above at doses of 1.0 ng. **C.** Spike frequency plot associated to ab3A firing (frequency settings: see A) for four different transgenic *D. melanogaster* flies expressing DsuzOR69aB tested to methyl salicylate at different doses (10 ng; 5.0 ng; 1.0 ng; 0.1 ng).

Figure 4

GC-SSR screening of DsuzOR69a-subunits tested to headspaces collected from *D. suzukii* females. Spike frequency plot (SSR = 80 Hz, Bin-width = 8.0 s, Filter = 2 Taps; above) associated with ab3A firing for transgenic *D. melanogaster* expressing DsuzOR69aA, -aB and aC subunits (N = 3 per subunit), screened to a collection of headspaces from *D. suzukii* female insects of different ages: 2-day-old, top; 4-day-old, middle; 5-day-old, bottom; GC 5.0 mV; Time: sec.

Figure 5

GC-SSR screening of DsuzOR69a-subunits tested to headspaces collected from *Hanseniospora uvarum*. A. Spike frequency plot (SSR = 80 Hz, Bin-width = 8.0 s, Filter = 2 Taps; above) associated with ab3A firing for transgenic *D. melanogaster* expressing DsuzOR69aA, -aB and aC subunits, tested with headspaces collected from *H. uvarum* (GC 5.0 mV; Time: sec; N = [3-5]) already available in our labs, that will be part of additional projects. Asterisks with letters (A1-C1) denote significant ab3A spiking per second (Additional File 5: Raw Data File 3); magnification of spike frequency plots in proximity to active retention times is reported in Additional File 8: Fig. S4. **B.** Box-plot generated upon analysis of spike counting for the effects elicited by the retention times indicated in A and Additional File 8: Fig. S4; asterisks denote significant effects (Additional File 5: Raw Data File 3). Blue, DsuzOR69aA (two-tailed paired T-test [□=0.05], N = 3: RT 14.953 [A1], p = 0.0373320); green, DsuzOR69aB (N = 5: RT 4.769-4.800 [B1], p = 0.030327506; RT 7.221 [B2], p = 0.02227182; RT 8.562 [B3], p = 0.014788452; RT 11.677 [B4], p = 0,019825885); red, DsuzOR69aC (N = 4: RT 14.953 [C1], p = 0.015551882).

Figure 6

Fluorescent *in situ* hybridization of DsuzOR69a subunits. A. Left: male (♂) and female (♀) antennae comparing bright field, Cy5 and merged channels for DsuzOR69aA (N_{male} = 11, N_{female} = 10), aB (N_{male} = 11, N_{female} = 10) and aC (N_{male} = 13, N_{female} = 9). Right: box-plots generated upon analysis of neuronal counting. Note: no significant differences in terms of the number of neurons were validated for the staining for each different transcript. **B.** Left: positive control DsuzOrco using a fluorescent probe stained with Fluo (laser: 488), comparing bright field, 488 and merged channels. Below, right: negative control DsuzIR60b using a fluorescent probe stained with DIG (laser: Cy5), comparing bright field and Cy5. **C.** Schematic representation of neuronal distribution on *D. suzukii* antenna: blue, DsuzOR69aA, green: DsuzOR69aB, red, DsuzOR69aC.

Supplementary Files

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