Structural and transcriptional evidence of mechanotransduction in the *Drosophila suzukii* ovipositor

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ABSTRACT

Drosophila suzukii is an invasive pest that prefers to lay eggs in ripening fruits, while most closely related *Drosophila* species exclusively use rotten fruit as oviposition substrates. This behaviour is allowed by an enlarged and serrated ovipositor that can pierce intact fruit skin, and by multiple contact sensory systems (mechanosensation and taste) that detect the optimal egg-laying substrates. Here, we tested the hypothesis that bristles present in the *D. suzukii* ovipositor tip contribute to these sensory modalities. Analysis of the ultrastructure revealed that four different types of cuticular elements (conical pegs type 1 and 2, chaetic and trichoid sensilla) are present on the tip of each ovipositor plate. All of them have a poreless shaft and are innervated at their base by a single neuron that ends in a distal tubular body, thus resembling mechanosensitive structures. Fluorescent labelling in *D. suzukii* and *D. melanogaster* revealed that pegs located on the ventral side of the ovipositor tip are innervated by a single neuron in both species. RNA-sequencing profiled gene expression of the terminalia of *D. suzukii* and of three other *Drosophila* species with gradual changes in their ovipositor structure (from serrated to blunt ovipositor: *Drosophila subpulchrella*, *Drosophila biarmipes* and *D. melanogaster*). Our results revealed few species-specific transcripts and an overlapping expression of candidate mechanosensitive genes as well as the presence of some chemoreceptor transcripts. These experimental evidences suggest a mechanosensitive function for the *D. suzukii* ovipositor, which might be of evolutionary importance across *Drosophila* species independently from ovipositor shape.

KEYWORDS

Spotted wing drosophila, mechanosensitive bristles, ultrastructure, comparative RNA-seq

INTRODUCTION

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 in 2008, and in Spain and Italy in 2009 (Cini et al., 2012; Hauser, 2011). Since then, it has spread quickly across several countries in both continents, where it is now a major threat for soft fruit production (Asplen et al., 2015). Differently from the majority of drosophilids, which thrive and lay eggs on already damaged or rotting vegetal substrates, *D. suzukii* can 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 *D. suzukii* with the aim to find innovative, effective, and eco-friendly methods to reduce its attacks (reviewed in (Cloonan et al., 2018)).

Several aspects of *D. suzukii* ecology and genetics have been analysed in a comparative framework across *Drosophila* species to identify key evolutionary innovations that allowed the transition from rotten to fresh fruit egg-laying behaviour (Atallah et al., 2014; Crava et al., 2016; Green et al., 2019; Hickner et al., 2016; Karageorgi et al., 2017; Muto et al., 2018; Ramasamy et al., 2016). The major morphological shift from rotten fruit-ovipositing *Drosophila* species (like the insect model *Drosophila melanogaster*) to fresh fruit-ovipositing species (*D. suzukii*) is the presence of an unusually enlarged and serrated ovipositor. Such structure is shared with the sister species *Drosophila subpulchrella* and allows the wounding of the intact skin of berries (Figure 1) (Atallah et al., 2014). 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) (Atallah et al., 2014). The ovipositor morphology correlates with the stiffness of the oviposition substrates, and serrated design facilitates egg-laying by attenuating the penetration force required to cut through the fruit skin; accordingly, *D. melanogaster* egg-laying is inhibited by stiff substrates, whereas *D. suzukii* has a broad tolerance, and *D. biarmipes* displays an intermediate behaviour (Karageorgi et al., 2017).

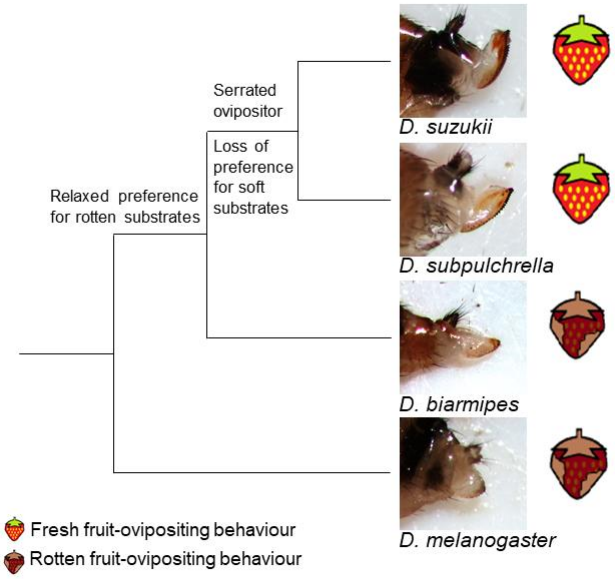

Figure 1 Evolutionary affinities and ovipositor shapes of *Drosophila suzukii* and other *Drosophila* species used in the study.

The currently most accepted scenario for fresh fruit-egg laying behaviour evolution (Karageorgi et al.,2017). Phylogeny is based on Atallah et al. (2014).

To detect substrate stiffness, insects rely on mechanosensitive receptors. In *Drosophila* spp., mechanosensitive organs are scattered throughout the body and can be of different type such as bristles, hair plates, campaniform sensilla, and chordotonal organs (Karkali and Martin-Blanco, 2017). Bristles are the main touch receptors, while the other three organs are proprioceptors, *i.e.* mechanosensitive structures that monitor the positions, and relative movements of the fly's own body parts (Keil and Steinbreicht, 1984; Tuthill and Wilson, 2016). A feature common to all mechanosensitive sensilla is the presence of mechanosensitive neurons (MNs), which have an outer dendritic segment that ends in a distal tubular body in contact with the flexible base of the sensillum shaft (Walker et al., 2000). Pure mechanosensitive sensilla accommodate a single MN whereas in poly-innervated taste sensilla the MN is coupled with multiple gustatory neurons and four non-neural cells (trichogen cell, tormogen cell, thecogen cell and glial cell) (Falk et al., 1976; Stocker, 1994). While the tip of mono-innervated mechanosensitive sensilla is smooth, the one of taste sensilla has

one or few pores that allow dendrites of gustatory neurons to get in contact with external fluid (Falk and Atidia, 1975). In *D. melanogaster*, poly-innervated taste sensilla have been found in the labial palps, the pharynx, the legs and the wings, and their presence on the external genitalia was suggested (Stocker, 1994). In *D. suzukii*, a single study describes the external morphology of the ovipositor tip (Atallah et al., 2014), but no information on the ultrastructrure of sensilla and pegs (which would provide clues for the physiological function of these structures) are available yet. Beside mechanosensation, also the assessment of the chemical composition of the oviposition substrate contributes to egg-laying behaviour in insects (Aranha and Vasconcelos, 2018). Accordingly, experiments with transgenic *D. suzukii* impaired in olfaction showed that they preferred ripe over rotten strawberry puree as wild-type flies when allowed to get in touch with the oviposition substrate (Karageorgi et al., 2017); thus in *D. suzukii* the contact chemosensory system (*i.e.* taste) is involved in the process for selecting the optimal egg-laying site, together with mechanosensation. Since the *D. suzukii* ovipositor pierces the fruit skin and comes into contact with fruit flesh, we hypothesize that this organ may carry taste sensilla, which contribute to the egg-laying decision. Alternatively, it is possible that D. *suzukii* ovipositor carries pure mechanosensitive sensilla, which may transfer information on the substrate stiffness and roughness, and on whether the ovipositor has penetrated it.

To select among these hypotheses, we analysed the ultrastructure of the pegs and sensilla present on *D. suzukii* ovipositor tip. We then used fluorescent antibody and GAL4 drivers to label neurons reaching these structures in both *D. suzukii* and *D. melanogaster*. Lastly, we performed RNA-seq experiment to understand if terminalia gene expression overlaps among *Drosophila* species characterised by gradual changes in their ovipositor structure (from blunt to serrated ovipositor) (Figure 1). Our results reveal the presence of mechanosensitive organs in *D. suzukii* ovipositor and suggest that mechanotransduction in ovipositor is conserved among *Drosophila* species independently from ovipositor shape.

METHODS

Insects

Insects used for transcriptomics, immunohistochemistry, and electron microscopy were taken from laboratory colonies maintained at the Fondazione Edmund Mach, S. Michele all'Adige (Italy). *Drosophila suzukii* and *D. melanogaster* strains were founded with individuals collected in 2010 in the Trento province (Italy) and periodically refreshed with insects caught from the same field sites. *Drosophila biarmipes* (genotype Dbii\wild-type, stock # 14023-0361.09) and *D. subpulchrella* (Dspc\wild-type, stock # 14023-0401.00) strains were obtained from the Drosophila Species Stock Center (San Diego, CA, US) in 2011. The four *Drosophila* species were reared on a standard diet (https://stockcenter.ucsd.edu/info/food_cornmeal.php), maintained at 23–25 °C, 65 ± 5% relative humidity, and under a 16:8 h light:dark photoperiod.

