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# Functional transcriptome analyses of *Drosophila suzukii* antennae reveal mating-dependent olfaction plasticity in females

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ABSTRACT

Insect olfaction modulates basal behaviors and it is often influenced by the physiological condition of each individual such as the reproductive state. Olfactory plasticity can be achieved by modifications at both peripheral and central nervous system levels. Here we performed a genome-wide transcriptomic analysis of the main olfactory organ, the antenna, to investigate how gene expression varies with female mating status in *Drosophila suzukii*, a destructive and invasive soft fruit pest. We observed a wide mating-induced up-regulation of chemosensory-related genes in females, especially odorant receptor (*Or*) genes. We then used a candidate gene approach to define the comprehensive dataset of antenna-expressed chemosensory receptors and binding proteins, which showed many similarities with *Drosophila melanogaster*. Candidate gene approach was also used to finely quantify differential expression at *Or* isoform level, suggesting post-mating transcriptional modulation of genes involved in the peripheral olfactory system. We identified 27 up-regulated *Or* transcripts encoded by 25 genes, seven of them were duplications specific to *D. suzukii* lineage. Post-mating olfactory modulation was further supported by electroantennogram recordings that showed a differential response according to mating status to one out of eight odors tested (isoamyl-acetate). Our study characterizes the transcriptional mechanisms driven by mating in *D. suzukii* female antennae. Understanding the role of genes differentially expressed in virgin or mated females will be crucial to better understand host finding and the crop-damaging oviposition behavior of this species.

## 1. Introduction

Chemoreception is a fundamental process for animals. In insects, the ability to respond to environmental chemical stimuli drives all the basic behaviors such as feeding, mating, predator avoidance and oviposition site choice (Hodgson, 1958). Mated females largely rely on the sense of smell to locate sites for egg-laying. They recognize specific odor signals with extraordinary sensitivity and specificity in a multi-component odor environment to find oviposition sites that support the development and fitness of their offspring (Renwick, 1989).

To detect olfactory signals and cues, insects depend on specialized structures called olfactory sensilla, which are located on the antennae and the maxillary palps. These appendages house olfactory sensory neurons (OSNs), whose dendrites stretch through the sensilla and express trans-membrane chemosensory receptors that detect volatile compounds. Each OSN expresses only one type or a very small combination of receptors. These mainly belong to the odorant receptor (OR) or ionotropic receptor (IR) families, whereas only a few gustatory receptors (GRs) are expressed in OSNs (Joseph and Carlson, 2015). ORs are always expressed in combination with the ubiquitous co-receptor Or83b, called Orco (odorant receptor co-receptor). Other classes of proteins involved in volatile recognition are the odorant binding proteins (OBPs) and the chemosensory proteins (CSPs), which are responsible for the transport of incoming odorants through the lymph of chemosensilla to their corresponding receptors, and the odorant-degrading enzymes (ODEs) that rapidly inactivate volatiles after binding to the receptors (Leal, 2013). Recognition of volatile compounds generates action potentials which transmit the signal to the primary olfactory center of the brain, the antennal lobe, and subsequently to the higher brain centers (Galizia and Sachse, 2010).

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Insect's responses to environmental stimuli must meet the physiological requirements of each individual: for example, foraging and reproductive needs of mated females are different from those of unmated insects. Hence, response to odors in a complex multi-odor environment must be coordinated with the organism's physiological state (Gadenne et al. 2016). This can be accomplished by modulation of the olfactory system to match response to odorants and physiological changes (Anton et al., 2007; Gadenne et al., 2016; Kromann et al., 2015). Generally, mating-dependent olfactory plasticity decreases female response to sex-attractants whereas responses to oviposition site cues are switched on. In the parasitic wasp *Nasonia vitripennis*, mated females are no longer attracted by male pheromones (Lenschow et al. 2018; Ruther et al. 2010, 2007; Steiner and Ruther, 2009). In the Mediterranean fruit fly, *Ceratitis capitata*, mated females become attracted to host fruit instead of male-emitted pheromones (Jang, 1995; Jang et al. 1999). A female enhanced behavioral response to oviposition cues after mating has been shown in the mosquito *Aedes aegypti* (Fernandez and Klowden, 1995; Naccarati et al. 2012) and in two species of moth (Martel et al. 2009; Masante-Roca et al. 2007; Saveer et al. 2012). Modulation of the olfactory responses may happen at both peripheral and at central nervous system level (Gadenne et al., 2016). In *Drosophila melanogaster*, post-mating modulation to high levels of poly-amines is triggered at the peripheral level of the olfactory system by the sex peptide receptor (SPR) and its neuropeptide ligands, and it is achieved by increased expression of SPR in the specific OSNs tuned to poly-amine odor recognition (Hussain et al. 2016). Olfactory post-mating changes are modulated at peripheral level also in *Spodoptera littoralis* females (Martel et al. 2009; Saveer et al. 2012). Vice versa, peripheral olfactory response of the lepidopteran *Pieris rapae* did not show post-mating changes, suggesting that in this species post-mating behavioral plasticity was modulated by the central nervous system (Itoh et al. 2018).

*Drosophila suzukii* is an Asiatic *Drosophila* species that invaded Europe and Americas, with a severe impact on commercial soft fruit production (Asplen et al., 2015; Cini et al., 2012; Rota-Stabelli et al., 2013). Egg-laying behavior of *D. suzukii* females diverges from the majority of *Drosophila* species, which oviposit on decaying substrates, since it is able to infest also healthy and undamaged ripening fruits thanks to a sclerotized and serrated ovipositor that can pierce intact fruit skin (Atallah et al., 2014). Attraction to fresh and ripe fruits has been associated with modification of olfactory system, since silencing of Orco-expressing neurons revealed that oviposition elicited by ripe fruit volatiles is an Orco-mediated olfactory process (Karageorgi et al., 2017). *D. suzukii* is attracted also by fermenting substrates (Mori et al., 2017; Scheidler et al., 2015; Wong et al., 2018), which may serve also as potential off-season oviposition sites (Bal et al., 2017). Considering the essential necessity of yeast for *D. melanogaster* and other drosophilids as well as the reported attraction to sweet baits, we assume that *D. suzukii* mainly uses fermenting substrates as food resource and fresh soft fruits as preferential oviposition substrates (Cloonan et al., 2018). This variety of behavioral patterns led us to hypothesize that mating modifies olfactory perception of *D. suzukii* females, leading to a shift in odorant response to match reproductive needs. To test the hypothesis, we used RNA-seq to look for transcriptional differences in antennae of mated and virgin females. Our results identified several differentially expressed genes that belong to the peripheral olfactory pathways. To test if differences in transcript abundances could affect the physiology, we measured antennal response of virgin and mated females to a panel of relevant odors. Our results suggest that mating modulates the olfactory response of *D. suzukii* at the peripheral nervous system level, and the differential expression of chemosensory-related genes may account for this shift.

