Exploring multiple sensory systems in ovipositors of *Drosophila suzukii*

and related species with different egg-laying behaviour

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19   ABSTRACT

20   *Drosophila suzukii* is an invasive agricultural pest species that lays eggs in fruit during ripening,

21   while most closely related *Drosophila* species use rotten matter as oviposition substrates. This

22   behaviour is allowed by an enlarged and serrated ovipositor that can pierce intact fruit skin. *D.*

23   *suzukii* combines multiple sensory systems (mechanosensation, olfaction, and taste) to select

24   oviposition sites. Here, we test the hypothesis that the *D. suzukii* ovipositor is involved in these

25   sensory modalities. We first investigate the ovipositor gene expression using a comparative

26   framework of four *Drosophila* species with gradual changes in ovipositor morphology to identify

27   evolutionary adaptations specific to *D. suzukii*. Results show transcription of chemoreceptors and

28   mechanoreceptors in the four species, with a common core of sensory receptors expressed in all

29   of them. Then, we demonstrate that sensory structures present in the distal tip of the *D. suzukii*

30   ovipositor are mechanosensory-like sensilla, and that the degenerin/epithelial sodium channel *ppk*

31   is expressed in homologous structures in *Drosophila melanogaster*. Our results suggest the *D.*

32   *suzukii* ovipositor playing a role in mechanosensation, which might be shared with other

33   *Drosophila* species.

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BACKGROUND

Drosophila suzukii (Matsumura) (Diptera Drosophilidae), also called spotted wing drosophila, is an

invasive South Eastern Asian fly species that was identified outside its native range in California

(US) in 2008 and in Spain and Italy (Europe) in 2009 (1,2). Since then, it has quickly spread across

both continents and settled down in several countries, where it represents a major threat for soft

fruit production (3). Differently from the majority of drosophilids, which thrive and lay eggs on

already damaged or rotting vegetal substrates, *D. suzukii* is able to pierce and lay eggs on healthy

ripening fruits before harvesting. Wherever it is present, this causes extensive agricultural damage

and has boosted research on the ecology and chemosensory behaviour of this pest with the aim to

find innovative, effective, and eco-friendly methods to reduce its attacks (reviewed in 4).

Several aspects of *D. suzukii* ecology and genetics have been analysed in a comparative

Drosophila framework to identify key evolutionary innovations that allowed the transition from

rotten to fresh fruit egg-laying behaviour (5–10). The major morphological shift from rotten fruit-

ovipositing *Drosophila* species (like the insect model *Drosophila melanogaster*) is the presence of

an unusually enlarged and serrated ovipositor, which is shared with the sister species *Drosophila*

subpulchrella, and allows the wounding of the intact skin of berries (Figure 1) (7). This feature is

not present in another closely related Asiatic spotted wing *Drosophila* species, *Drosophila*

biarmipes, whose ovipositor shows intermediate features between *D. suzukii* and *D. melanogaster*

(Figure 1).

The ovipositor morphology correlates with the stiffness of the oviposition substrates, and

serrated designs facilitate egg laying by attenuating the required penetration forces to cut through

the fruit skin. In fact, *D. melanogaster* egg-laying is inhibited by stiff substrates, whereas *D. suzukii*

has a broad tolerance, and *D. biarmipes* displays an intermediate behaviour (9). Besides

mechanosensation, also the chemical composition of the egg-laying substrate contributes to *D.*

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59   *suzukii* oviposition behaviour: strawberry odours are sufficient to elicit oviposition, suggesting that

60   olfaction has an important role in egg-laying site choice decision. Moreover, olfaction-impaired *D.*

61   *suzukii* performed as wild-type flies (they prefer ripe over rotten strawberries puree) when

62   allowed to get in touch with the oviposition substrate (9). Hence, contact chemosensory systems,

63   such as taste, are likely used by *D. suzukii* for egg-laying site selection together with olfaction and

64   mechanosensation. Insects detect non-volatiles compounds mainly through the gustatory receptor

65   neurons (GRNs), which are scattered over various locations across their body. In *D. melanogaster*,

66   GRNs house either gustatory receptors (GRs) or ionotropic receptors (IRs), and in adults they are

67   mainly located at the labellum and along the legs (11). It has long been suggested that GRNs are

68   also present in the ovipositor of *D. melanogaster* (12,13) as it was demonstrated in other insect

69   species (14–17). However, a functional characterization of these structures is lacking. An intact

70   hydrophobic cuticle, that prevents water loss and microbial contamination, covers healthy

71   undamaged fruits (18). We therefore conjecture that, differently from other drosophilids, which

72   can easily taste rotten fruit substrates with legs and mouthparts, fruit cuticle of ripening fruits

73   likely prevents *D. suzukii* probing with these appendages. The ovipositor pierces fruit skin and

74   comes into contact with fruit flesh, thus the possibility that this organ may carry chemosensory

75   organs which contribute to egg-laying decision is suggestive.

76   Here, we test this hypothesis using RNAseq to map the transcriptional profile of the female

77   abdominal distal tip into a phylogenetical framework composed by *D. suzukii* and other three

78   *Drosophila* species characterised by gradual changes in their ovipositor structure (from blunt to

79   serrated ovipositor) (Figure 1). One of our aims is to understand if the presence of chemosensory-

80   related transcripts might be a common *Drosophila* feature or something unique to fresh-fruit-

81   ovipositing *Drosophila* species. We found expression of a set of chemosensory and

82   mechanosensory-related mRNA conserved among the four species. This prompted a detailed

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83   analysis of the ultrastructure of pegs and sensilla present at the ovipositor of *D. suzukii*, and the

84   use of transgenic *D. melanogaster* GAL4-drivers to provide evidence of sensory neuron-specific
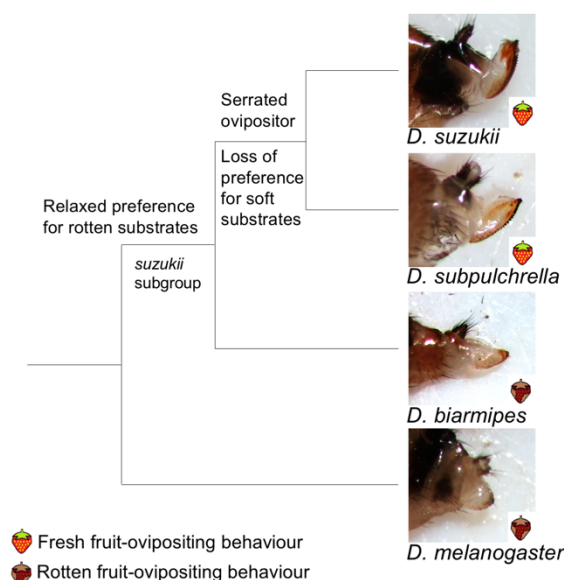
85   genes expressed in ovipositor pegs.

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88   **Figure 1 Evolutionary affinities and ovipositor shapes of *Drosophila suzukii* and other *Drosophila* species**
89   **used in the study**.
90   The currently most accepted scenario for fresh fruit-egg laying behaviour evolution (9). Phylogeny is based
91   on (7).