Drosophila melanogaster transgenic strains used for imaging were obtained from the Bloomington Drosophila Stock Center (BDSC) (Bloomington, IN, US) and reared under the same conditions as described above.

Drosophila suzukii *ovipositor scanning electron microscopy*

Adult females of *D. suzukii* were anaesthetized by exposure to cold temperatures (-18°C) until death, then they were immediately soaked in 60% alcohol. The ovipositor of each individual was dissected from the abdomen. Specimens were dehydrated in a series of graded ethanol, from 60% to 99%, 15 min for each step. After dehydration, 99% ethanol was substituted with pure HMDS (Hexamethyldisilazane, Sigma-Aldrich) and the specimens were allowed to dry under a hood at room temperature (RT); this step was repeated twice. Up to five samples were mounted on aluminium stubs, with different orientations, in order to obtain a clear view on the ventral and lateral sides of the ovipositor. Mounted specimens were gold-sputtered using a Balzers Union SCD 040 unit. The observations were carried out using a Philips XL 30 scanning electron microscope (SEM) operating at 7-10 KV, working distance 9-10 mm.

Drosophila suzukii *ovipositor transmission electron microscopy*

Ten *D. suzukii* female individuals were anesthetized by exposure to cold temperatures (-18°C) for 60 s, then immediately immersed in a solution of glutaraldehyde and paraformaldehyde (PFA) 2.5% in 0.1 M cacodylate buffer (pH 7.2-7.3) plus 5% sucrose. The ovipositor was detached from the abdomen, reduced in size to help fixative penetration, and left at 4°C for 24 h. Then, the specimens were washed twice in cacodylate buffer for 10 min, post-fixed in 1% OsO_4 for 1 h at 4°C, and rinsed in the cacodylate buffer. They were dehydrated in graded ethanol series from 60% to 99% and embedded in Epon-Araldite with propylene oxide as bridging solvent. Thin sections were taken with a diamond knife on an LKB Bromma ultramicrotome and mounted on formvar-coated 50 mesh grids. Then, sections on grids were stained with uranyl acetate (20 min, RT) and with lead citrate (5 min, RT). Finally, the sections were imaged with a Philips EM 208 transmission electron microscopy (TEM). A digital camera MegaViewIII (SIS) provided high-resolution images.

Drosophila suzukii *ovipositor immunohistochemistry*

Drosophila suzukii adult females were anesthetized using CO_2. Abdominal distal tips were cut with a razor blade and fixed in 4% PFA in phosphate-buffered saline (PBS, pH 7.4) (Sigma-Aldrich) for 40 min on ice. Samples were then washed three times with PBS for 20 min, incubated in 10% sucrose (Sigma-Aldrich) solution, and kept rotating for 1 h at RT. Sucrose solution was increased to 25%, and samples were kept rotating overnight at 4°C. Samples were then embedded in OCT (OCT mounting medium Q PATH, VWR), and mounted on a sample holder. Sections of 15 µm thickness were cut with a CM 1510-3 cryostat (Leica) and collected on a SuperFrost glass slide (ThermoFisher Scientific). Slides were washed in PBS-T (PBS + 0.1% Triton-X-100, Sigma-Aldrich) for 5 min, and then blocked in 5% normal goat serum (Sigma-Aldrich) in PBS-T for 30 min. Anti-horseradish peroxidase (HRP) cyanine-conjugated antibodies (Cy3 AffiniPure Rabbit Anti-HRP, Jackson ImmunoResearch) diluted 1:300 were used to stain the neurons. Slides were kept in a moist chamber at 4°C overnight in dark. The next day, antibodies were removed, and the slides were washed three times with PBS-T for 5 min and then mounted using Vectashield (Vector Laboratories).

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

The native GFP signal was observed at the level of the ovipositor of females expressing the super bright 6xGFP UAS-reporter (UAS-6xGFP; BDSC accession number 52262) under the pattern of the pan-neuronal nsyb-GAL4 driver (GMR57C10-GAL4; BDSC accession number 39171). Flies were anesthetized using CO_2, abdominal distal tips were cut, embedded in 70% glycerol and immediately imaged.

Confocal imaging

Images were acquired using a Leica TCS SP8 confocal microscope, equipped with HC PL FLUOTAR 20x/0.55 DRY and HC PL APO CS2 63x/1.40 OIL objectives. For *D. suzukii*, Cy3 excitation was performed using a 522-nm solid-state laser, and fluorescence was detected at 561-591 nm. Single images of 1024 × 1024 pixels (pixel size of 0.072 × 0.072 μm) and stacks with z-steps of 2 μm were acquired. For *D. melanogaster*, GFP excitation was performed using a 488-nm solid-state laser, and fluorescence was detected at 499-524 nm. Stacks of 1024 × 1024 pixels (pixel size of 0.06 × 0.06 μm) optical section were generated with a z-interval of 2 μm. Images were analyzed using Fiji-ImageJ software (Schindelin et al., 2012).

RNA extraction and sequencing

RNA was extracted from the terminalia of 3- to 10-day old mated females. Dissection was done with forceps and included both the genitalia and the analia (Supplementary Figure S1). Dissected tissues were stored at −80 °C in RNAlater (ThermoFisher Scientific) until extraction. Each species sample was composed of RNA extracted from around 60-80 individuals. Samples were homogenized using TissueLyser (Qiagen) 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 to concentrate samples. The total RNA (∼1 μg/sample) was sent to Beckman Coulter Genomics (Danvers, MA USA) for library preparation and Illumina sequencing. Library preparation was carried out through polyA + selection, and paired-end (PE) sequencing was run on an Illumina HiSeq 2500 System with V3 chemistry that generated 100 bp reads. Raw reads are accessible from the Genbank SRA database (BioProject number PRJNA526247) (Supplementary Table S1).

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

Raw reads were trimmed with Trimmomatic (Bolger et al., 2014). Both paired and unpaired reads were used for a *de novo* assembly of the transcriptome for each species with Trinity v2.0.6 (Grabherr

et al., 2011), using the normalization step and flag --min_kmer_cov 2. The transcriptome quality was checked by mapping the paired reads against the assembled transcriptome with Bowtie2 with default parameters (Langmead and Salzberg, 2012). The four transcriptomes were annotated using Standalone Blast+. Blast searches were run with the command blastx using the predicted proteins from the *D. melanogaster* genome (version r6.25) as the database. The top hit for each sequence was retained when the E-value was less than 1×10^{-10}. PANTHER version 14.0 (Mi et al., 2017) was used to extract gene ontology (GO) terms (Panther GO-Slim) for each annotated transcriptome. Venn diagrams were created using Venny 2.1.0 (Oliveros, 2015).

Annotation of mechanosensory channels described in *D. melanogaster* have been perfomed with iterative blastx searches against *D. suzukii* (accession number AWUT00000000) and *D. biarmipes* genomes (accession number AFFD00000000) using *D. melanogaster* sequences as query. The intron–exon structure was manually determined using BioEdit (Hall, 1999) and full-length coding sequences were extracted. Quantification of mechanosensory channel expression as well as as of chemoreceptor genes, which we previously annotated in *D. suzukii* and *D. biarmipes* (Crava et al., 2016; Ramasamy et al., 2016) was done mapping reads to the gene set for each species except for *D. subpulchrella*, where we use the gene set from its sister-species *D. suzukii*. Mapping was perfomed with Bowtie2 wth default parameters and transcript per million (TPM) were quantified by RSEM (Li and Dewey, 2011).

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

Expression of chemosensory receptor genes in the *D. suzukii* terminalia by RNA-seq analysis was confirmed by reverse transcription PCR (RT-PCR). *Orco* and *Gr64*, which were not found to be expressed by RNA-seq, were used as negative control and genomic DNA as positive control. The used primers are listed in Supplementary Table S2. RNA was extracted with Trizol and treated with DNAse I as described before. 1 μg RNA was then retrotranscribed to cDNA with SuperScript III Reverse Transcriptase (ThermoFisher Scientific) following the manufacturer's protocol. To control

for genomic DNA contamination, RNA underwent a parallel mock reverse transcription step, in which the reverse transcriptase was omitted. Amplifications were carried out with GoTaq Green Master Mix (Promega) in a final volume of 25 µl containing 1 µl of cDNA diluted 1:10 and 0.4 µM of each primer at the following 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 at 72°C, followed by a final elongation step of 5 min at 72°C. PCR amplicons were run on 1% agarose gel stained with Midori Green Advance (Nippon Genetics).