## 2. Materials and methods

### 2.1. Insect rearing

*D. suzukii* individuals used in all the experiments were taken from a laboratory colony established in 2010 in Fondazione Edmund Mach from wild insects collected in Trento province (Italy) and maintained at laboratory conditions with periodical refreshments with insects caught from the same field sites. Flies were reared on a standard diet (*Drosophila* species stock center, https://stockcenter.ucsd.edu/info/food_cornmeal.php) and maintained at 23–25 °C, 65 ± 5% relative humidity and under a 16:8 h light:dark photoperiod. Three days-old flies were used for all the experiments. To control for mating state of females, newly emerged unmated *D. suzukii* were collected in the morning, anaesthetized with $CO_2$ and sorted by sex. To obtain virgin experimental flies, sexed females were kept in isolation in standard *Drosophila* vials (VWR, Radnor, PA US) filled with 3 cm of diet (ten females per vial) for three days. Mated females were obtained by keeping flies of both sexes together for three days (ten females and ten males per vial). To confirm that females were mated, we took randomly twenty females and we placed them individually in a single vial to control for oviposition and larvae hatching. All the twenty mated females laid fertile eggs.

### 2.2. RNA extraction and sequencing

RNA was extracted from third antennal segments of virgin or mated females. Dissected tissues were stored at −80 °C in RNAlater (ThermoFisher Scientific, Waltham, MA USA) until RNA extraction. Three independent biological replicates were collected per mating status, and each replicate was composed of RNA extracted from around 300 antennal segments. Antennae were homogenized using TissueLyser (Qiagen, Hilden, Germany) and total RNA was extracted with TRIzol reagent (ThermoFisher Scientific), following the manufacturer's protocol. DNA contamination was removed with a DNase I (ThermoFisher Scientific) incubation step. A second RNA extraction with PureLink™ RNA Mini Kit (ThermoFisher Scientific) was performed to remove DNase and concentrate samples. Total RNA (~0.5 μg/sample) was sent to Genewiz (South Plainfield, NJ USA) for library preparation and Illumina sequencing. Library preparation was carried out through polyA + selection and paired-end (PE) sequencing was run on an HiSeq 2500 System with V4 chemistry that generated 100 bp reads. Raw reads are accessible at the Genbank SRA database (BioProject accession number PRJNA490636) (Supplementary Table S1).

### 2.3. Whole genome differential expression analysis

Raw reads were trimmed by Trimmomatic (Bolger et al., 2014) to remove low quality reads and adapter sequences, and they were mapped to the *D. suzukii* reference genome (version 1.0) (Chiu et al., 2013) using Bowtie2 (v2.2.5)-based Tophat (v2.1.0) with –-no-novel-juncs flag (Kim et al., 2013). Only proper PE reads after trimming were used in the mapping (Supplementary Table S1). Annotated count matrix was obtained with htseq-count (Anders et al., 2014) and used as input in the Bioconductor package EdgeR (McCarthy et al., 2012) to estimate differentially expressed (DE) genes. Counts were filtered to retain transcripts with more than 1 count per million in at least three replicates, and then normalized with the default TMM-method (Trimmed Mean of *M*-values). Trended dispersion was estimated, and the data were fitted to a negative binomial generalized linear model. Differences between mating status were estimated with likelihood ratio tests. Genes were considered significantly DE when the false discovery rate (FDR) was lower than 0.05.

Genes tested for differential expression were annotated using Standalone Blast+ (Altschul et al., 1990). Blast searches were run using the command Blastp specifying the predicted proteins from the *D.*

*melanogaster* genome (version r6.13) (Marygold et al., 2013) as the database and the predicted proteins from *D. suzukii* genome (version 1.0) as the query. The top best hit for each sequence was retained when the E-value was less than $1 \times 10^{-5}$. PANTHER version 13.1 was used to run over-representation test analysis of gene ontology (GO) terms to identify the most significant up- and down-regulated processes (Mi et al., 2017).

### 2.4. Candidate gene approach

We used the dataset of manually curated annotations for *Or*, *Gr*, *Ir*, *Obp* and *Csp* genes (Crava et al., 2016; Ramasamy et al., 2016) to estimate a reliable expression of each isoform. Proper PE trimmed reads were mapped to the annotated isoforms using Bowtie v1.0.0 (Langmead et al., 2009) and the read counts were estimated by RSEM v1.2.20 (Li and Dewey, 2011). Counts were first normalized by the total mapped reads for each library to obtain the reads per million reads mapped (RPM) for each gene. Genes were defined as antenna-expressed if they had ≥1 RPM in at least three samples. RPM were then normalized by gene length to obtain the fragment per kilobase per million reads (FPKM). Results were visualized with heatmap.2 implemented in package gplots (R Core Team, 2015). To identify *Or* isoforms differentially expressed between mating status, we used the Welch *t*-test to compare FPKM from mated and virgin females for each *Or* gene considered to be expressed in the antenna. Differential expression was considered statistically significant when $p < 0.05$. DE *Or* genes were then ranked by fold change and the expression variations of the top *Or* genes were confirmed by reverse transcription quantitative PCR (RT-qPCR).

### 2.5. Reverse transcription quantitative PCR

DNase-treated RNA was directly used in one-step RT-qPCR using the KAPA SYBR FAST One-step qRT-PCR Kit (KAPA Biosystems, Wilmington, MA USA). Total RNA was quantified with Qubit (Thermofisher) and 1 ng was used for each one-step reverse transcription and real-time PCR reaction. Each 20 μl reaction was composed by 10 μl of 2X KAPA SYBR FAST qPCR Master Mix, 0.4 μl of 50X KAPA RT Mix, 1 μl RNA, 4 μl of primer mix (1 μM each) and 4.6 μl $H_2O$. The one-step RT-qPCR reaction was carried out on a LightCycler 480 (Roche, Basel, Switzerland) and consisted of a reverse transcription step (5 min at 42 °C and 3 min at 95 °C), an amplification step (40 cycles of 95 °C for 10 s, 60 °C for 20 s, and 72 °C for 1s) and a final dissociation analysis. To control for genomic DNA contamination, each batch of total RNA underwent a parallel mock reverse transcription step in which the reverse transcriptase was omitted. Expression levels were calculated as Δct (Δct = ct[reference genes] - ct[gene of interest]) using the mean of two housekeeping genes as ct[reference genes]. ΔΔct was calculated as mean(Δct[virgin]) - mean(Δct[mated]). Primers used are listed in Supplementary Table S2. We tested expression of nine of the top DE *Or* genes ranked for fold-change (FC). We excluded from the analysis *Or23a3* and *Or49a2* (the first and the seventh *Or* genes, respectively, from the fold change ranking) because their expression was detected at cycle threshold (Ct) higher than 30.