92   METHODS

93   *Insects*

94   Insects used for transcriptomics, immunohistochemistry, and electron microscopy were taken

95   from laboratory colonies maintained at the Fondazione Edmund Mach (Italy). *Drosophila suzukii*

96   and *D. melanogaster* strains were founded with individuals collected in 2010 in the Trento

97   province (Italy) and periodically refreshed with insects caught from the same field sites. *Drosophila*

98   *biarmipes* (genotype Dbii\wild-type, stock # 14023-0361.09) and *Drosophila subpulchrella*

99   (Dspc\wild-type, stock # 14023-0401.00) strains were obtained from the Drosophila Species Stock

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100   Center in 2011. The four *Drosophila* species were reared on a standard diet

101   (https://stockcenter.ucsd.edu/info/food_cornmeal.php), maintained at 23–25 °C, 65 ± 5% relative

102   humidity and under a 16:8 h light:dark photoperiod.

103   *Drosophila melanogaster* transgenic strains used for imaging were obtained from the

104   Bloomington Drosophila Stock Center and reared under the same conditions as described above.

105   GAL4 lines are listed in Supplementary Table S1 and the reporter line expressing hexameric green

106   fluorescent protein (6GFP) was #52262. Three to ten days old mated females were used for the

107   analysis.

108   *RNA extraction and sequencing*

109   RNA was extracted from the abdominal distal tip of 3-10 days old mated females. Dissected tissues

110   were stored at −80 °C in RNAlater (ThermoFisher Scientific) until extraction. Each species sample

111   was composed of RNA extracted from around 60-80 individuals. Samples were homogenized using

112   TissueLyser (Qiagen) and total RNA was extracted with TRIzol reagent (ThermoFisher Scientific),

113   following the manufacturer's protocol. DNA contamination was removed with a DNase I

114   (ThermoFisher Scientific) incubation step. A second RNA extraction with PureLink RNA Mini Kit

115   (ThermoFisher Scientific) was performed to remove DNase and concentrate samples. Total RNA

116   (∼1 µg/sample) was sent to Beckman Coulter Genomics (Danvers, MA USA) for library preparation

117   and Illumina sequencing. Library preparation was carried out through polyA + selection and

118   paired-end (PE) sequencing was run on an Illumina HiSeq 2500 System with V3 chemistry that

119   generated 100 bp reads. Raw reads are accessible from the Genbank SRA database (BioProject

120   number PRJNA526247) (Supplementary Table S2).

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121   De novo *transcriptome assembly, annotation, and gene ontology*

122   Raw reads were trimmed with Trimmomatic (19). Both paired and unpaired reads were used for a

123   *de novo* assembly of the transcriptome for each species with Trinity v2.0.6 (20), using the

124   normalization step and flag --min_kmer_cov 2. The transcriptome quality was checked by mapping

125   the paired reads against the assembled transcriptome with Bowtie (21) (Supplementary Table S2).

126   The four transcriptomes were annotated using Standalone Blast+ (22). Blast searches were run

127   with the command blastx using the predicted proteins from the *D. melanogaster* genome (version

128   r6.25) as the database. The top hit for each sequence was retained when the E-value was less than

129   1×10^{-10}. PANTHER version 14.0 (23) was used to extract gene ontology (GO) terms (Panther GO-

130   Slim) for each annotated transcriptome. Venn diagrams were created using Venny 2.1.0 (24).

131   *Quantification of gene expression of species-specific chemosensory-related transcripts*

132   To confirm the identity of chemosensory-related genes in each species and to estimate their

133   expression level in each *Drosophila* species, we mapped trimmed PE reads against the dataset of

134   manually curated annotations for *D. biarmipes*, *D. suzukii*, and *D. melanogaster Or*, *Gr*, *Ir*, and *Obp*

135   genes (5,6). For *D. subpulchrella*, we estimated expression only for *Or* and *Gr* genes using an in-

136   house curated dataset. Proper PE trimmed reads were mapped to the annotated isoforms using

137   Bowtie v1.0.0 (21), and the read counts were estimated by RSEM v1.2.20 (25). Genes were defined

138   as expressed if they had at least twenty RSEM estimated expected counts. Transcripts per million

139   reads (TPM) were visualized with heatmap.2 implemented in package gplots (R Core Team, 2015)

140   to compare expression levels among species.

141   *Reverse transcription PCR of* D. suzukii *chemosensory-related genes*

142   Expression of chemosensory receptor genes, found to be expressed in the *D. suzukii* abdominal

143   distal tip by RNAseq analysis, was confirmed by reverse transcription PCR (RT-PCR). *Orco* and *Gr64*,

144   which were not found to be expressed by RNAseq, were used as negative control and genomic

145 DNA as positive control. The used primers are listed in Supplementary Table S3. RNA was

146 extracted with Trizol and treated with DNAse I as described before. 1 μg RNA was then

147 retrotranscribed to cDNA with SuperScript III Reverse Transcriptase (ThermoFisher Scientific)

148 following the manufacturer's protocol. To control for genomic DNA contamination, RNA

149 underwent a parallel mock reverse transcription step in which the reverse transcriptase was

150 omitted. Amplifications were carried out with GoTaq Green Master Mix (Promega) in a final

151 volume of 25 μl containing 1 μl of cDNA diluted 1:10 and 0.4 μM of each primer, at the following

152 conditions: 2 min at 95°C, then 25 cycles composed by a 30 s step at 95°C, 30 s at 55°C, and 1 min

153 at 72°C, followed by a final elongation step of 5 min at 72°C. PCR amplicons were run on 1%

154 agarose gel stained with Midori Green Advance (Nippon Genetics).

155 Drosophila suzukii *ovipositor immunohistochemistry*

156 *Drosophila suzukii* adult females were anesthetized using CO_2. Abdominal distal tips were cut with

157 a razor blade and fixed in 4% PFA in PBS (Sigma-Aldrich) for 40 min on ice. Samples were then

158 washed three times with PBS for 20 min, incubated in 10% sucrose (Sigma-Aldrich) solution, and

159 kept rotating for 1 h at room temperature (RT). Sucrose was changed to 25% solution, and

160 samples were kept rotating overnight at 4°C. Samples were then embedded in OCT (OCT mounting

161 medium Q PATH, VWR) and mounted on a sample holder. Sections of 15 μm thickness were cut

162 with a CM 1510-3 cryostat (Leica) and collected on a SuperFrost glass slide (ThermoFisher

163 Scientific).

164 Slides were washed in PBS-T (PBS + 0.1% Triton-X-100, Sigma-Aldrich) for 5 min and then

165 blocked in 5% normal goat serum (Sigma-Aldrich) in PBS-T for 30 min. Anti-horseradish peroxidase

166 (HRP) cyanine-conjugated antibody (Cy3 AffiniPure Rabbit Anti-Horseradish Peroxidase, Jackson

167 ImmunoResearch) diluted 1:300 was used to stain the neurons. Slides were kept in a moist chamber

168 at 4°C overnight in dark. The next day, antibodies were removed, and the slides were washed three

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169   times with PBS-T for 5 min, mounted using Vectashield (Vector Laboratories), and imaged on a

170   confocal microscope TCS SP8 (Leica).