RESULTS AND DISCUSSIONS

D. suzukii *ovipositor carries four types of mechanosensilla-like structures*

The ovipositor of *D. suzukii* is positioned at the very tip of the abdomen, and, at rest, is held hidden within the last abdominal segments. With a gentle pressure on the abdomen it is possible to expose the ovipositor, which comprises two elongated sub-triangular plates ending in a tip. The ovipositor of *D. suzukii*, as well as of its sister species *D. subpulchrella*, has been defined as "serrated" because of the presence of well-evident modified bristles, mostly arranged along its outer margin (*i.e.* the ventral side of the ovipositor, when considered in its resting position), giving to the ovipositor itself a jagged profile (Supplementary Figure 2) (Atallah et al., 2014). The *D. suzukii*'s ovipositor length and morphology were analysed in a comparative framework in previous studies (Atallah et al., 2014; Green et al., 2019). Of them, Atallah et al. (2014) described the external morphology and the number of bristles in *D. suzukii* and *D. subpulchrella*. Yet, ultrastructure observations or functional studies are lacking, and no information about the role these structures is known. Here, we fill the gap analysing the modified bristles present in the distal tip of the ovipositor, which are thought to come into contact with the fruit flesh (Supplementary Figure 2), whereas the more proximal unmodified bristles have not been analysed (see Atallah et al., 2014 for bristle classification). We identified four types of cuticular elements: conical pegs type 1 (CP1, also defined modified lateral bristles by Atallah et al., 2014), conical pegs type 2 (CP2, also defined modified marginal bristles by Atallah et al., 2014), and two categories of previously undescribed apical sensilla: trichoid sensilla (TS), and chaetic

sensilla (CS) (Figure 2, Supplementary Figure 2). The number, placement, and types of bristles on drosophilids ovipositor valves as well as the ovipositor size and shape vary greatly among species (Craddock et al., 2018). In general, all *Drosophila* species seem to have a more or less regular row of sensilla distributed on the ventral side of each valve (also called thorn bristles in *D. melanogaster*, (Chen and Baker, 1997)) and a distinctive chaetic sensilla projecting from the ventral side of the ovipositor (Craddock et al., 2018). In *D. melanogaster,* this latter is referred to as the "long bristle" (Taylor, 1989a). Other features which are not displayed by all ovipositors are dorsal or lateral sensilla and microbristles or trichoidea sensilla into the ovipositor apex (Craddock et al., 2018; Taylor, 1989a).

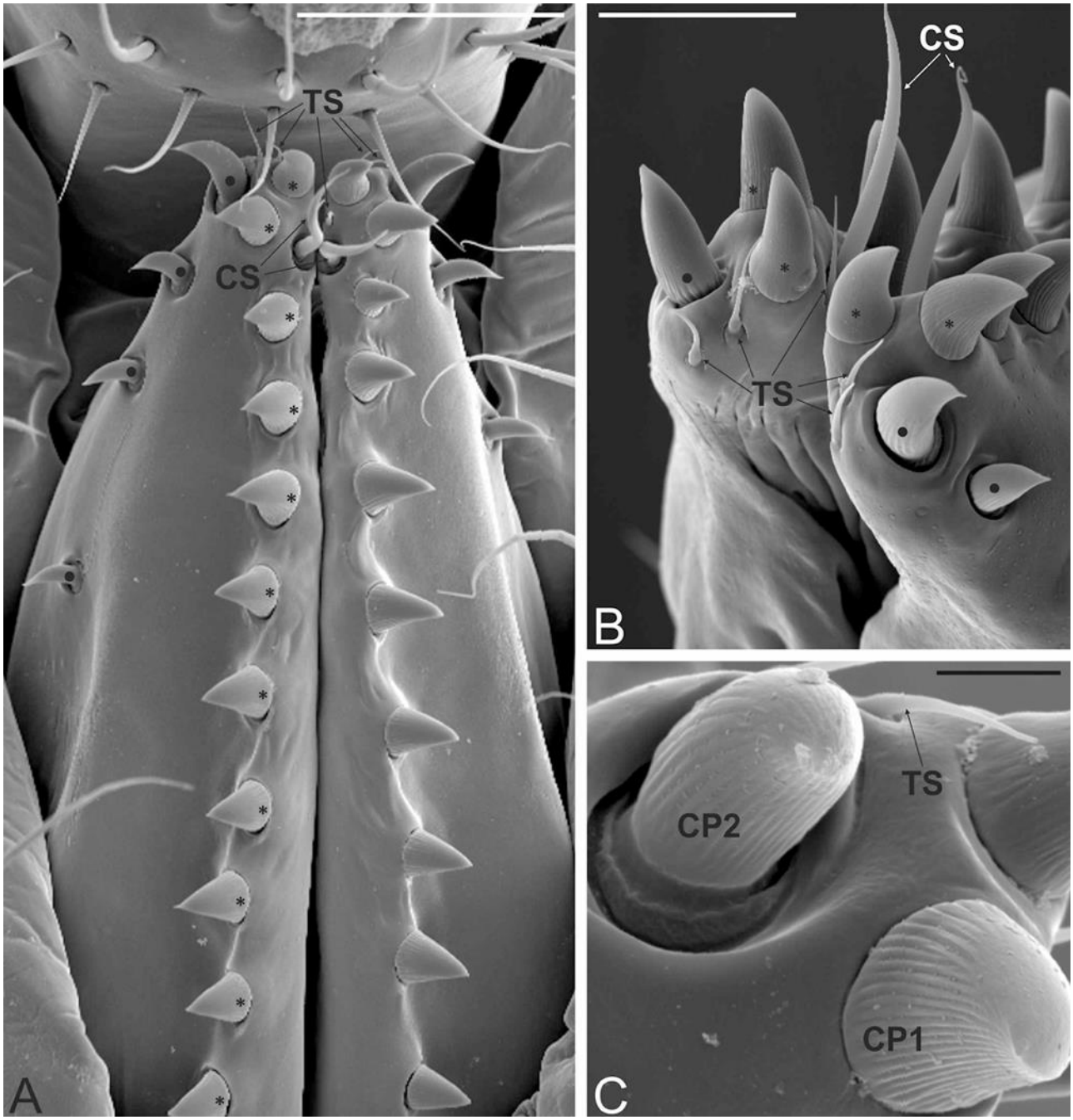

Figure 2. *Drosophila suzukii* ovipositor pegs and sensilla

(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 of 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. Scale bars: A, 50 µm; B, 20 µm; C, 5 µm.

Our observations estimate an average of 17 CP1s (n = 50, SD =0.99) sitting on narrow sockets in the cuticle on the ventral margin of each ovipositor plate arranged in a single row. Their number is slightly higher than what is reported by Atallah et al (2014) in two different *D. suzukii* strains, suggesting that the number of such structures may vary among individuals. Likewise, in *D. melanogaster*, the observed number of thorn bristles (the structures homologous to CP1s) varies from 11 to 17 (Chen and Baker, 1997; Taylor, 1989a). CP1 number did not differ significantly between *D. suzukii* and *D. subpulchrella* (Atallah et al., 2014). Our observations revealed that CP1s are about 15 µm long with a base diameter of 10 µm and are characterised by a cuticular shaft slightly bent towards the external side of the plate (Figure 2A). The cuticle is grooved externally all along (Figure 3A). Each structure ends in a sharp tip, although in some specimens the tip appears worn, having a blunt shape. The analysis of ultrathin sections shows that the internal structure is characterised by a solid, poreless cuticular shaft (Figure 3B). Micrographs taken at the level of the medial peg show a thick and continuous cuticular wall with a small lumen without sensory neurons (Figure 3C). Imaging at the socket level shows the presence of a single sensory neuron embedded in an electron-dense dendrite sheath, and ending in a tubular body (Figure 3D and E). The tubular body is located at the base of the peg, where the socket with suspension fibres is evident (Figure 3B). In insects, the tubular body is a cellular feature of mechanoreceptors since it is required for mechanotransduction and its absence causes a loss of mechanosensitivity (Erler, 1983; Keil and Steinbreicht, 1984; Marshall and Lumpkin, 2012). In *D. melanogaster* the tubular body is typical of type I sensory neurons that construct cilia or flagella, which include bristle mechanoreceptors and propioreceptors such as chordotonal organs and campaniform sensilla (Gillespie and Walker, 2009; Kernan, 2007). All these evidences strongly suggest that CP1s are mechanosensilla.