### 2.6. Chemicals

Compounds used in electroantennography were all greater than 98% pure with exception of β-cyclocitral that was ≥95% pure. Isopentyl acetate (CAS number 123-92-2, Sigma-Aldrich, St. Louis, MI USA) (referred hereinafter as isoamyl acetate, IAA), methyl hexanoate (106-70-7, SAFC, Madison, WI USA), ethyl hexanoate (123-66-0, Sigma-Aldrich), β-cyclocitral (432-25-7, Sigma-Aldrich), (Z)-3-hexen-1-ol (928-96-1, Sigma-Aldrich), acetic acid (64-19-7, Sigma-Aldrich), ethanol (64-17-5, Honeywell, Morris Plains, NJ USA) and (−)-linalool (126-91-0, Fluka, Buchs, Switzerland).

### 2.7. Electroantennography (EAG)

Electrodes were composed of a silver wire inserted into a pulled glass capillary filled with Kaissling solution [NaCl (7.5 $gL^{-1}$); $CaCl_2$ (0.21 $gL^{-1}$); KCl (0.35 $gL^{-1}$); and $NaHCO_3$ (0.2 $gL^{-1}$l)] (Kaissling and Thornson, 1980). Stimuli were prepared as ten-fold dilutions in hexane and doses between 25 ng and 2.5 μg of each were applied in a volume of 25 μl on pieces of filter paper (Whatman, Maidstone, UK). Five minutes later, after evaporation of the solvent, the filter papers loaded with the individual stimuli, were separately inserted into Pasteur pipettes. Similarly, filter paper treated with hexane only was prepared for control. Pipettes were wrapped with parafilm and kept at 4 °C while not in use. No pipette was kept for longer than two hours after preparation and generally one pipette was used for testing two to three individual insects. Prior the start of the experiment, a single *D. suzukii* was anaesthetized in ice for few minutes and the head was excised with a scalpel. The reference electrode was placed at the base of the head and the recording electrode was firmly put into contact with the tip of the antenna using MP22 micromanipulators (Syntech, Buchenbach, Germany). Only one randomly chosen antenna for each insect was used. A humidified stream of charcoal filtered air was continuously passed over the fly head and individual stimuli were delivered into this stream as a 400 ms puff through a stimulus controller CS 55 (Syntech). EAG responses were amplified and recorded by an IDAC-2 acquisition controller (Synthech). A stimulus series consisted of three concentrations applied in ascending order. The recovery time between two consecutive stimuli was 1 min. From each antenna we recorded responses to two concentration series (two compounds per antenna).

EAG responses were analyzed by the EAG2000 software version 2.7 (Syntech). Absolute responses were normalized to the control. All statistical analyses were performed with the computing environment R (R Core Team, 2015). Data were log transformed and Levene's test for homogeneity of variance was performed. Parametric data were compared with two-way repeated measures ANOVA (TW-RM-ANOVA) from package car. If variance was not homogeneous following transformation, data were compared by generalized least squares (GLS) from package nlme.

## 3. Results

### 3.1. Whole genome expression patterns

Transcriptional patterns of genes expressed in the third antennal segment of *D. suzukii* females clustered by mating status (Fig. 1A). Differential expression analysis revealed 1893 genes up-regulated and 2116 genes down-regulated in mated females with false discovery rate (FDR) $< 0.05$ (Fig. 1B, Supplementary Dataset S1). Fold change were small for the majority of DE genes: when the threshold was set to two, up- and down-regulated genes decreased to 25 and 330, respectively. GO over-representation analysis was performed with Panther and grouped genes with similar GO annotation terms to identify functions associated with differences in mating status. Among genes up-regulated in mated females, we found over-representation of GO terms related to structural constituent of ribosome (Molecular Function), ribosomes, membrane and mitochondrial inner membrane (Cellular Component), oxidative phosphorylation and sensory perception of smell (Biological Process) (Fig. 1C, Supplementary Table S3). Among genes down-regulated, we found over-representation of GO terms associated with kinase activity and protein binding (Biological Process) (Supplementary Table S4). Over-represented GO terms related to sensory perception of smell (and to the correspondent sensory perception of stimulus and sensory perception) (Supplementary Fig. S1) among up-regulated genes were mirrored by an under-representation of GO terms associated to sensory perception of stimulus in down-regulated genes (Supplementary Table S4). This expression pattern suggests a shift in chemosensory perception that matches female physiological state. To better understand these