171   Drosophila suzukii *ovipositor scanning electron microscopy*

172   Adult females of *D. suzukii* were anaesthetized by exposure to cold temperatures (-18°C) until

173   death, then they were immediately soaked in 60% alcohol. The ovipositor of each individual was

174   dissected from the abdomen. Specimens were dehydrated in a series of graded ethanol, from 60%

175   to 99%, 15 min for each step. After dehydration, 99% ethanol was substituted with pure HMDS

176   (Hexamethyldisilazane, Sigma-Aldrich) and the specimens were allowed to dry under a hood at

177   room temperature; this step was repeated twice. Up to five samples were mounted on aluminium

178   stubs, with different orientations, in order to obtain a clear view on the ventral and lateral sides of

179   the ovipositor. Mounted specimens were gold-sputtered using a Balzers Union SCD 040 unit

180   (Balzers). The observations were carried out using a Philips XL 30 scanning electron microscope

181   (SEM) operating at 7-10 KV, WD 9-10 mm.

182   Drosophila suzukii *ovipositor transmission electron microscopy*

183   Ten female *D. suzukii* individuals were anesthetized by exposure to cold temperatures (-18°C) for

184   60 s, then immediately immersed in a solution of glutaraldehyde and PFA 2.5% in 0.1 M cacodylate

185   buffer plus 5% sucrose, pH 7.2-7.3. The ovipositor was detached from the abdomen, reduced in

186   size to help fixative penetration, and left at 4°C for 24 h. Then, the specimens were washed twice

187   in cacodylate buffer for 10 min, post-fixed in 1% OsO_4 for 1 h at 4°C, and rinsed in the cacodylate

188   buffer. Dehydration in a graded ethanol series from 60% to 99%, was followed by embedding in

189   Epon-Araldite with propylene oxide as bridging solvent. Thin sections were taken with a diamond

190   knife on an LKB Bromma ultramicrotome (LKB) and mounted on formvar-coated 50 mesh grids.

191   Then, sections on grids were stained with uranyl acetate (20 min, RT) and with lead citrate (5 min,

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192   RT). Finally, the sections were imaged with a Philips EM 208 transmission electron microscopy

193   (TEM). A digital camera MegaViewIII (SIS) provided high-resolution images.

194   *Examination of GAL4-driven GFP expression patterns in* D. melanogaster *ovipositor*

195   The native GFP signal was observed at the level of the ovipositor of females expressing the super

196   bright 6GFP reporter under the pattern of several GAL4-drivers. Flies were anesthetized using CO_2,

197   abdominal distal tips were cut, embedded in 70% Glycerol, and imaged with the confocal

198   microscope TCS SP8 (Leica).

RESULTS

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200   *Sequencing data and annotation of sensory genes*

201   Illumina RNA-seq libraries from the abdominal distal tip of four *Drosophila* species generated an

202   average of 60M (±2.4M SD) 100 bp paired-end reads. This resulted in four *de novo* assembled

203   transcriptomes with contig counts ranging from 31'315 (*D. subpulchrella*) to 40'162 (*D. suzukii*)

204   (Supplementary Table S2). On average, 70% of contigs from each of the four transcriptomes had a

205   blastx hit against *D. melanogaster* predicted proteome (Supplementary Dataset S1). In particular,

206   *D. suzukii* hit 11'840 unique *D. melanogaster* predicted proteins, whereas *D. subpulchrella*, *D.*

207   *biarmipes,* and *D. melanogaster* had 10'973, 11'687, and 12'734 unique *D. melanogaster* hits,

208   respectively. Panther functional classification revealed that the Gene Onthology (GO) composition

209   of assembled contigs was similar among the four species (Figure 2A and Supplementary Figure S1).

210   The most represented Molecular Function GO-slim terms were binding and catalytic activity,

211   whereas the metabolic process, biological regulation, and cellular component organization were

212   the most represented Biological Process GO-slim terms (Figure 2A). Of 10'774 *D. melanogaster*

213   genes encoding for proteins that hit assembled contigs, 7294 were common among the four

214   *Drosophila* species, thus representing the conserved transcriptional core for the distal abdominal

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215   tip (which was enriched for the ovipositor, but also included the internal genitalia, the anal plate,

216   and some abdominal tissues). On the contrary, 131, 163, 187, and 682 genes hit specifically *D.*

217   *subpulchrella*, *D. suzukii, D. biarmipes,* or *D. melanogaster* contigs, respectively (Figure 2B).
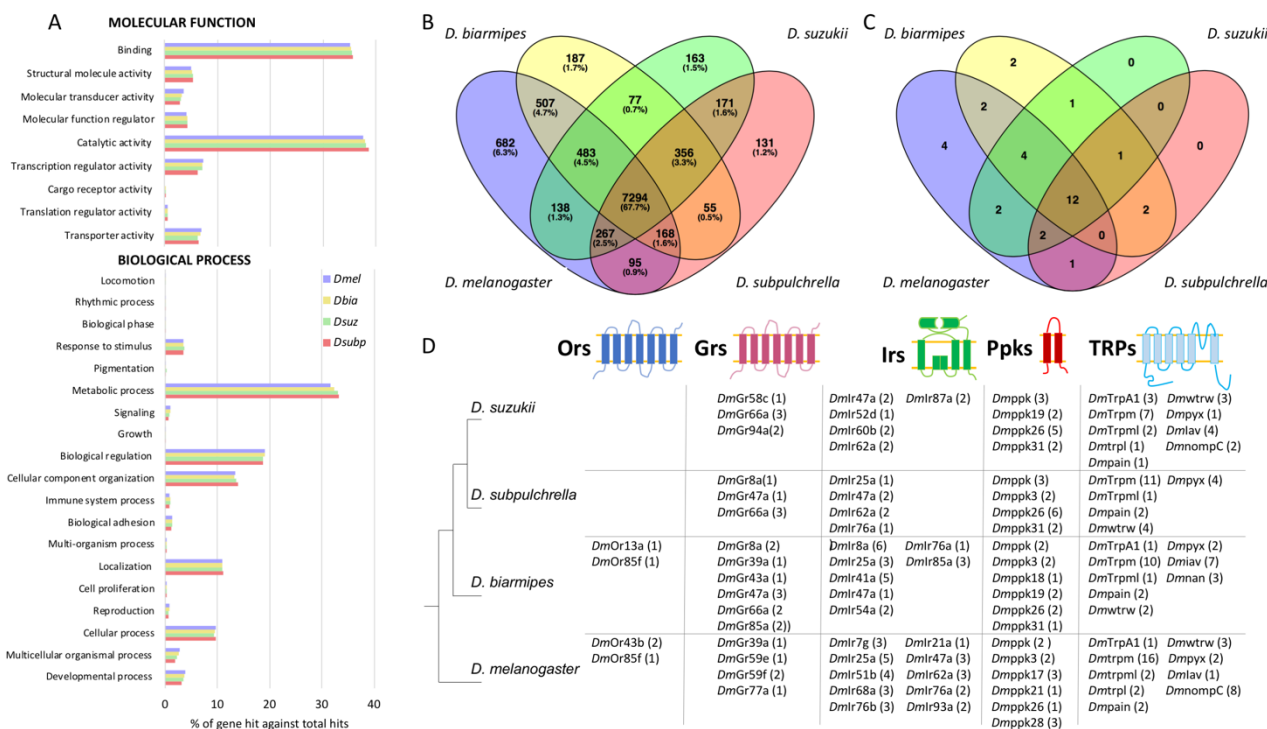

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Figure 2. Annotation of transcriptomes from the abdominal distal tip of four *Drosophila* species reveals a core of conserved transcripts involved in sensory perception.