In *D. melanogaster*, the anti-HRP staining of the ventral thorn bristles corresponding to *D. suzukii* CP1s provided evidences of their sensory nature (Taylor, 1989a, 1989b). Labelling of *D. suzukii* ovipositor tip with anti-HRP is consistent with ultrastructure observations and shows the presence of a single sensory neuron that terminates at the base of each CP1 (Figure 4). *D. melanogaster* thorn bristles were labelled with the pan-neuronal marker *n-syb* revealing the presence of a single neuron ending at the base of each bristle, likewise to the anti-HRP staining in *D. suzukii* (Figure 4). This anatomical similarity between *D. suzukii* and *D. melanogaster* suggests that also in the latter species, ventral ovipositor bristles could be mechanosensitive structures. Hence, CP1 function could be conserved in the ovipositor of *Drosophila* species independently of the ovipositor shape. This is consistent with results from Craddock et al. (2018) that did not identify any pore on the surface of ventral bristles in 41 Hawaiian drosophilids with dramatic differences in ovipositor morphology. However, further functional and structural studies are needed to rigorously falsify this hypothesis in *D. melanogaster* and other *Drosophila* species.

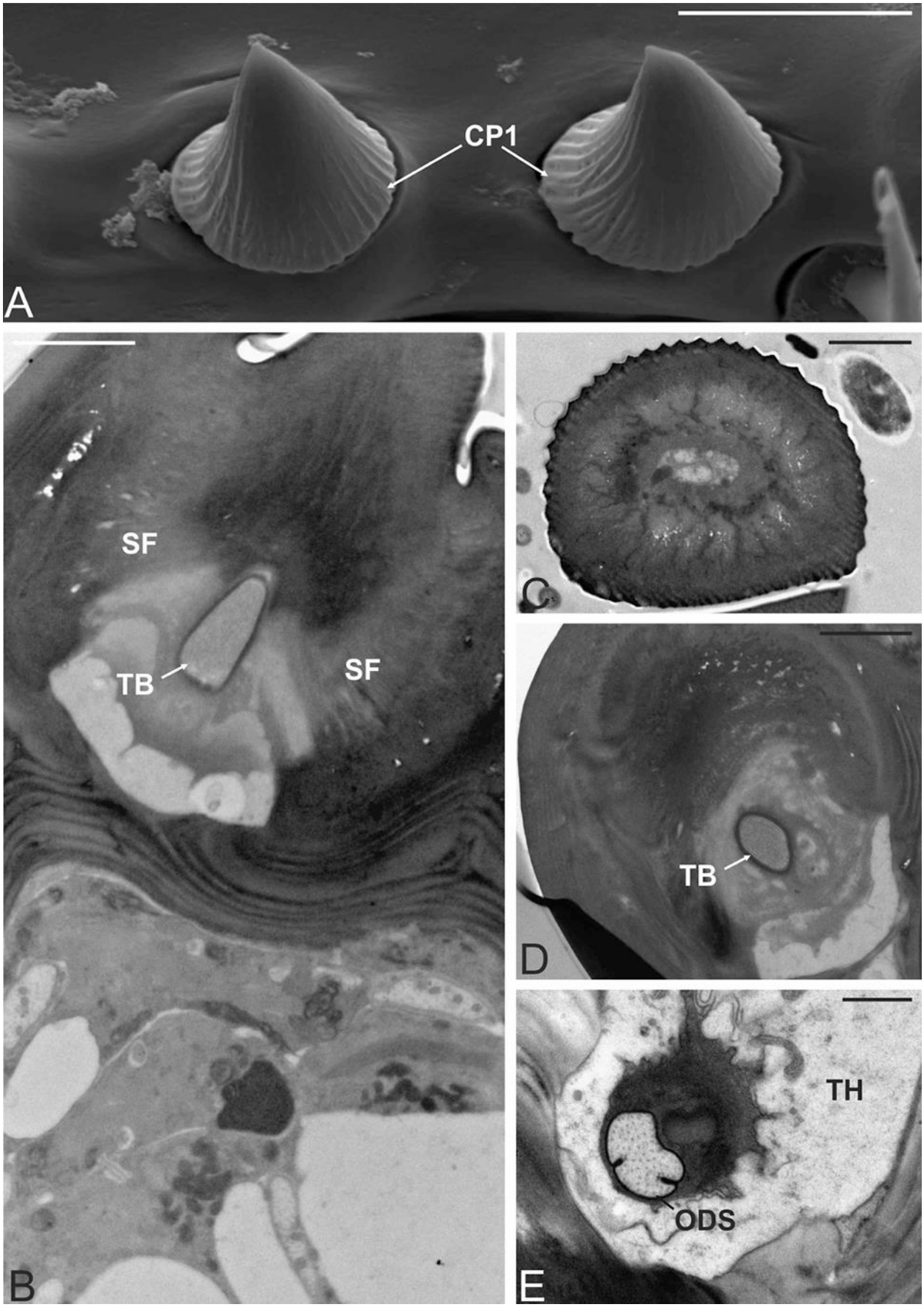

Figure 3. Micrographs showing details of the conical pegs type 1 of the *Drosophila suzukii* ovipositor.

(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, holding the peg and giving flexibility to the structure. The single sensory neuron associated with the CP1 terminates in a tubular body (TB) ending just at the base of the peg. **(C-E)** Serial TEM micrographs of a CP1 cross sections, taken at different levels, show the solid cuticular structure of the peg **(C),** the presence of the tubular body (TB) at the socket level **(D),** and the outer dendritic segment (ODS) of the sensory neuron enclosed by the thecogen cell (TH) **(E)**. Scale bars: A, 10 µm; B-D, 2 µm; E, 1 µm.

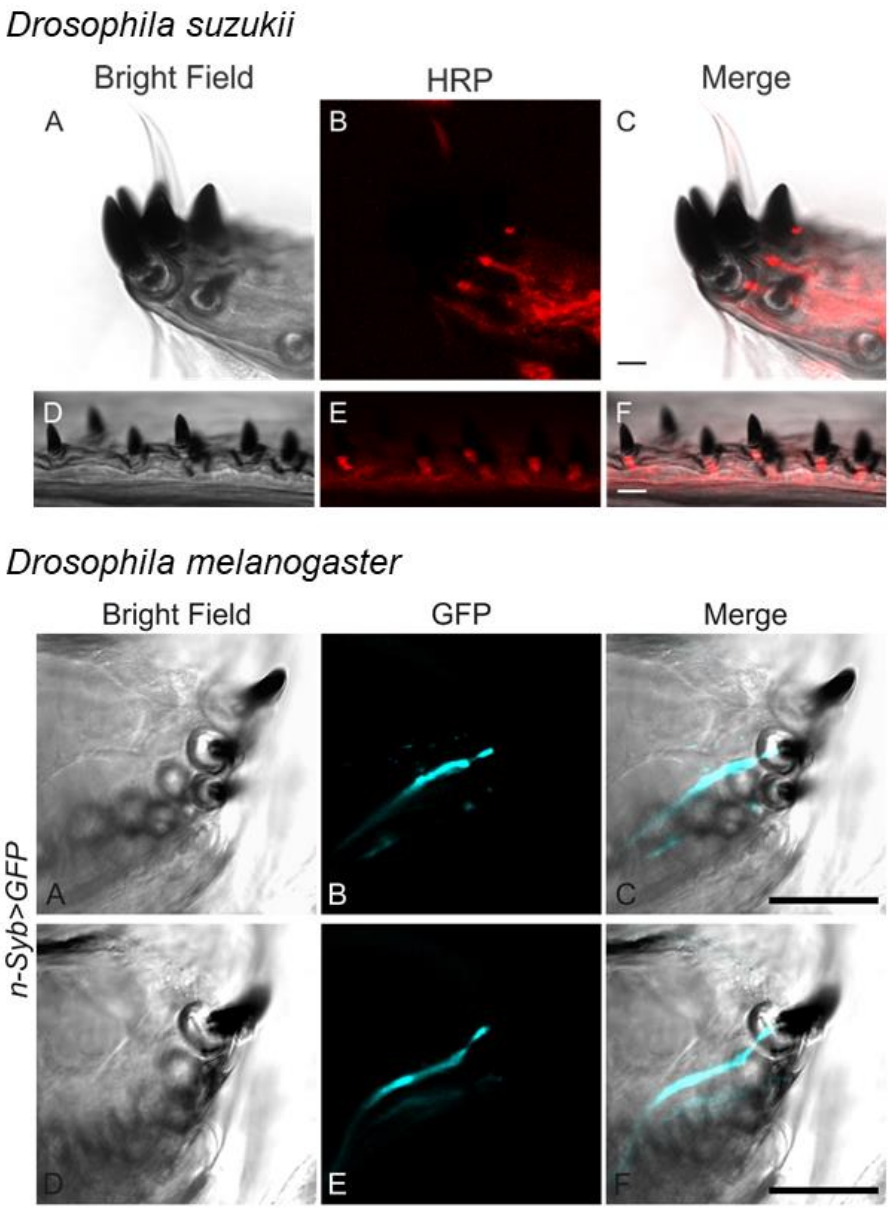

Figure 4. Conical pegs are innervated by single neurons in both *Drosophila suzukii* and *D. melanogaster* ovipositors.