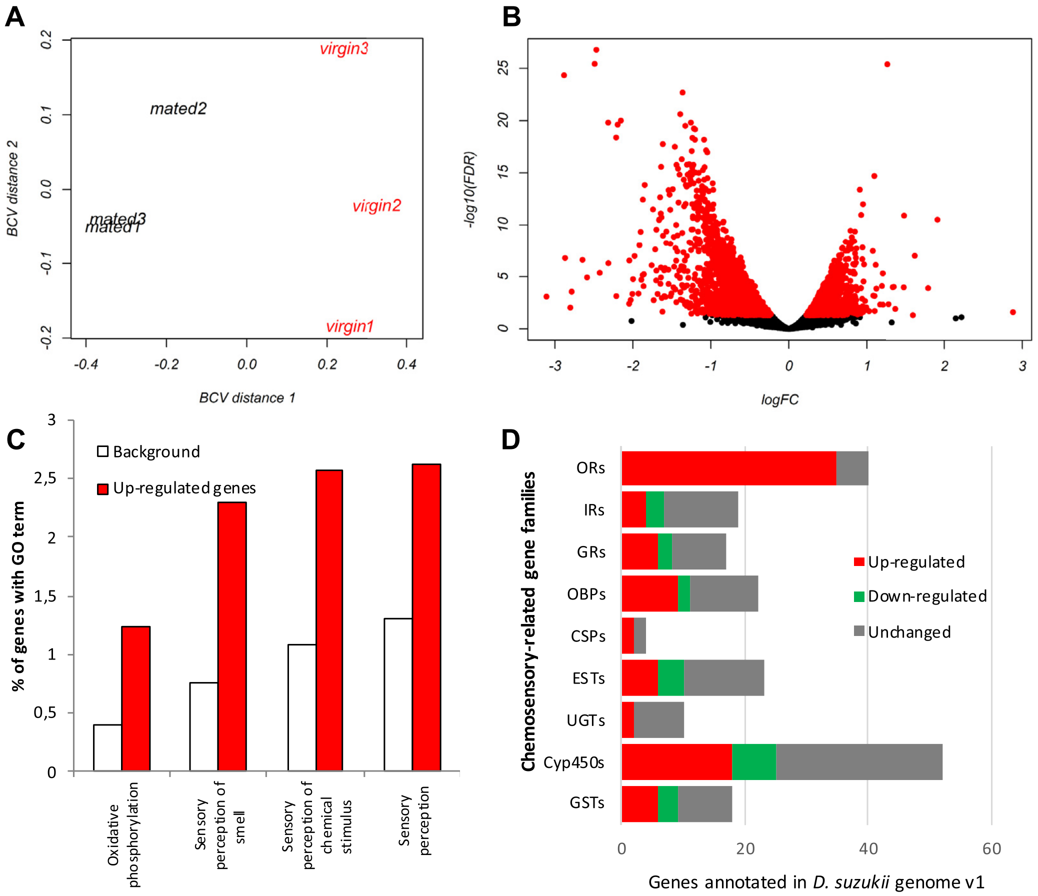

**Fig. 1.** Mating alters gene expression patterns in the *Drosophila suzukii* third antennal segment. A) Multidimensional scaling plot showing that replicates are separated according to mating status (BCV, biological coefficient of variation). B) Vulcano plot highlighting differential expressed genes (red dots) at false discovery rate (FDR) $< 0.05$, (FC, fold-change). C) Bar plot showing frequencies of Gene Ontology (GO) terms (Panther-GO slim Biological process) for functions over-represented among genes up-regulated in mated females. D) Bar plot showing the number of up-regulated, down-regulated and unchanged genes after mating for each family of chemosensory-related genes: chemosensory receptors (odorant receptors, ORs; ionotropic receptors, IRs; gustatory receptors, GRs), binding proteins (odorant binding proteins, OBPs; chemosensory binding proteins, CSPs) and odorant-degrading enzymes (esterases, ESTs; UDP-glycosyltransferases, UGTs; cytochrome P450, Cyp450s; glutathione-S-transferases, GSTs). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

changes, we manually inspected transcription of genes encoding chemosensory receptors, binding proteins and ODEs, to find genes DE between mating status. We found that expression of *Or* genes was largely increased in mated females: 35 genes were up-regulated (corresponding to the 87.5% *Or* genes tested for differential expression in whole genome analysis) whereas no *Or* was down-regulated and only five were unchanged (Fig. 1D). A less dramatic but still broad up-regulation was observed in mated females also for the other gene families analyzed (GRs, IRs, OBPs, CSPs, esterases, cytochromes p450, UDP-glucuronosyltransferase and glutathione-S-transferases). In all of them, up-regulated genes were more numerous than down-regulated but less than unchanged ones (up-regulated genes ranged from 21 to 50% the total of antenna-expressed genes from the same family) (Fig. 1D). These results suggested an overall post-mating up-regulation of the transcription of genes involved in peripheral chemosensory perception. The largest up-regulation of *Or* genes in mated females prompted a more detailed analysis of their expression profiles.

### 3.2. Candidate gene approach

#### 3.2.1. Chemoreceptor repertoire of D. suzukii antennae

The expression of transcripts coding for chemoreceptors in the third antennal segment was profiled by mapping reads to the manually curated annotations of *D. suzukii Or*, *Gr* and *Ir* genes (Crava et al., 2016; Ramasamy et al., 2016). Only expression detected at ≥ 1 RPM in at least three samples was considered biologically relevant and those genes were included in the set of the antenna-expressed genes (Supplementary Dataset S2).

We detected expression of 47 transcripts encoded by 43 *Or* genes (Fig. 2A). The most expressed transcript codified for Or83b, the olfactory receptor co-receptor (Orco). It was expressed at ~1000 FPKM whereas the expression of the other *Or* genes ranged from 1 to 207 FPKM. *Orco* expression was 46-fold higher than the mean level of the other 47 *Or* transcripts (22 FPKM) supporting a 1:1 stoichiometry as suggested in *D. melanogaster* (Menuz et al., 2014). Comparison of *D. suzukii Or* gene expression with that of their ortholog in *D. melanogaster* revealed a clear correlation between the two species: of the 34 *D. melanogaster* genes whose expression had been mapped on the third antennal segment both by transgenic fly lines expressing GFP (Fishilevich and Vosshall, 2005; Laissue and Vosshall, 2008) and transcriptomics (Menuz et al., 2014), only *Or33a, Or33c* and *Or85b* were not expressed in *D. suzukii* (the latter is pseudogenized in *D. suzukii* genome, Ramasamy et al., 2016). Of the 12 genes unique to *D. suzukii* lineage (Ramasamy et al., 2016), eight were expressed in the third antennal segment (*Or19a2*, *Or23a2*, *Or23a3*, *Or49a2*, *Or65c2*, *Or67a2*, *Or67a3* and *Or67a4*). No genes ortholog to those mapped to maxillary

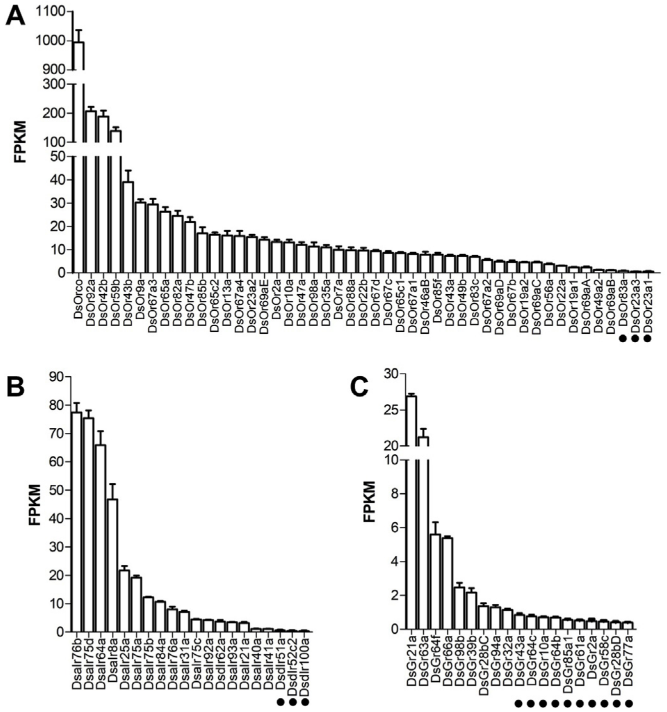

**Fig. 2.** Chemosensory receptor gene repertoire of *Drosophila suzukii* third antennal segment. Bar plots showing expression levels of genes encoding chemosensory receptors: A) Odorant receptors (Ors); B) Ionotropic receptors (Irs: antennal Irs, aIrs; divergent Irs, dIrs); C) Gustatory receptors (Grs). Black dots highlight transcripts expressed less than 1 FPKM (fragments per kilobase per million mapped reads). Bars represent average (N = 6) ± SEM.

palp OSNs in *D. melanogaster* (Laissue and Vosshall, 2008) or specific to larval OSNs (Fishilevich et al., 2005; Kreher et al., 2005) were found in the *D. suzukii* antennal transcriptome, with the exception of *Or46 aB* and *Or83a*. Correlation between *Or* gene expression in *D. suzukii* and *D. melanogaster* also emerged from the comparison of normalized expression levels (Supplementary Fig. 2S).