(A) Gene Ontology (GO) classification for the four transcriptomes referred to Molecular Function and Biological Process (Panther GO-Slim terms). Abbreviations: Dmel, *D. melanogaster*; Dbia, *D. biarmipes*, Dsuz, *D. suzukii*, Dsubp, *D. subpulchrella*. **(B)** Venn diagram representing the unique *D. melanogaster* gene hits retrieved by blastx searches using contigs from each transcriptome as query. **(C)** Venn diagram representing the unique *D. melanogaster* odorant binding protein (Obp) gene hits retrieved by blastx searches for each transcriptome. **(D)** Schematic representation of the number of unique *D. melanogaster* gene hits encoding for sensory receptors (ORs: odorant receptors; GRs: gustatory receptors; IRs: ionotropic receptors; Ppks: pickpocket proteins; TRPs: transient receptor potential channels) retrieved in each *Drosophila* transcriptome by blastx searches. Between parentheses the number of contigs matching the same *D. melanogaster* gene hit.

232   Among contigs annotated by blastx searches, we various transcripts encoding for sensory

233   receptors belonging to different classes: chemoreceptors such as odorant receptors (ORs),

234   gustatory receptors (GRs), and ionotropic receptors (IRs), as well as other sensory receptors such

<div align="center">11</div>

235   as pickpocket proteins (ppks) from the degenerin/epithelial sodium channels (DEG/ENaC) gene

236   superfamily (which contribute to mechanosensation) and transient receptor potential channels

237   (TRPs) (which virtually contribute to every sensory modality from chemosensation to

238   mechanosensation and thermotransduction) (Figure 2D). Of classical chemosensory receptors, 4

239   gustatory receptors (GRs), 4 ionotropic receptors (IRs), and 1 odorant receptor (OR) were

240   expressed in more than one species. We also found a consistent number of contigs annotated as

241   odorant binding proteins (OBPs) in the four transcriptomes (from 18 putative unique OBPs in *D.*

242   *subpulchrella* to 27 in *D. melanogaster*). Likewise chemosensory receptors, the identity of these

243   contigs was largely shared between the four species (Figure 2C). Of 13 *trp* genes present in *D.*

244   *melanogaster* genome (26), 5 were found putatively expressed in *D. subpulchrella*, 8 in *D.*

245   *biarmipes,* and 9 in *D. melanogaster* and *D. suzukii*. Of these, 5 were conserved among the four

246   species (Figure 2D). Unique pickpocket proteins that hit assembled contigs varied from 4 (*D.*

247   *suzukii* and *D. subpulchrella*) to 6 (*D. melanogaster* and *D. biarmipes*), and 3 of them were shared

248   among all four species (Figure 2D).

249   *Species-specific RNAseq expression patterns of chemosensory-related transcripts*

250   We profiled the expression levels of chemosensory-related genes in the four *Drosophila*

251   abdominal tips by mapping their reads against available manually-annotated chemosensory gene

252   datasets for the four species (5,6). This led us to finely define which isoform was expressed in each

253   species. Only genes that had ≥ 20 RSEM expected counts were considered expressed and

254   designated as the set of abdominal distal tip genes (Supplementary Dataset S2).

255   We found expression of 14 chemosensory receptors in both *D. melanogaster* (3 *Grs*, 1 *Or*,

256   and 10 *Irs*) and *D. biarmipes* (4 *Grs*, 2 *Ors*, and 8 *Irs*), and 8 in *D. suzukii* (2 *Grs*, 1 *Or*, and 5 *Irs*)

257   (Figure 3 and Supplementary Figure S2). In *D. subpulchrella,* we tested only for *Gr* and *Or*

258   transcription, and 2 *Grs* and 1 *Or* met our formal criteria for abdominal tip expression (Figure 3).

259   Our analysis identified *Gr66a* and *Or43b* as expressed in the four *Drosophila* species, as well as 2 *Ir*

260   genes were found in the three species tested for this gene family: *Ir47a* and *Ir62a*. These 4 genes

261   likely represent a conserved core of chemoreceptors expressed in the abdominal distal tip of

262   *Drosophila* species. We could not detect no expression of the co-receptor *Orco* in any *Drosophila*

263   species and its absence was also confirmed in *D. suzukii* by RT-PCR (Supplementary Figure S2).

264   Expression patterns of other chemosensory receptor genes were variable, and most of them

265   showed a species-specific expression. Only *Gr94a* was expressed in both *D. suzukii* and *D.*

266   *subpulchrella*, *Ir75d* in *D. suzukii* and *D. melanogaster,* and *Ir25a* in *D. melanogaster* and *D.*
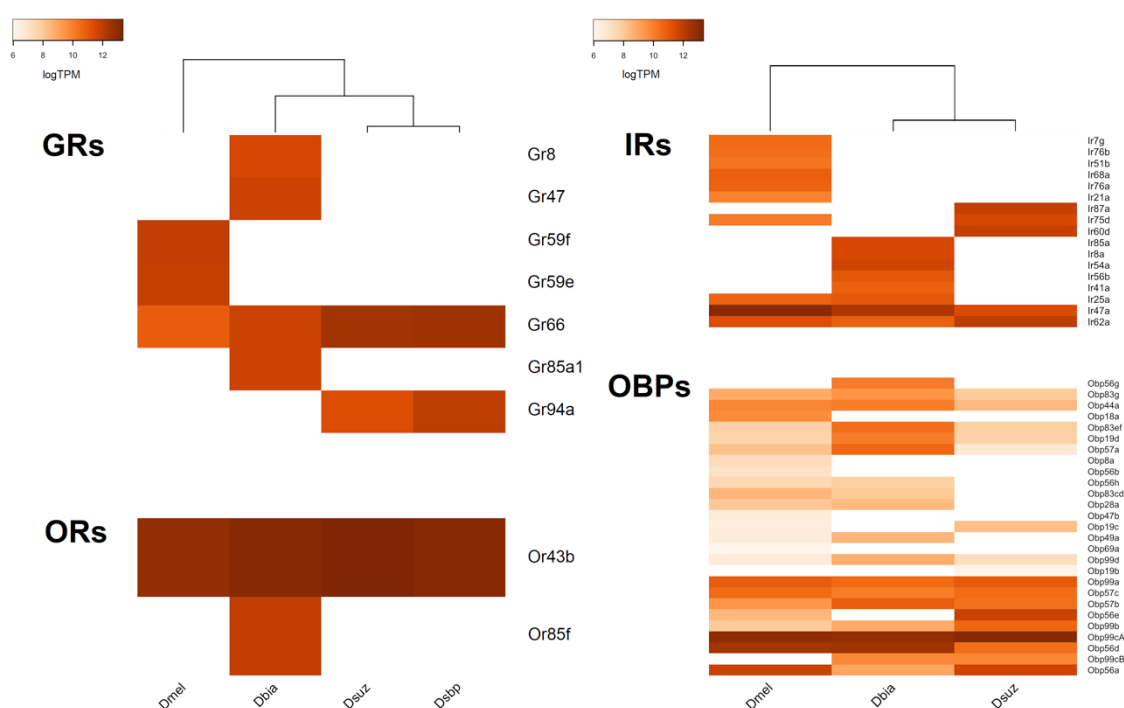
267   *biarmipes*.