Upper panel: Immunostaining of cryosection of the *D. suzukii* ovipositor plate: **(A and D)** bright-field, **(B and E)** counter staining with anti-horseradish peroxidase (HRP) to visualize the neuron, **(C and F)** merged pictures.

Scale bars: 10 µm. Lower panel: The pan-neuronal marker *n-syb* showed a single neuron innervating all ovipositor pegs in *D. melanogaster*. **(A and D)** bright-field, **(B and E)** green fluorescent protein (GFP) visualization, **(C and F)** merged pictures. Scale bars: 20 µm.

Our observations reveal four to five CP2s arranged in line starting from the tip of each ovipositor plate (Figure 2A). They are smaller than CP1s and show decreasing sizes from the most apical (14.5 µm long and 6 µm of base diameter) to the proximal one (12.5 µm long and 3.5 µm of base diameter). Atallah et al. (2014) reported that size differences between CP1s and CP2s are less pronounced in *D. subpulchrella* than in *D. suzukii*, while in *D. melanogaster* no bristles equivalent to CP2s have been found (Taylor, 1989a). The cuticular shaft of CP2 is slightly grooved along the longitudinal axis for most of its length, although grooves are not as evident as in CP1 (Figure 2C). Each CP2 ends in a fine tip that is absent in case of mechanical abrasion. The peg is sitting on an evident socket within the cuticular wall of the plate (Figure 5A). TEM investigation revealed an internal structure similar to CP1, *i.e.* the presence of a solid cuticular shaft, devoid of pores (see inset in Figure 5A), a small internal lumen without sensory neurons, and a single sensory neuron with a distal tubular body attached at the base of the peg (Figure 5B-D). The peg itself is attached flexibly to the cuticle through a large socket with an abundance of suspension fibres (Figure 5B). Anti-HRP staining highlights the presence of a single sensory neuron that stops at the base of each CP2 (Figure 4). All these evidences suggest that CP2s are mechanosensitive structures likewise CP1s.

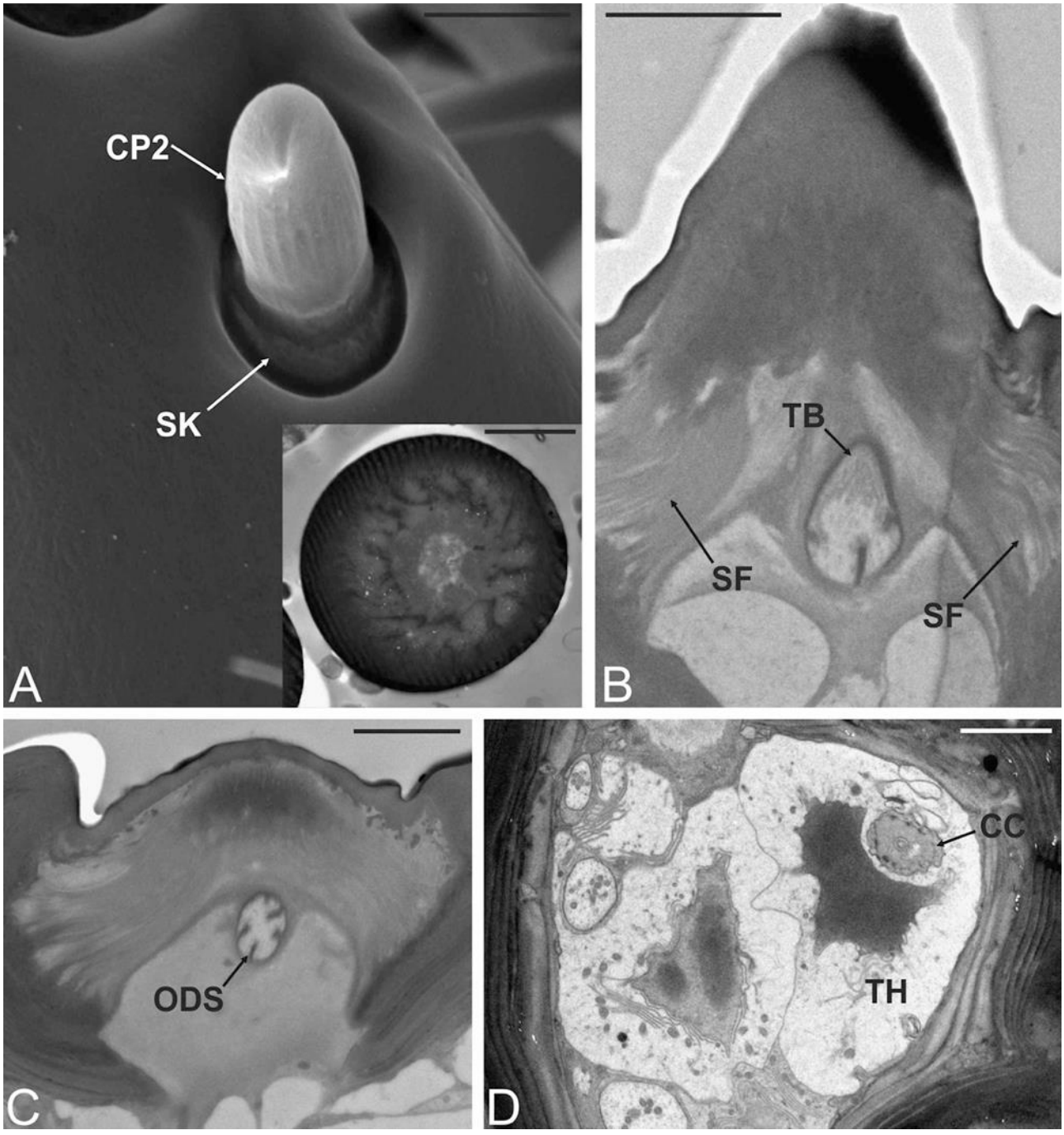

Figure 5. Micrographs showing details of the conical pegs type 1 of the *Drosophila suzukii* ovipositor (A) SEM ventral view of the ovipositor plate, showing one of the conical pegs type 2 (CP2), with a slightly grooved cuticle and a sharp tip. Noticeable is also the large socket (SK) on which the CP2 is sitting in the cuticular wall of the ovipositor plate. The inset in **(A)** shows the TEM micrograph of a CP2 cross section, taken at half of the length of the peg: the peg is made of solid, thick cuticle, and presents a reduced lumen, devoid of sensory neurons. **(B-D)** Serial TEM micrographs of a CP2, longitudinally and cross sections taken at different levels. They show in **(B)** the base of a CP2 with a large flexible socket and several suspension fibres (SF). The single sensory neuron ends in a tubular body (TB) at the base of the peg. In **(C)** the large socket is visible, as well as the outer dendritic segment (ODS) of the sensory neuron. In **(D)** a cross section is imaged at a lower level

respect to the previous: the sensory neuron appears at the ciliary constriction level (CC), and it is enclosed by the thecogen cell (TH). Scale bars: A, 5 µm; inset in A, 2 µm; B-D, 2 µm.

At the apex of each *D. suzukii* ovipositor plate, there are three small TS (Figure 2B). Two are located on the dorsal side of the plate, whereas the third one is located apically (Figure 6A). In *D. melanogaster*, three trichoid ovisensilla have also been reported (Taylor, 1989a). In *D. suzukii*, they are slender and finely tipped sensilla with a smooth cuticular shaft devoid of cuticular pores (12.5 µm long and 1.5 µm of base diameter). These sensilla are sitting in the cuticular wall on distinct sockets, running almost parallel to the plate cuticular wall itself. TEM images reveal that TS are made of solid cuticle, there are no pores on the cuticle and no sensory neurons entering the peg lumen (Figure 6B). A single sensory neuron is associated with each TS, reaching the sensillum base through a distal tubular body (Figure 6C and Supplementary Figure S3). In *D. melanogaster*, these three trichoidea sensilla appeared innervated by multiple neurons and have been hypothesized to be endowed with chemosensory functions (Taylor, 1989b). However, our observations in *D. suzukii* clearly discard a role in chemosensation.