Expression of twenty *Ir* genes was detected in *D. suzukii* antennae (Fig. 2B). Sixteen were orthologs to the antennal *Ir* (*aIr*) genes of *D. melanogaster* (Croset et al., 2010; Menuz et al., 2014) and their expression varied from 1 to 78 FPKM. The remaining four *Ir* genes belonged to the group of the divergent *Ir* (*dIr*) genes (*dIr62a*, *dIr51a*, *dIr52c2*, *dIr100a*) (Croset et al., 2010; Koh et al., 2014). Of these, three were expressed at lower level than the least expressed *aIr* gene (less than 1 FPKM) whereas *dIr62a*, which has been found expressed also in *D. melanogaster* antenna (Menuz et al., 2014), was expressed at ~ 4 FPKM.

Nineteen *Gr* genes met our formal criteria for expression in the third segment of *D. suzukii* antennae (Fig. 2C). Of them, ten were expressed less than 1 FPKM (in comparison, three *Or* and no *aIr* genes were transcribed at less than 1 FPKM). The other *Gr* gene expression ranged from 1 to 6 FPKM with the exception of *Gr21a* and *Gr63a*, which were expressed at high levels (more than 20 FPKM).

### 3.2.2. Binding proteins repertoire of D. suzukii antenna

Odorant-binding proteins (OBPs) and chemosensory proteins (CSPs) are two classes of proteins identified in the lymph of insect chemosensilla. *Obp* genes are grouped in a wide family which is composed by 53 members in *D. suzukii* (Ramasamy et al., 2016). Overall, candidate gene approach detected expression of 30 *Obp* genes in *D. suzukii* third antennal segments, which could be clustered in two groups: 19 were expressed at higher expression level (from 19 to 3494 FPKM) whereas other 11 at lower level (from 0.6 to 2.6 FPKM) (Fig. 3A). *Csp* genes belong to a smaller gene family with only four members in *Drosophila* species (Ramasamy et al., 2016). All of them were expressed in the *D. suzukii* third antennal segment, although *Csp2* and *Csp3* were expressed around 1350-fold more than *Csp1* and *Csp4* (Fig. 3B).

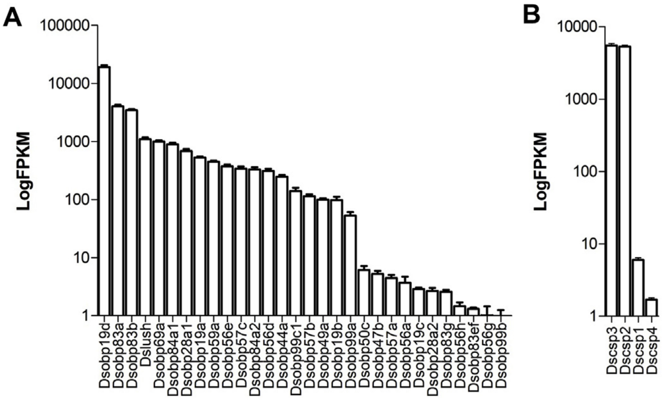

**Fig. 3.** Binding protein gene repertoire of *Drosophila suzukii* third antennal segment. Bar plots showing expression levels of genes encoding biding proteins: A) Odorant binding proteins (Obps); B) Chemosensory proteins (Csps). FPKM: fragments per kilobase per million mapped reads. Bars represent average (N = 6) ± SEM.

### 3.2.3. Odorant receptors up-regulated in the antennae of mated D. suzukii females

To finely identify *Or* isoforms which were differentially expressed in mated *D. suzukii* females, we used a candidate gene approach based on the manually curated annotation of *D. suzukii Or* genes. Our candidate gene approach yielded more reliable quantifications of gene expression levels for manually annotated genes than the whole genome approach that used an automatic annotation (Chiu et al., 2013). We identified 27 transcripts encoded by 25 genes which were up-regulated in mated females at $p < 0.05$ (Fig. 4A). Among them seven were paralogs duplicated in *D. suzukii* (*Or23a2*, *Or23a3*, *Or49a2*) or in *D. suzukii/Drosophila biarmipes* lineage (*Or67a2*, *Or67a3*, *Or67a4*, *Or65c2*) and thus suspected to play a role in relaxed preference for fresh fruit egg-laying behavior (Karageorgi et al., 2017; Ramasamy et al., 2016). We then chose nine of the top up-regulated genes to test their expression by RT-qPCR. Results were consistent with RNA-seq and confirmed the small fold-change between mating status (Figs. 4B and Supplementary Fig. 3S). Of the nine genes tested, Welch-t test identified six genes whose differential expression remained statically significant: *Or7a*, *Or13a*, *Or42b*, *Or46 aB*, *Or85b* and *Or67a4*.