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Figure 3. RNAseq expression levels of chemosensory-related genes in the abdominal distal tip of four

Drosophila species.

271   Heatmaps depict the expression level (calculated as transcripts per million, TPM) for each chemosensory-

272   related gene found to be expressed in the most abdominal tip of *D. melanogaster* (Dmel), *D. biarmipes* (Dbia),

273   *D. suzukii* (Dsuz), and *D. subpulchrella* (Dsbp). Expression levels were calculated using the manually curated

274   datasets from (5,6). For *D. subpulchrella,* we used an in-house dataset containing only GRs and ORs

13

275   sequences. Abbreviations: gustatory receptors (GRs), ionotropic receptors (IRs), odorant receptors, (ORs),

276   and odorant binding proteins (OBPs).

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278   The dynamic range of chemosensory receptor expression in *Drosophila's* abdominal distal

279   tip was similar among species. Within each species, the most expressed or the second most

280   expressed gene was *Or43b*. More generally, in each species mean expression levels of genes that

281   shared transcription across species were substantially higher than the mean levels of the species-

282   specific expressed genes (from 2 to 4-fold higher) (Supplementary Dataset S2).

283   RNAseq revealed the expression from 17 to 24 *Obp* genes in the three *Drosophila* species

284   tested. The expression patterns of the three species largely overlapped since 13 *Obps* were

285   expressed in *D. melanogaster*, *D. biarmipes,* and *D. suzukii*. On the other way around, species-

286   specific expressed *Obps* were few: 5 in *D. melanogaster* and only 1 in both *D. biarmipes* and *D.*

287   *suzukii*. Their expression levels were substantially lower than those of chemosensory receptor

288   genes. Within each species, the mean level expression of *Obps* was from 3 to 4-fold lower than the

289   mean level of chemoreceptors. Only *Obp99cA* had expression levels that were comparable to

290   *Or43b* (Figure 3).

291   *Ultrastructure of sensilla and pegs located on* D. suzukii *ovipositor*

292   Transcriptional profiles supported the hypothesis of chemosensory and mechanosensory

293   perception in the abdominal distal tip of the four *Drosophila* species. To investigate the

294   contribution of these sensory modalities in *D. suzukii* egg-laying behaviour, we analysed at the

295   ultrastructural level the small bristles and pegs located at the tip of the ovipositor. Our

296   observations revealed the presence of four different types of structures: conical pegs type 1 ,

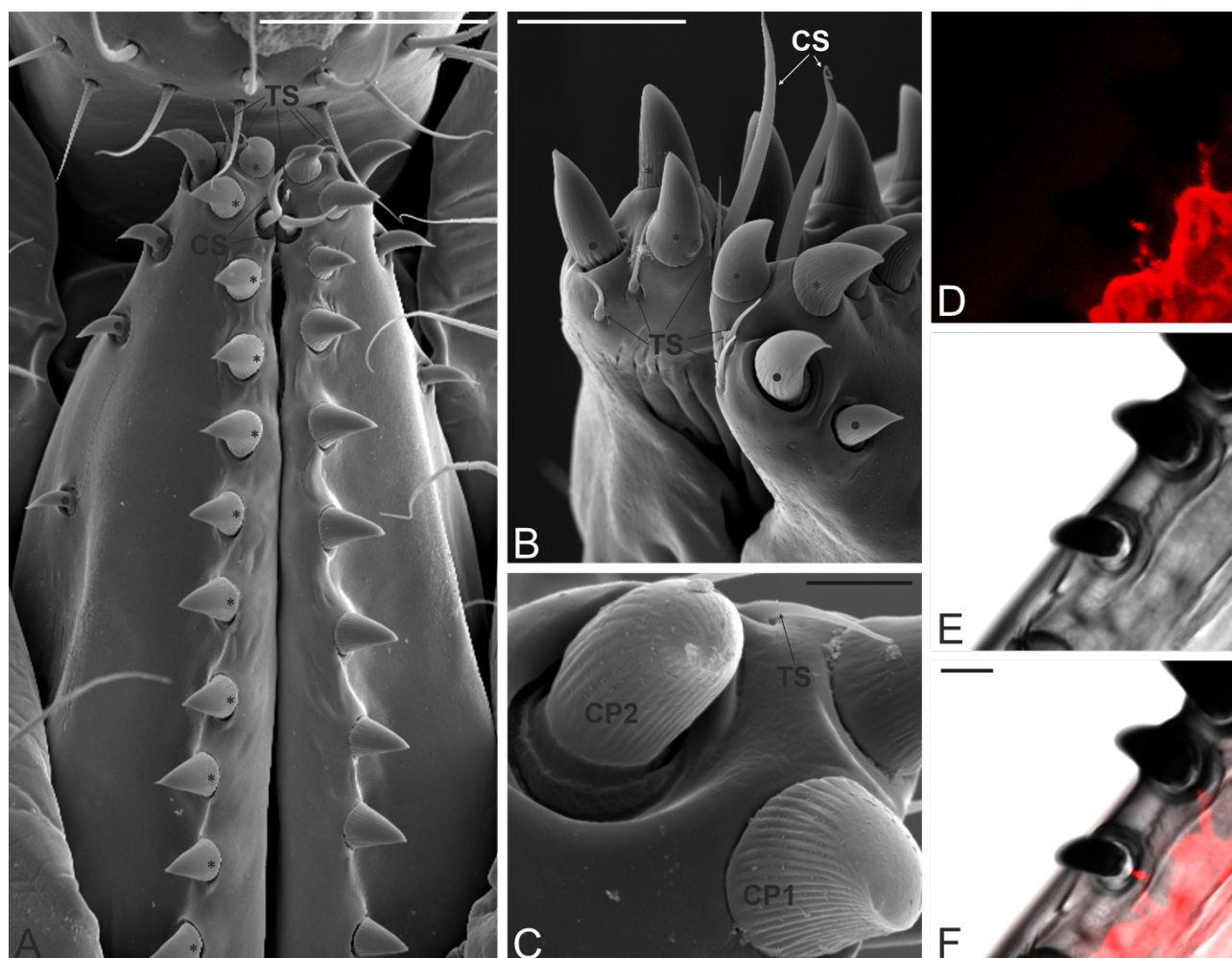297   conical pegs type 2, trichoid sensilla and chaotic sensilla (Figure 4).

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Figure 4. *Drosophila suzukii* ovipositor pegs and sensilla are innervated sensory structures

(A) Ventral view of the ovipositor of *D. suzukii* showing the two ovipositor plates and the different structures that are present. The tip of each plate presents three trichoid sensilla (TS) and a chaetic sensillum (CS). A single row of conical pegs type 1 (*) is found, with the structures arranged along the ventral edge of each ovipositor plate. Four conical pegs type 2 (●) are present, with the first one sitting at the very tip of the ovipositor plate, while the others are positioned along a medial line of the ovipositor plate. **(B)** Detailed view on the tip of the ovipositor plates. The two apical TS are clearly visible, as well as the third, inserted just behind the most apical CP1. The CS are located very close to the TS. **(C)** Close-up view of the ovipositor plate tip. The CP1 is sitting on a narrow socket; it presents a grooved cuticle that smoothens at the tip. The CP2 is sitting on a large socket; it shows a grooved cuticle as well, but with less evident grooves and a pointed tip. **(D-E)** Immunostaining of cryosection of the ovipositor plate: **(D)** neuronal anti-horseradish peroxidase signal, **(E)** bright-field, **(F)** Merged picture. Scale bars: A: 50 μm, B: 20 μm, C: 5 μm, D-F: 10 μm.