Each ovipositor plate shows the presence of a single CS inserted into the inner face of each valve tip. It is long (38 µm) and slender (2.5 µm of base diameter), with a typical curved shape and a very fine tip (Figure 6A). It is sitting on a large socket in the plate wall. Externally, the CS wall is smooth. Serial ultrathin sections revealed that the CS cuticular shaft is made of solid cuticle and shows a central lumen without neurons (Figure 6D). At the base, the shaft is attached to the cuticle and suspended through an elaborated socket with numerous suspension fibres (Figure 6E). A single sensory neuron ends in a tubular body that attaches at the sensillum base (Figure 6F). This is a typical feature of mechanosensitive-like organs, and together with the absence of pores on the surface of the *D. suzukii* CS clearly points out a likely function in mechanotransduction. However, in two groups of Hawaiian *Drosophila* species, the apical CS (referred to as "long subapical sensillum") is uniporous, thus suggesting that in some species it may have taste function that helps to exploit specific ecological microniches (Craddock et al., 2018).

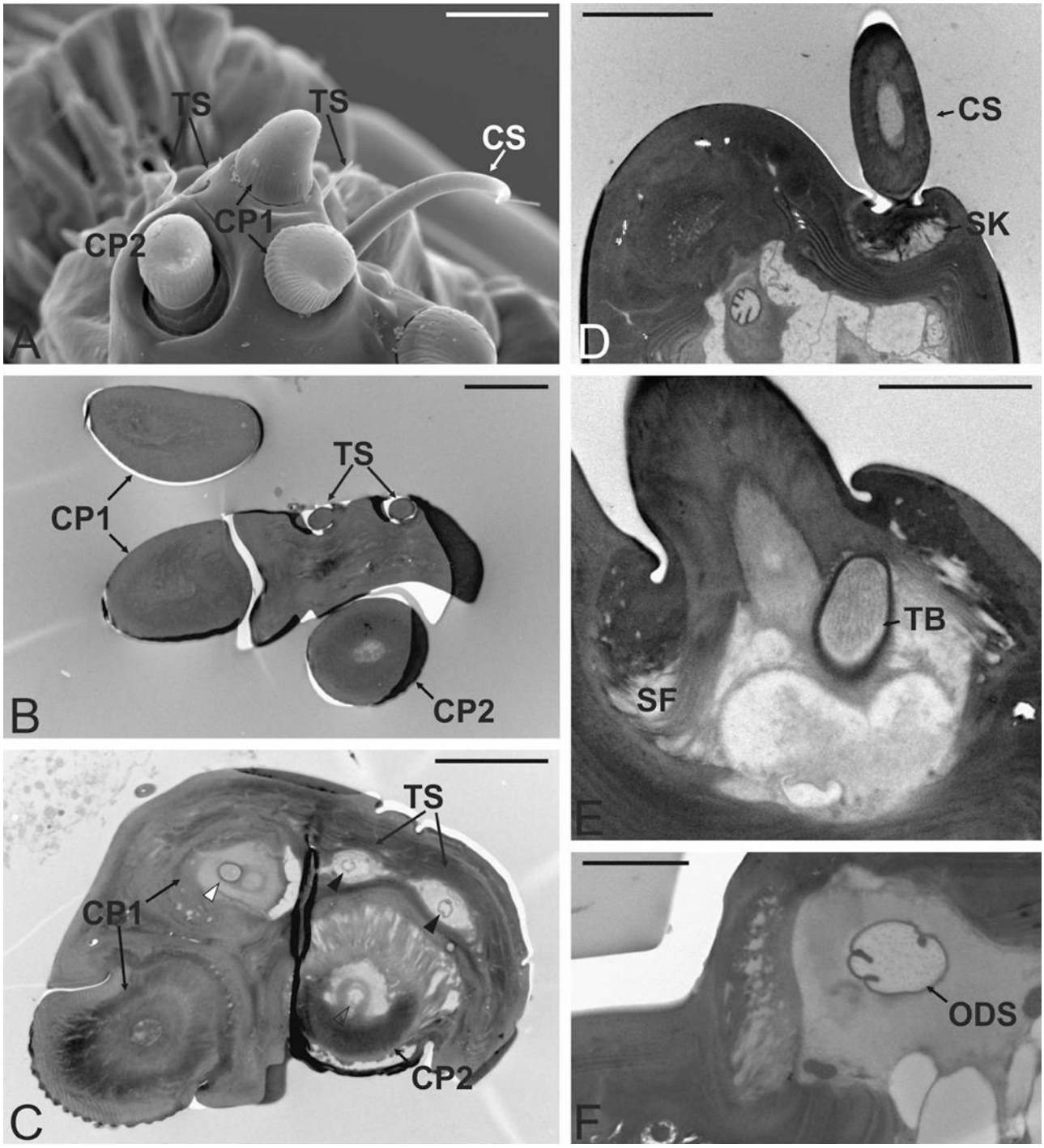

Figure 6. Micrographs showing details of the trichoid sensilla and chaetic sensilla.

(A) Scanning electron microscopy (SEM) picture of the ovipositor plate tip showing the three trichoid sensilla (TS) and the single chaotic sensilla (CS). **(B-C)** Serial transmission electron microscopy (TEM) cross sections of the ovipositor plate. In **(B)** the section is taken most apically and shows the two TS made of solid cuticles entering the cuticular wall, no sensory neurons were detected at this level. In **(C)** the two TS are pictured more proximally, the peg is no longer visible but two sensory neurons (one per each TS) are visible (black arrowheads). White and blank arrowheads show the sensory neurons associated with conical pegs type 1 (CP1)

and type 2 (CP2), respectively**. (D-F)** Serial TEM longitudinal sections showing the main ultrastructural features of a CS: in **(D)** the CS is taken at the socket level (SK) and shows a thick cuticle with a central lumen without sensory neurons; in **(E)** a single sensory neuron ending in a tubular body (TB) inserted at the CS base is visible. The socket presents numerous suspension fibres (SF). In **(F)** the outer dendritic segment (ODS) of the sensory neuron is visible. Scale bars: A, 10 µm; B-C-D, 5 µm; E-F, 2 µm.

RNA-seq characterization of the terminalia of four Drosophila *species*

Since variation in egg-laying sites and ovipositor morphology among *Drosophila* species exists, we were interested in determing whether there is a corresponding level of variation in gene-expression comparing *de novo* assembled transcriptomes from the terminalia of four *Drosophila* species with different ovipositor shapes and egg-laying behaviors (Figure 1). Female terminalia can be divided in two parts, genitalia and analia. The genitalia comprise the external ovipositor plates and the internal ducts of the reproductive system, whereas the analia are made by the external anal plates and the posterior hindgut (Taylor, 1989a). Thus, our RNA-seq analysis reflects transcripts expressed in all of these tissues.

Illumina RNA-seq libraries from the terminalia of four *Drosophila* species generated an average of 60M (±2.4M SD) 100 bp paired-end reads that led to four *de novo* assembled transcriptomes with contig counts ranging from 31,315 (*D. subpulchrella*) to 40,162 (*D. suzukii*) (Supplementary Table S1). Although reference genomes for three out of four *Drosophila* species studied were available, in the present study, transcripts were *de novo* assembled instead of mapping to genomic sequences because of the dramatic differences in genome completeness and resolution that would affect the detection of several transcripts. Our strategy has yelded four assemblies of comparable number of contigs (34884 ± 4017), N50 (2728 ± 4588) and percentage of reads successfully aligned to the assemblies (80 ± 6) (Supplementary Table S1). Thus, they are appropriate for a comparative analysis aimed to study transcript composition among assemblies, and presence or absence of specific transcripts. We used blastx to identify homologous genes in *D. melanogaster* and assign gene ontology (GO) terms to our contigs. On average, 70% of contigs from each of the four

transcriptomes has a blastx hit against the *D. melanogaster* predicted proteome (Supplementary Dataset S1). Unique hits retrieved for each species vary from 8,537 (*D. subpulchrella*) to 9,634 (*D. melanogaster*). Of these, 7,294 hits are common among the four *Drosophila* species, and represent the conserved transcriptional core for the *Drosophila* terminalia (Figure 7A). GO analysis shows that the common terminalia genes are mostly involved in cellular processes (32%), metabolic processes (29%), and biological regulation (15%) (Figure 7B). Species-specific hits are few, namely 163 for *D. suzukii*, 131 for *D. subpulchrella*, 187 for *D. biarmipes*, and 682 for *D. melanogaster*, respectively. Unique *D. suzukii* hits have 811 unique GO terms (Figure 7C, and Supplementary Dataset S2). Among them there are contigs homologous to genes involved in pigmentation, cellular component organization, and response to stimulus compared to the other species (Figure 7D). Contigs annotated with pigmentation are homologous to *yellow-g2*. Proteins belonging to yellow gene family are involved in the synthesis of melanic pigment (Ferguson et al., 2011; Gompel et al., 2005), which may be related to the phenotypic plasticity of *D. suzukii*, whose winter morph phenotype is characterized by darker pigmentation (Shearer et al., 2016). Contigs annotated to cellular component organization are homologous to genes related to ribosome biogenesis. The ovipositor of *D. suzukii* has bigger ovipositor cells than *D. melanogaster* (Green et al., 2019), and this expression of contigs related to ribosome biogenesis may reflect the cost of cell maintenance.