### 3.3. Electroantennogram recordings

We recorded electroantennogram responses of mated and virgin females to a panel of eight odors relevant for *D. suzukii* biology (Fig. 5). Responses of mated females were higher than those of virgin females for isoamyl acetate (IAA) (TW-RM-ANOVA on log-transformed data: concentration $F_{2,54} = 0.084$, $p = 0.919$; mating status $F_{1,54} = 14.363$, $p < 0.001$; mating status * concentration: $F_{2,54} = 0.237$, $p = 0.79$) whereas there was a marginally significant tendency showing a higher response in virgin females for acetic acid (TW-RM-ANOVA on log-transformed data: concentration $F_{2,54} = 0.59$, $p = 0.558$; mating status $F_{1,54} = 4.097$, $p = 0.048$; mating status * concentration: $F_{2,54} = 0.664$, $p = 0.519$). There were no differences for (−)-linalool (TW-RM-ANOVA on log-transformed data: concentration $F_{2,54} = 0.006$ $p = 0.994$; mating status $F_{1,54} = 6.447$, $p = 0.425$; mating status * concentration: $F_{2,54} = 0.043$, $p = 0.958$), for methyl hexanoate (TW-RM-ANOVA on log-transformed data: concentration $F_{2,54} = 0.099$ $p = 0.906$; mating status $F_{1,54} = 0.233$, $p = 0.631$; mating status * concentration: $F_{2,54} = 0.021$, $p = 0.979$), ethyl hexanoate (TW-RM-ANOVA on log-transformed data: concentration $F_{2,54} = 0.727$

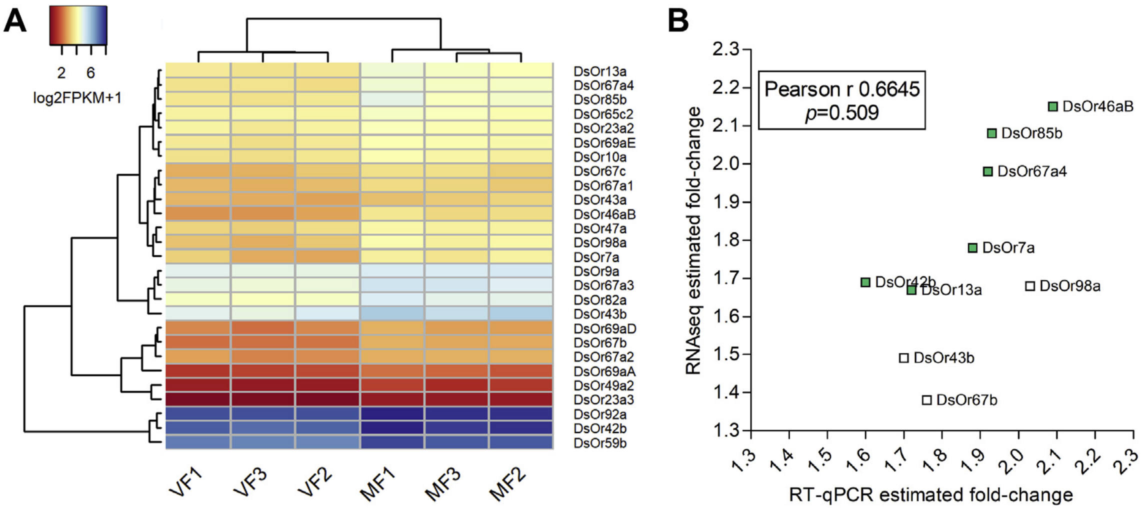

**Fig. 4.** Odorant receptors are differently expressed according to *Drosophila suzukii* female mating status. A) Differentially expressed odorant receptors (Ors) identified by RNA-seq candidate gene approach (VF = virgin females, MF = mated females). B) Scatter plot showing the correlation between gene expression fold-change estimated by RNA-seq candidate gene approach and RT-qPCR for nine *Or* genes. Solid boxes depict *Or* genes found differentially expressed by both RT-qPCR and RNA-seq.

$p = 0.488$; mating status $F_{1,54} = 2.115$, $p = 0.152$; mating status * concentration: $F_{2,54} = 0.039$, $p = 0.962$), (Z)-3-hexen-1-ol (TW-RM-ANOVA on log-transformed data: concentration $F_{2,54} = 0.150$, $p = 0.861$; mating status $F_{1,54} = 0.002$, $p = 0.963$; mating status * concentration: $F_{2,54} = 0.679$, $p = 0.512$) β-cyclocitral (TW-RM-ANOVA on log-transformed data: concentration $F_{2,54} = 5.767$ $p = 0.005$; mating status $F_{1,54} = 1.921$, $p = 0.171$; mating status * concentration: $F_{2,54} = 1.31$, $p = 0.878$) and ethanol (GLS: concentration $F_2 = 3.47$, $p = 0.038$; mating status $F_1 = 1.588$, $p = 0.213$; mating status * concentration: $F_2 = 1.452$, $p = 0.243$).