CONICAL PEG TYPE1 (CP1): The outer margin of each ovipositor plate presents a single row of stout, conical pegs sitting on narrow sockets in the cuticle (Figure 4A). They are present in a

314   number of about 15 per each plate, with a base diameter of about 10 µm and 15 µm long. CP1 are

315   characterised by a cuticular shaft slightly bent towards the external side of the plate. The cuticle is

316   grooved externally all along. Each structure ends in a sharp tip, although in some specimens the

317   tip appears worn, having a blunt shape (Figure 4C and 5A). The analysis of ultrathin sections shows

318   the internal structure of CP1, characterised by a solid, poreless cuticular shaft (Figure 5B-E).

319   Micrographs taken at the level of the medial peg region show a thick and continuous cuticular wall

320   with a small lumen without sensory neurons (Figure 5C). Imaging at the socket level shows as

321   remarkable feature the presence of a single sensory neuron, typically embedded in an electron-

322   dense dendrite sheath and ending in a tubular body (Figure 5D-E). The tubular body is located at

323   the base of the peg, where a large socket with suspension fibres is evident (Figure 5B-D).

324
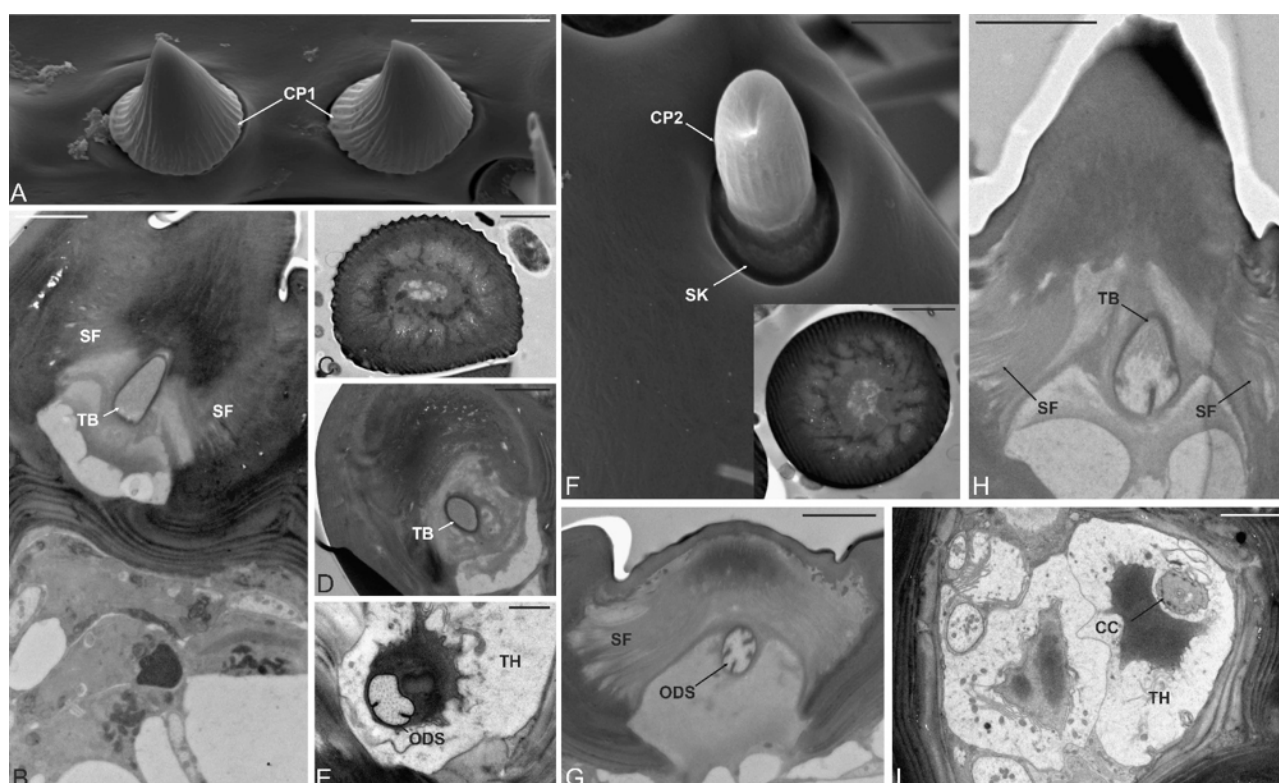
Figure 5. Micrographs showing details of the conical pegs type 1 and type 2 of the *Drosophila suzukii* ovipositor.

(A) Scanning electron microscopy (SEM) ventral view of parts of the ovipositor plate ridge, showing two

conical pegs type 1 (CP1). **(B)** Transmission electron microscopy (TEM) longitudinal section at the socket

level. The peg is sitting on a narrow socket made of thick cuticle. Suspension fibres (SF) are apparent,

330   holding the peg and giving flexibility to the structure. The single sensory neuron associated with the CP1

331   terminates in a tubular body (TB) ending just at the base of the peg. **(C-E)** Serial TEM micrographs of a CP1

332   cross sections, taken at different levels, show the solid cuticular structure of the peg **(C),** the presence of

333   the tubular body (TB) at the socket level **(D),** and the outer dendritic segment (ODS) of the sensory neuron

334   enclosed by the thecogen cell (TH) **(E)**. **(F)** SEM ventral view of the ovipositor plate, showing one of the

335   conical pegs type 2 (CP2), with a slightly grooved cuticle and a sharp tip. Noticeable is also the large socket

336   (SK) on which the CP2 is sitting in the cuticular wall of the ovipositor plate. The inset in **(F)** shows the TEM

337   micrograph of a CP2 cross section, taken at half of the length of the peg: the peg is made of solid, thick

338   cuticle and presents a reduced lumen, devoid of sensory neurons. **(G-I)** Serial TEM micrographs of a CP2,

339   longitudinally and cross sections taken at different levels. They show: in **(G)** the base of a CP2 with a large

340   flexible socket and several suspension fibres (SF). The single sensory neuron ends in a tubular body (TB) at

341   the base of the peg. In **(H)** the large socket is visible, as well as the outer dendritic segment (ODS) of the

342   sensory neuron. In **(I)** a cross section is imaged at a lower level respect to the previous: the sensory neuron

343   appears at the ciliary constriction level (CC), and it is enclosed by the thecogen cell (TH). Scale bars: A: 10

344   μm, B-D: 2 μm, E: 1 μm, F: 5 μm, inset in F: 2 μm, G-I: 2 μm.

345

346   CONICAL PEG TYPE2 (CP2): Starting from the tip of each plate, a series of 4 curved sensilla

347   are found. They are arranged in line and show decreasing sizes from the most apical one (the

348   larger, 14.5 μm long and 6 μm of base diameter) to the proximal one (12.5 μm long and 3.5 μm of

349   base diameter) (Figure 4A). The cuticular shaft is slightly grooved along the longitudinal axis for

350   most of its length, although the grooves are not as evident as in CP1 (Figure 4C). Each CP2 ends in

351   a fine tip that can be absent in case of mechanical worn. The peg is sitting on an evident socket

352   within the cuticular wall of the plate (Figure 5F). TEM investigation revealed also for CP2 an

353   internal structure similar to the one described for CP1, *i.e.* the presence of a solid cuticular shaft,

354   devoid of pores (see inset in Figure 5F), a small internal lumen without sensory neurons, and a

355   single sensory neuron with a distal tubular body attached at the base of the peg (Figure 5G-I). The

356   peg itself is attached flexibly to the cuticle through a large socket with an abundance of

357   suspension fibres (Figure 5G-H).