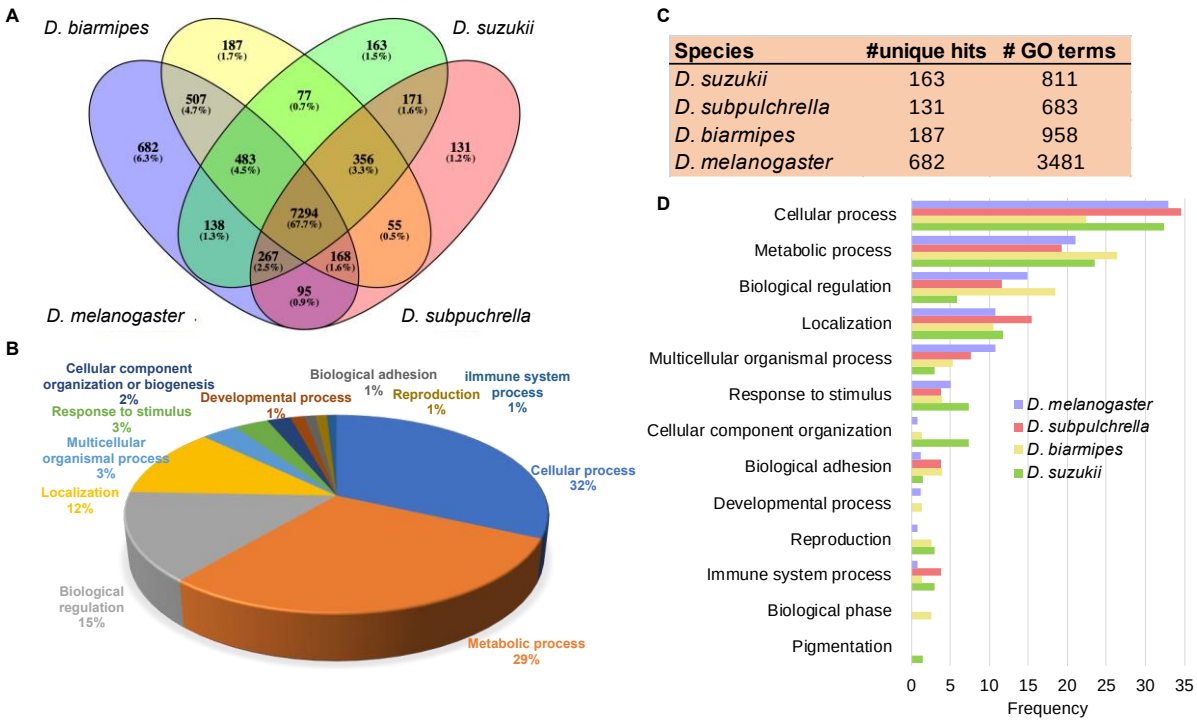

Figure 7. Annotation of transcriptomes from the terminalia of four *Drosophila* species.

(**A**) Venn diagram representing the unique *D. melanogaster* gene hits retrieved by blastx searches using contigs from each assembly as query. **(B)** Gene Ontology (GO) classification for thde 7294 gene hits common to the four assemblies referred to Biological Process (Panther GO-Slim terms). **(C)** Number of species-specific hits and associated GO terms **(D)** GO classification for species-specific hits referred to biological processes (Panther GO-Slim terms). Abbreviations: Dmel, *D. melanogaster*; Dbia, *D. biarmipes*; Dsuz, *D. suzukii*; Dsubp, *D. subpulchrella.*

Conserved set of transcripts associated with mechanotransduction in Drosophila *terminalia*

Given the ultrastructure analysis revealed that *D. suzukii* ovipositor pegs and sensilla are mechanosensitive-like structures, we were interested in determining which mechanosensitive channels and other sensory-related transcripts are expressed in terminalia. Our data allow us to explore in detail the presence-absence of transcripts in the different species. We specifically looked for contigs orthologous to mechanosensitive genes described so far in *D. melanogaster* (Karkali and Martin-Blanco, 2017): the putative metazoan mechanotransduction channels, *i.e.* degenerin/epithelial Na^+ channel C (DeG/eNaC) (Adams et al., 1998; Gorczyca et al., 2014; Guo et al., 2014; Jang et al., 2019; Tsubouchi et al., 2012; Zhong et al., 2010), the transient receptor potential (TRP) channels (Cheng et al., 2010; Gong et al., 2004; Göpfert et al., 2006; Tracey et al., 2003; Tsubouchi et al.,

2012; Walker et al., 2000), the two-pore domain K^+ channel proteins (K2P) (Tabarean and Morris, 2002), as well as *piezo* (Kim et al., 2012), *piezo-like* (*pzl*) (Hu et al., 2019) and *transmembrane channel-like* (*tmc*) genes (Guo et al., 2016).

Consistent with the results of the ultrastructural analysis, a conspicuous number of mechanosensitive-related transcripts are present in the *D. suzukii* assembly (Figure 8). However, all the candidate mechanosensitive genes present in *D. suzukii* terminalia transcriptome are commonly present in more than one of the other *Drosophila* assemblies (Table 1), pointing out that mechanosensation is a feature equally present in the terminalia of all species. Among genes whose expression is completely shared among the four species there are three DeG/eNaC proteins (which in *Drosophila* are commonly referred to as pickpocket proteins): *ppk*, *rpk*, and *ppk26* (Adams et al., 1998; Gorczyca et al., 2014; Tsubouchi et al., 2012; Zhong et al., 2010). Only transcripts encoding a fourth pickpocket protein (ppk30), which has been recently related to mechanotransduction in *D. melanogaster* (Jang et al., 2019), are absent in all samples. Also *piezo*, its paralog *pzl*, as well as *shaker* and *painless* (*pain*) have a presence-absence pattern conserved across species. The four assemblies contain contigs homologous to *piezo*, that is a transmembrane protein involved in mechanosensitive nociception in *D. melanogaster* (Kim et al., 2012), but not to *pzl*. Contigs homologous to *shaker*, which is a K2P stretch-sensitive ion channel (Tabarean and Morris, 2002), are present in the four assemblies as well as contigs homologous to *pain*, which codes for a TRP channel required for both thermal and mechanical nociception (Tracey et al., 2003).

In a subset of assemblies, we could detect trascritps of *tmc* and TRP channels other than *pain* involved in mechanotransduction. *tmc* is expressed in *D. melanogaster* larval peripheral sensory neurons, and it is involved in proprioception and sensory control of larval locomotion (Guo et al., 2016). Contigs homologs to this gene were found only in *D. melanogaster* and *D. biarmipes* assemblies, while they were absent in transcriptomes from species with serrated ovipositors. TRP proteins are membrane proteins that mediate many forms of sensory perception, including mechanosensation. Among TRPs involved in mechanotransduction, *no mechanoreceptor potential C*

(*nompC*), which is the *bona fide* mechanotransduction channel in *Drosophila* (Walker et al., 2000) is present only in *D. suzukii* and *D. melanogaster* assemblies. Contigs homologous to *inactive* (*iav*) are absent in *D. subpulchrella* assembly, and contigs homologous to *nanchung* (*nan*) are present only in the *D. biarmipes* assembly. Since *iav* and *nan* encode interdependent channel subunits and require each other for expression in *D. melanogaster* (Gong et al., 2004), we sought to understand why we found transcripts corresponding to only one of the two transcripts in the assemblies of *D. suzukii* and *D. melanogaster*. One reason might be due to the their specific expression in few cells that has been diluted by the sequencing of a composite tissue such as the terminalia (Johnson et al., 2013). To explore if the absence of some transcripts is due to artifacts, we quantified expression of manually annotated mechanosensitive-related genes in each species (Figure 8, Supplementary Dataset S3). Although the absence of replicates does not allow us to study in depth species-specific expression patterns, expression levels may indicate if a specific gene has similar mRNA levels among the four species. Our data reveal that all genes showing an inconsistent presence-absence pattern across species (*iav*, *nan*, *nompC* and *tmc*) have a low expression signal in all Drosophilas. Thus, their absence from some *de novo* assembled transcriptome may be due to limiting number of reads available for assembling. Interestingly, likewise genes with an inconsistent presence-absence pattern, also genes present in the four assemblies have similar mRNA levels across species.