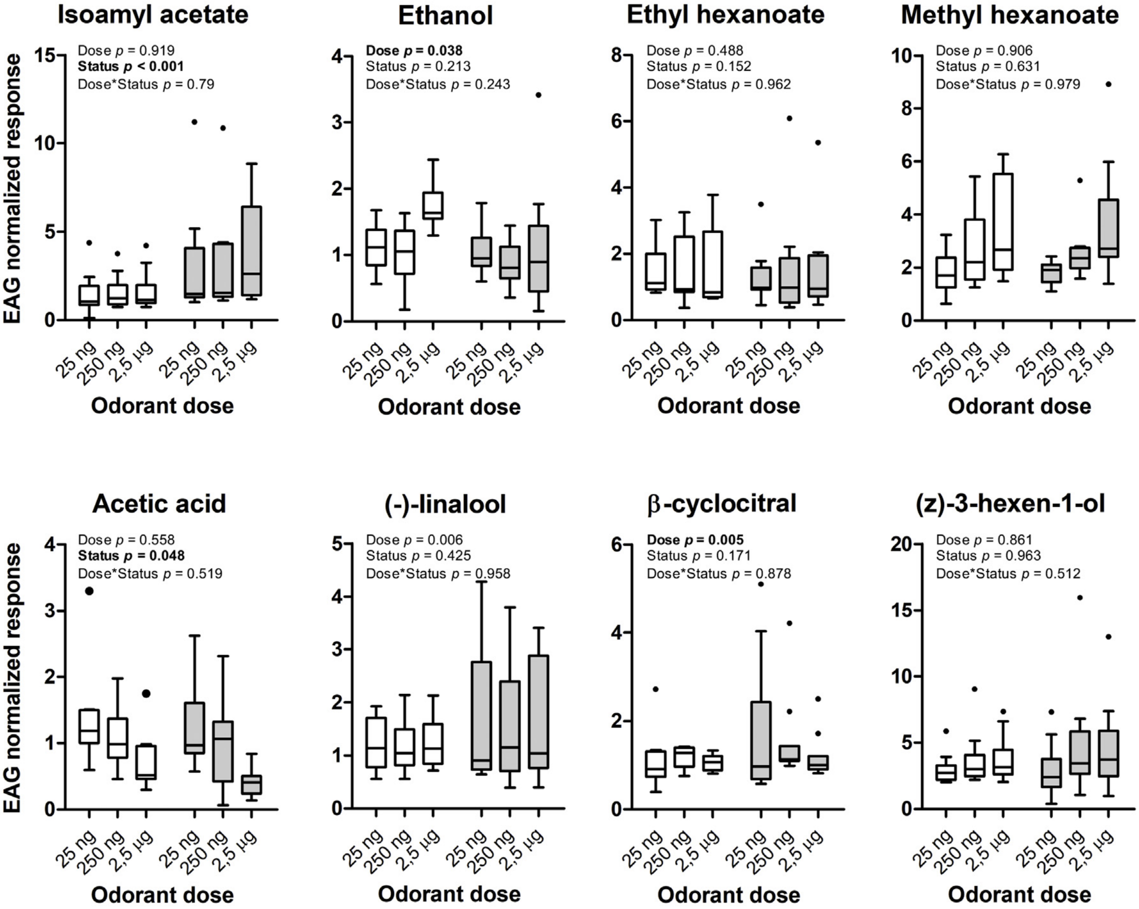

**Fig. 5.** Electroantennogram responses of mated and virgin *Drosophila suzukii* females. Tukey box plots represent electroantennogram (EAG) responses of virgin (white bars) and mated (grey bars) females to three concentration-series of eight odors. *p*-values obtained with two-way repeated measures ANOVA or generalized least squares are reported for each odorant tested (N ≥ 10). For raw data see Supplementary Dataset S3.

## 4. Discussion

Our results have i) provided the quantitative and comprehensive overview of gene expression in the main olfactory organ of *D. suzukii;* ii) identified antennal genes differentially expressed after mating in *D. suzukii* females; iii) shown that post-mating changes occur at the peripheral level of the olfactory system in *D. suzukii* females.

### 4.1. The antennal transcriptome of D. suzukii reveals marked similarities with D. melanogaster

The results of our RNA-seq analysis were consistent with the transcriptional profiling analysis of the *D. melanogaster* antenna (Menuz et al., 2014). When we compared the expression of chemosensory receptors and binding proteins between the two species, we found a clear correlation at both qualitative and quantitative levels, suggesting a conserved role of most of these chemosensory-related proteins in both organisms.

Among orthologous *Or* and *Ir* genes, only three transcripts expressed in *D. suzukii* antennae and likely involved in olfaction had not been previously found in the *D. melanogaster* antennal transcriptome (Menuz et al., 2014): *Or83a*, *Ir51a* and *Ir100a*. Vice versa *Or33a*, *Or33c*, *Ir60a* and *Ir51b* were found expressed in *D. melanogaster* but not in *D. suzukii*. Expression levels of all the genes identified in only one species were extremely low (less than 0.9 FPKM in *D. suzukii* and 2.9 RPKM in *D. melanogaster,* data from Menuz et al., 2014) suggesting that these discrepancies may arise from the different criteria for expression used in the two studies. Among similarities observed between the two species, we found a single splicing variant of the gene *Or46a* (*Or46 aB*) expressed in *D. suzukii* antennae, in a way consistent to what was observed in *D. melanogaster*, where *Or46 aB* was the only splicing variant expressed in the antenna (Menuz et al., 2014) and *Or46 aA* had been previously mapped to maxillary palp (Couto et al., 2005). It will be interesting to localize the expression of *Or* and *Ir* genes in *D. suzukii* at sensillum level and confirm if the same sensillum type expresses the same chemosensory receptors as in *D. melanogaster*. So far, comparative electrophysiology studies have shown that response to odorants are conserved within the same sensillum type across the two species, with the exception for neurons that house odorant receptors that are pseudogenized in the *D. suzukii* genome. For example, Keesey et al. (2015) did not detect differences among the fly species with single-sensillum recordings of sensillum ab1, whose cognate neurons ab1A, ab1B and ab1D in *D. melanogaster* express *Or42b*, *Or92a* and *Or10a*, respectively. Only *D. suzukii* neuron ab1C (that expresses *Gr21a* and *Gr63a* in *D. melanogaster*) was more sensitive to $CO_2$ than *D. melanogaster* (Keesey et al., 2015; Krause Pham and Ray, 2015). Responses of sensillum ab2 were also similar for neuron ab2A, which expresses *Or59b* in *D. melanogaster*. On the contrary neuron ab2B, which expresses *Or85a* in *D. melanogaster* and whose ortholog in *D. suzukii* is pseudogenized and not expressed in our antennal transcriptome, showed a strong response to 2-heptanone only in this latter species (Keesey et al., 2015; Ramasamy et al., 2016). Similarly, one of the two neurons of sensillum ab3 (ab3A, which expresses *Or22b* and *Or22a* in *D. melanogaster* and the second is a pseudogene in *D. suzukii* genome) showed a specific *D. suzukii* response to β-cyclocitral whereas response of the other neuron ab3B (expressing *Or85b* in *D. melanogaster*) was consistent between the two species (Keesey et al., 2015). Also the trichoid sensillum T1, which houses the Or67d-expressing neurons in *D. melanogaster*, responds to the same compound, the pheromone cVA, in both species (Dekker et al., 2015). However, T1 sensilla are rare in *D. suzukii* antennae: Dekker et al. (2015) identified only around 7–10 T1s per individual, compared to 55–60 found in *D. melanogaster.* Consistent with the reduction in the number of T1 sensilla, we found that the normalized expression levels of *Or67d* in *D. suzukii* were around three times lower than normalized expression levels in *D. melanogaster* (data extrapolated from Menuz et al., 2014).

Among *Gr* genes, we found a higher number of transcripts expressed in *D. suzukii* than previously observed in *D. melanogaster*. Of the twelve *Gr* genes expressed in *D. melanogaster* (Menuz et al., 2014) ten were also expressed in *D. suzukii*, and most of them encode receptors expected to bind sugar or bitter compounds. The most striking difference was that the two most expressed *Gr* genes in *D. melanogaster*, *Gr10a* and *Gr64f*, whose function in antennae remains unexplored, were expressed in *D. suzukii* four to five-fold less than the broadly tuned antennal $CO_2$ receptors *Gr21a* and *Gr63a* (Jones et al., 2007; Kwon et al., 2007). Such different ranking of expression levels may be correlated with the differences in sensitivity to $CO_2$ observed in neuron ab1C, which houses *Gr21a* and *Gr63a* in *D. melanogaster* (Krause Pham and Ray, 2015).

Expression overlap between *D. suzukii* and *D. melanogaster* could be observed also for *Obp* genes. In both species, some OBPs were encoded by the most abundant mRNAs in the antenna (Larter et al., 2016; Menuz et al., 2014). Of the 27 *Obp* genes expressed in *D. melanogaster*, 22 were found also in the *D. suzukii* antennal transcriptome. In this latter species, we observed a large dynamic range of expression, as observed in *D. melanogaster*. Interestingly, all the most abundant *D. suzukii Obp* genes were also found to be expressed in *D. melanogaster*, with the exception of *Obp84a2* that represents a gene gain occurred in the *D. suzukii* lineage (Ramasamy et al., 2016). The *Obp* genes whose expression did not match between the two species (seven genes expressed in *D. suzukii* but not in *D. melanogaster*, and conversely five genes expressed in *D. melanogaster* but not in *D. suzukii*) were all expressed at low levels.