17

358   TRICHOID SENSILLA (TS): At the very tip region of each ovipositor plate, we observed 3

359   small TS (Figure 4A). Two of them are located on the lower side of the plate (just below and almost

360   in between the largest CP2 and the apical CP1). The third TS is located apically on the medial

361   region of the plate, posteriorly to the apical CP1 (Figure 4B and Supplementary Figure S3). TS are

362   slender and finely tipped sensilla with a smooth cuticular shaft devoid of cuticular pores (12.5 μm

363   long and 1.5 μm of base diameter). These sensilla are sitting in the cuticular wall on distinct

364   sockets, under an angle that makes them run almost parallel to the plate cuticular wall itself. TEM

365   images revealed that TS are made of solid cuticle, there are no pores on the cuticle and no sensory

366   neurons entering the peg lumen (Supplementary Figure S3). A single sensory neuron was found to

367   be associated with TS sensilla, reaching the sensillum base (Supplementary Figure S3).

368   CHAETIC SENSILLA (CS): Each ovipositor plate shows the presence of a single CS. This

369   sensillum is positioned apically, located medially on the ovipositor plate right behind the most

370   apical CP1 and very close to TS. CS is long (38 μm) and slender (2.5 μm of base diameter), with a

371   typical curved shape and a very fine tip (Figure 4B and Supplementary Figure S3). It is sitting on a

372   large socket in the plate wall. Externally, the CS wall is smooth. Serial ultrathin sections revealed

373   that the CS cuticular shaft is made of solid cuticle and shows a central lumen not innervated

374   (Supplementary Figure S3). At the base, the shaft is attached to the cuticle and suspended through

375   an elaborated socket with numerous suspension fibres (Supplementary Figure S3). A single

376   sensory neuron was found in this area, it ends in a tubular body that attaches at the sensillum

377   base (Supplementary Figure S3).

378   *Neuronal staining of* D. suzukii *ovipositor pegs*

379   Anti-HRP was used as a marker to stain the neuronal membrane of the peripheral nervous system

380   (27). HRP labelling showed the presence of a single neuron innervating each CP1s and CP2s (Figure

381   4D-E and Supplementary Figure S4). Neurons stopped at the CP base level, as evidenced by TEM

18

382   imaging (Figure 5B and 5G). Altogether, these results suggest that the structures located on *D.*

383   *suzukii* ovipositor tip host mechanosensory and not chemosensory neurons. If this inference is

384   correct, then these neurons should not express any of the chemosensory-related genes, whose

385   expression was found in the abdominal distal tip by RNAseq analysis, but only mechanosensory-

386   related genes. To test this hypothesis, we moved to the model organism *D. melanogaster*, whose

387   available transgenic toolbox allowed to drive GPF expression in its ovipositor pegs tip (Figure 1).

388   *GAL4-driven GFP expression patterns of sensory genes in* D. melanogaster *ovipositor pegs*

389   In our systematic GAL4 expression analysis, we examined 5 genes encoding chemosensory

390   receptors (*Or43b*, *Gr66a*, *Ir47a*, *Ir62a,* and *Ir75d*) and 4 mechanosensory-related genes (*iav*,

391   *nompC*, *pain,* and *ppk*), which were found to be expressed by RNAseq analyses in both *D.*

392   *melanogaster* and *D. suzukii* abdominal distal tip. One mechanosensory-related gene (*nan*) which

393   was found to be expressed only in *D. melanogaster* and the odorant receptor co-receptor *Orco*,

394   whose expression was not found in any transcriptome. We also examined 7 GAL4 drivers

395   commonly used as markers for mechanosensory neurons and one pan-neuronal marker (28,29).

396   Using these GAL4 lines to drive 6GFP expression, we examined expression in the pegs and sensilla-

397   like structures present on the ovipositor tip of *D. melanogaster.*

398   No *GAL4* drivers representing any gene encoding chemosensory receptors or *Orco* showed

399   expression in sensory-like structure present in *D. melanogaster* ovipositor. Only *GAL4* drivers

400   representing the pan-neuronal marker *n-syb* (Figure 6A-C) and the sodium channel *ppk* (Figure 6D-

401   E) were expressed in the pegs, thus supporting a role in mechanosensory perception for *D.*

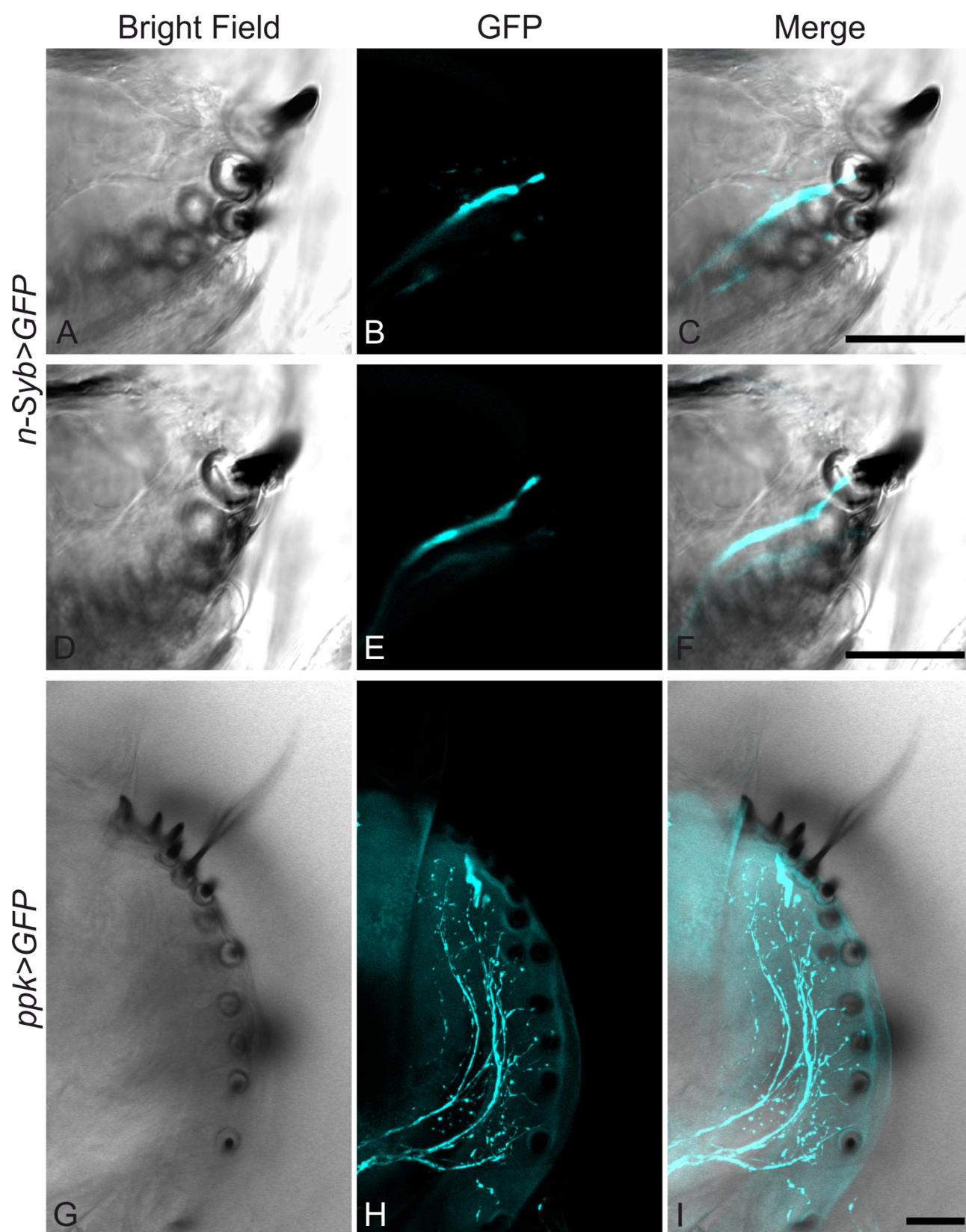402   *melanogaster* ovipositor pegs very similar to *D. suzukii*.