Gene symbol	*D. suzukii* assembly	Other *Drosophila* assemblies
Degenerin/epithelial Na+ channel C (DeG/eNaC)		
ppk	Present	Present
rpk	Present	Present
ppk26	Present	Present
ppk30	Absent	Absent
Transient receptor potential (TRP) channels		
pain	Present	Present
nompC	Present	*D. melanogaster*
iav	Present	*D. biarmipes, D. melanogaster*
nan	Absent	*D. biarmipes*
Two-pore domain K+ channel proteins (K2P)		
Sh	Present	Present
Piezo proteins		
Piezo	Present	Present
Pzl	Absent	Absent
Transmembrane channel-like (TMC) proteins		
Tmc	Absent	*D. biarmipes, D. melanogaster*

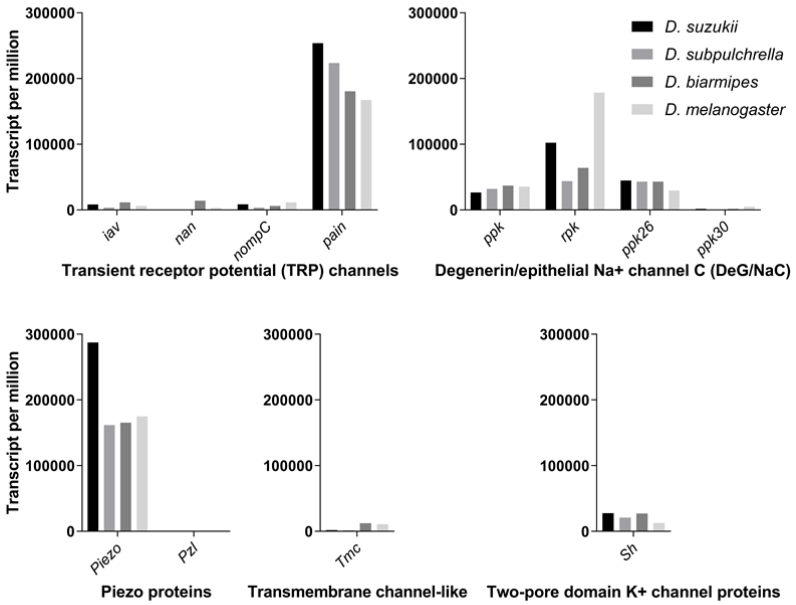

Figure 8. Annotation of transcriptomes from the terminalia of four *Drosophila* species.
(Left panel) Table highlighting the presence or absence of contigs orthologous to *Drosophila melanogaster* mechanosensitive genes in *D. suzukii de novo* transcriptome assembly and in the other three assemblies. **(Right panel)** Quantification of mRNA levels of mechanosensitive genes in *D. melanogaster*, *D. suzukii*, *D. biarmipes* and *D. subpulchrella* terminalia. Trimmed reads were mapped against manually annotated mechanosensitive genes except for *D. subpulchrella*, whose reads were mapped against the sister-species *D. suzukii* annotation set.

Expression of chemosensory-related genes in terminalia

The four assemblies contain contigs homologous to genes involved in chemosensory perception, *i.e.* several gustatory receptors (GRs), ionotropic receptors (IRs), and odorant receptors (ORs) (Supplementary Table 3). In particular, contigs homologous to *Ir47a* are present in all four assemblies, and contigs homologous to *Ir62a* and *Gr66a* are present in three out of four assemblies. Interestingly, these three genes are among the most expressed ones in all species (Supplementary Figure S4). Among the other highest expressed chemoreceptor genes there is *Or43b*. Surprisingly, we did not find any trace of the olfactory receptor co-receptor (*Orco*) expression, which is required to have functional odorant receptors (Joseph and Carlson, 2015). In *D. suzukii*, *Orco* absence has been confirmed by RT-PCR (Supplementary Figure S5).

The genomes of *D. suzukii* or *D. biarmipes* contain several paralogous genes that encode chemoreceptor and are not shared with *D. melanogaster* (Crava et al., 2016; Ramasamy et al., 2016). Gene duplication may be source of novel properties, *e.g.* paralogs may be expressed in body parts other than the ancestral expression site where they take care of new functions (Kaessmann, 2010). No paralogous genes are present in *D. suzukii* and *D. biarmipes* terminalia transcriptomes, pointing out that duplicated chemoreceptor genes identified in these species do not carry out any new function in this appendage.

Expression of chemoreceptor genes in *D. suzukii* terminalia should not be related to the presence of sensilla and pegs on the tip of the ovipositor valves, since ultrastructure analysis detailed features compatible only with mechanosensitive-like organs. However, chemosensory structures may be present in other tissues in the terminalia, such as the analia (Supplementary Figure S1). In *D. melanogaster*, analia carry several small and long bristles. In addition, the eight tergite, which wraps the anal plate, is scattered with thin bristles (Taylor, 1989a). Moreover, it has been shown that, besides the main function in taste, some GRs (and likely some IRs) may have other non-gustatory functions, such as the detection of internal ligands. For example, internal sensory neurons are present in the reproductive tract of *D. melanogaster* to sense sex peptide (Lee et al., 2016; Naccarati et al., 2012), and this may be a location, where chemoreceptor transcripts are expressed. *Gr43a*, which is involved in fructose detection in the *D. melanogaster* brain, is also expressed in the female uterus, possibly for the detection of fructose in the seminal fluid (Sato et al., 2011). *Gr43a* was found only in *D. biarmipes* assembly, and its expression levels in the four species are quite low, possibly due to an expression pattern limited to few cells. The other taste receptors present in the assemblies may have similar physiological roles in internal sensing.

CONCLUSIONS

Our results represent the first step towards a full molecular, anatomical, and physiological characterization of sensory perception in the ovipositor of *D. suzukii*. In particular, these findings (i)

show that pegs and sensilla housed in the tip of the *D. suzukii* ovipositor have a mechanosensilla-like structure, (ii) indicate that such feature may be shared with *D. melanogaster*, another species with blunt-end ovipositor, (iii) provide a qualitative overview of genes expressed in the terminalia of four *Drosophila* species with different ovipositor shapes. We propose that the sensilla and pegs present on the ovipositor tip of *D. suzukii* are the sensory structures responsible to probe substrate stiffness for the egg-laying site selection. It is also possible that these sensory structures work together with other sensory organs in assessing when the ovipositor has penetrated the substrate and peristaltic waves can start pushing down the egg. Previous research has demonstrated that fruit stiffness is the crucial component in host selection and is negatively related to oviposition and, as a consequence, fruit susceptibility to *D. suzukii* (Baser et al., 2018; Ioriatti et al., 2015; Kinjo et al., 2013; Lee et al., 2011). In this wider context, our work provides a necessary starting point to elucidate the molecular and physiological basis of the mechanotransduction system in the ovipositor of *D. suzukii*, which might allow for the development of mechanotransduction-based control strategies. Furthermore, we suggest that ovipositor mechanosensitive-like organs are likely to be of evolutionary importance across *Drosophila* species independently of the ovipositor shape. Future functional studies will clarify the relationship between the structure and the function of the ovipositor sensilla.

AUTHOR CONTRIBUTIONS

CMC participated in the design of the study, carried out the RNA-seq analysis, participated in the neuronal staining experiments, and drafted the manuscript; RR participated in the design of the study, carried out the ultraimaging experiments and critically revised the manuscript; DZ participated in the design of the study, participated in the neuronal staining experiments, carried out the visualization of the GFP expression, and critically revised the manuscript; SA carried out the molecular work, and participated in the neuronal staining experiments and the visualization of GFP expression; GS, RC, AH, MP, VRS, ORS, and GT participated in the design of the study and critically revised the

manuscript, GA conceived of the study, designed the study, coordinated the study, and critically revised the manuscript.

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