### 4.2. Transcriptional variations of antennal chemosensory receptors after mating

Here we found a large number of antenna-expressed genes that were specifically up- or down-regulated after mating in *D. suzukii* females. The majority of these genes underwent a 2-fold or lower change in RNA abundance. Consistent with this observation, a previous study in *D. melanogaster* which was aimed to compare whole body gene expression of mated and unmated females showed a similar narrow dynamic range of variation (Delbare et al., 2017).

Annotation of DE genes in virgin and mated females suggests the occurrence of post-mating modulation of the olfactory system at the peripheral level, since many chemosensory-related genes showed a mating-biased expression. Of the 205 genes in the *D. suzukii* genome (Chiu et al., 2013) that were annotated by blast searches as proteins involved in chemoreception (which include chemosensory receptors, odorant-binding proteins, chemosensory proteins and odorant-degrading enzymes), 83 were up-regulated in mated females and 20 were down-regulated. Of these, the most up-regulated family was that of the odorant receptors of which 35 out of 40 genes were up-regulated and none down-regulated. In addition, of the 20 most significantly up-regulated genes, two were olfactory receptors (*Or7a* and *Or13a*). This expression pattern is in sharp contrast with that observed in *Bactrocera dorsalis*, where 39 out of 43 *Ors* were down-regulated after mating in adult females (Jin et al., 2017). In *D. melanogaster*, post mating changes in females triggered a reduction of transcript abundances of four *Gr* and twelve *Or* genes (Zhou et al., 2009). This latter observation was recorded using whole body RNA and investigating changes in *D. melanogaster* specifically at antennal level would be interesting.

The broad up-regulation of *Ors* in *D. suzukii*, and in general all chemosensory-related genes, may be associated to the post-mating female behaviors observed in this species. Ferguson et al. (2015) described that mated *D. suzukii* females were more active during daylight hours than males and virgin females. They proposed that elevated activity is a post-mating response in female *D. suzukii* that likely correlates to the need to obtain additional resources for egg production and finding oviposition sites. In accordance with these observations, mated *D. suzukii* females were more attracted than virgin females to both yeast and blueberry cues, likely for foraging and egg-laying respectively (Mori et al., 2017). The broad up-regulation of chemosensory-related genes may underlie the olfactory plasticity that triggers the elevated attraction in mated females. Under this point of view, it will be interesting to test the hypothesis that increased attraction to both feeding and egg-laying sites is correlated with altered perception of these stimuli.

The biological significance of the up-regulated *Ors* in mated *D. suzukii* remains to be demonstrated however comparisons with the orthologous *Ors* deorphanized in *D. melanogaster* revealed some interesting points. Of the 25 up-regulated *Ors* identified by our candidate gene approach, seven were lineage duplications that are absent in the *D. melanogaster* genome and hence likely related to the innovative *D. suzukii* egg-laying behavior (Ramasamy et al., 2016). The remaining eighteen up-regulated *Ors* had an ortholog in *D. melanogaster*, and in this latter species fifteen of them responded to fruit volatiles (*Or7a*, *Or9a*, *Or10a*, *Or13a*, *Or42b*, *Or43b*, *Or46a*, *Or47a*, *Or59b*, *Or67a*, *Or67b*, *Or69a*, *Or82a*, *Or85b*, *Or98a*) whereas the other three are not well characterized (*Or43a*, *Or67c* and *Or92a*) (Dweck et al., 2018). Even more interesting, among *D. melanogaster Or* orthologs to the genes that were not differentially expressed between mating status in *D. suzukii*, seven belonged to ecologically labelled lines. No orthologs to *Or47b*, *Or65a*, *Or67d*, *Or83c* and *Or88a*, which respond to pheromone compounds in *D. melanogaster* (van der Goes van Naters and Carlson, 2007; Ronderos et al., 2014; Dweck et al., 2015), were differentially expressed in *D. suzukii* as well as *Or85f* which governs parasitoids avoidance (Ebrahim et al., 2015) and *Or56a* which detects geosmin (Stensmyr et al., 2012). Based on this scenario, one can speculate that post-mating up-regulation of *Ors* in *D. suzukii* is specifically tuned to the need to localize fruit and oviposition sites, and thus to the specific post-mating female behavior of this species.

### 4.3. *Post-mating modulation at peripheral level of the olfactory system*

We used EAG to correlate differential transcript expression to physiological responses. We observed that one out of eight odors tested (IAA), triggered a differential antennal response thus supporting the hypothesis of post-mating peripheral modulation of olfaction in *D. suzukii* females. Interestingly, IAA significantly attracted mated *D. suzukii* females in olfactometer bioassays at a release dose comparable to that of fresh fruits (Revadi et al., 2015). Taken together, these observations suggest a role for IAA in the egg-laying behavior of *D. suzukii*. Similar post-mating peripheral modulations of olfaction have been reported for other insect species like *S. littoralis* (Martel et al., 2009; Saveer et al., 2012) and *D. melanogaster* (Hussain et al., 2016). In this latter species, post-mating attraction to high levels of poly-ammine is triggered by the increased expression of the sex peptide receptor (SPR) in specific OSNs tuned to poly-ammine recognition. It seems unlikely that the same mechanism is involved in the broad up-regulation of chemosensory-related genes observed in *D. suzukii*, since the transcript level of SPR were unchanged between mated and virgin *D. suzukii* females. However, it is interesting to note that among up-regulated genes in mated females there is the gene homolog to the short neuropeptide F (sNPF), which is involved in modulation of olfactory sensitivity in *D. melanogaster* (Root et al., 2011).

## 5. Conclusions

Our study demonstrates post-mating modulation at transcriptional levels of the genes involved in the peripheral chemosensory systems of *D. suzukii*. This does not exclude that alterations in higher processing centers may contribute to egg-laying behavior, but it represents an important contribution to disentangle the mechanisms underlying olfactory modulation in insects exposed to a multi-stimuli environment. Further studies of the *Ors* up-regulated in mated females may reveal odors relevant for *D. suzukii* egg-laying behavior.

## Data statement

Raw reads are available at Genbank Sequence Read Archive (SRA) under the accession number PRJNA490636.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ibmb.2018.12.012.

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