403

Figure 6. Gene expression at the _Drosophila melanogaster_ ovipositor pegs

(A-F) The pan-neuronal marker _n-syb_ showed a single neuron innervating all ovipositor pegs. **(G-I)** _ppk-GAL4_ driver that labels ovipositor pegs. All scale bars 20 μm.

DISCUSSION

409   Our results represent the first step toward a full molecular, anatomical, and physiological

410   characterization of sensory perception in the *D. suzukii* ovipositor and its closely related species. In

411   particular, these findings have (i) provided a qualitative overview of sensory genes expressed in

412   the abdominal distal tip of four *Drosophila* species with different ovipositor shapes and egg-laying

413   behaviours, have (ii) shown that pegs and sensilla present in the *D. suzukii* ovipositor tip have a

414   mechanosensilla-like structure, and have (iii) identified the sodium channel *ppk* as the sensory

415   receptor expressed in the *D. melanogaster* ovipositor pegs.

416   It has long been suggested that chemoreceptors are present in *D. melanogaster* female

417   genitalia (12,13,30) but to our knowledge, this hypothesis has never been fully proven. Our

418   transcriptome results have revealed that transcription of few chemoreceptors in the abdominal

419   distal tip is a common feature in *Drosophila* species. However, both the ultrastructure of *D. suzukii*

420   ovipositor's pegs and sensilla, as well as the GAL4-driven GFP expression in *D. melanogaster*

421   ovipositor's pegs pointed out that these sensory structures host mechanoreceptors but not

422   chemoreceptors. This suggests that the distal tip of *Drosophila* ovipositors is not used to evaluate

423   the chemical composition of the egg-laying substrate.

424   On the other hand, the presence of a common transcriptional core of several homologous

425   genes encoding for chemoreceptors suggests a conserved functional role of these proteins in the

426   abdominal distal tip of *Drosophila* species. Of these, *Gr66a* is among the best characterized *D.*

427   *melanogaster* GRs. It is normally expressed in broadly-tuned bitter-sensing neurons and its

428   canonical function is the detection of aversive compounds (31–34). Its expression is not restricted

429   to taste neurons (30), but it has been also identified in multidendritic neurons in the adult

430   *Drosophila* abdomen (35), which may be the origin of the transcripts detected in our study. Other

431   taste receptors for which we observed a transcriptional overlap among species are *Ir47a* and

21

432 *Ir62a*, whose expression in *D. melanogaster* have been identified by GAL4-driven GFP expression

433 in taste neurons in leg and labellum (36). Recently, *Ir62a* has been shown to be required together

434 with *Ir25a* and *Ir76b* in specific GRNs to taste Ca^{2+} in food (37). Consistent with this function, in the

435 *D. melanogaster* transcriptome we found the expression of those three transcripts. It has been

436 shown that, besides their main function in taste, some GRs (and likely some IRs) may have other

437 non-gustatory functions, such as the detection of internal ligands. For example, *Gr43a* is involved

438 in fructose detection in the *D. melanogaster* brain, and it is also expressed in the female uterus,

439 possibly to sense fructose presence in the seminal fluid (38). We did not detect expression of

440 *Gr43a* in any of the four transcriptomes generated in this study, but the expression of the other

441 taste receptors found may have similar physiological roles in internal sensing. Internal sensory

442 neurons are present in the reproductive tract of *D. melanogaster* to sense sex peptide (39,40), and

443 this may be another location where chemoreceptor transcripts are expressed. Interestingly, in all

444 *Drosophila* species we could detect the expression of *Or43b* homologs (which in *D. melanogaster*

445 is normally expressed in the ab8A antennal sensilla) but not of *Orco*, whose co-expression is

446 normally required for odour responsiveness in olfactory organs (41). As discussed before for taste

447 receptors, *Or43b* expression localized outside its characteristic pattern may be associated with the

448 detection of internal ligands, such as monitoring the internal nutritional state in the final

449 gastrointestinal tract or seminal fluid evaluation.

450   *Drosophila suzukii* females use mechanosensation to choose the oviposition site (9,42,43).

451 However, the localization of mechanosensory neurons responsible for this behaviour had not been

452 shown yet. Here, we propose that the sensilla and pegs present on the ovipositor tip of *D. suzukii*

453 are the sensory structures responsible to probe substrate stiffness for the egg-laying site selection.

454 It is also possible that these sensory structures work together with other sensory organs, providing

455 the information when the ovipositor has penetrated the substrate and peristaltic waves can start

456 to push down the egg. Our transcriptome analysis found the expression of several genes encoding

457 for receptors known to be expressed in proprioceptive neurons and nociceptors (44–49). The

458 expression of most of these transcripts seems to be conserved among the four *Drosophila* species

459 suggesting a conserved functional role. These genes represent the best candidates to be expressed

460 in the ovipositor pegs and sensilla of *D. suzukii*.

461 Mechanosensation is important also for *D. biarmipes* and *D. melanogaster* egg-laying site

462 choice (9), and we hypothesized that pegs and sensilla present in the tip of all *Drosophila*

463 ovipositors may host mechanosensory-like neurons similar to those observed in *D. suzukii*. Hence,

464 we used GAL4-driven GFP expression to identify genes expressed in *D. melanogaster* ovipositor

465 pegs. Expression of *nsyb* showed that these appendages are innervated by a neuron that stopped

466 at the base of the peg, likewise in *D. suzukii*, thus supporting the presence of mechanosensory

467 neurons. We also showed that these neurons express *ppk*, a mechanosensitive channel in

468 proprioceptive neurons (45).

469 Taken together, these findings represent an important step in delineating the function of

470 ovipositor pegs and sensilla in *Drosophila* species with different egg-laying behaviour. Our work

471 builds a necessary starting point for functional studies, to assess the relationship between the

472 structure and the function of the ovipositor sensilla, and to better understand how the egg-laying

473 behaviour diverged across *Drosophila* species. Experiments aimed to further elucidate the

474 mechanosensory system in the ovipositor of *D. suzukii* might allow for a development of

475 mechanosensory-based control strategies.

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