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 lays 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 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 thricoid 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 mechanosensory structures. Fluorescent labelling in *D. suzukii* and *D. melanogaster* (a species with a blunt-end ovipositor) revealed that pegs located in the ovipositor tip are innervated by a single neuron in both species. We then used RNA-seq to profile gene expression of the abdominal distal tip of *D. suzuki*i and compared it with that 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 a overlapping expression of candidate mechanosensory genes. These experimental evidence suggest a mechanosensory function for the *D. suzukii* ovipositor, which might be of evolutionary importance across *Drosophila* species independently from ovipositor shape.

KEYWORDS

Spotted wing drosophila, mechanosensory 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* 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 *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; 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). 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).

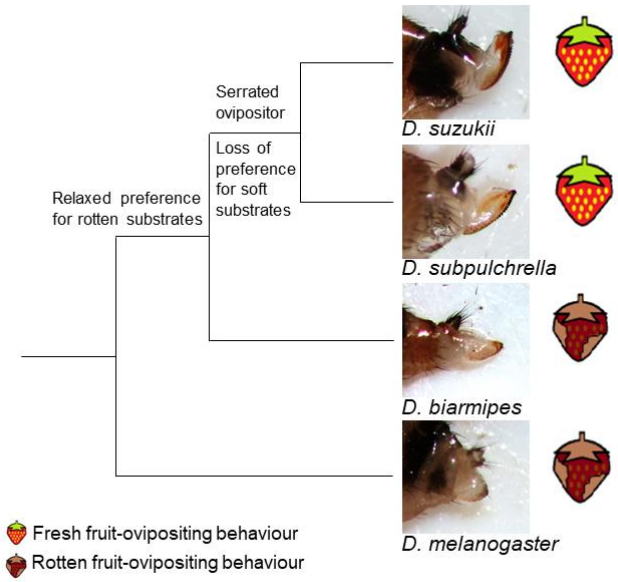


Figure 1 Evolutionary affinities and ovipositor shapes of *Drosophila suzukii* and other *Drosophila* species used in the study.
The currently most accepted scenario for fresh fruit-egg laying behaviour evolution (Karageorgi et al.,2017). Phylogeny is based on Atallah et al. (2014).

To detect substrate stiffness, insects rely on mechanosensitive receptors. In *D. melanogaster*, 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 (Tuthill and Wilson, 2016). A feature common to all mechanosensitive sensilla is that each individual sensillum is innervated by a single sensory neuron, which has 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). In addition to mono-innervated mechanosensitive sensilla, insects also possess poly-innervated external sensory organs accommodating multiple gustatory

neurons and a single mechanosensory neuron with four non-neural cells (trichogen cell, tormogen cell, thecogen cell and glial cell) (Falk et al., 1976; Stocker, 1994). While the tip of mechanosensitive sensilla is smooth, the one of taste sensilla presents a single or few pores. Dendrites of a taste neuron reaches the terminal pore opening, where physical contact with external fluid takes place (Falk and Atidia, 1975). Such taste organs have been found in the labial pals, the pharynx, the legs and the wings of *D. melanogaster*, and their presence on the external genitalia was suggested (Stocker, 1994).

Chemical composition of the egg-laying substrate also contributes to *D. suzukii* oviposition behaviour (Tait et al., 2020). 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, the contact chemosensory system, such as taste, is likely used by the flies to find 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 hypothesized that this organ may carry taste organs, 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 hypotehses, we analysed the ultrastructure of the pegs and sensilla present on *D. suzukii* ovipositor. 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 ovipositor 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 mechanosensation 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-Horseradish Peroxidase, 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, mounted using Vectashield (Vector Laboratories), and imaged with a laser scanning confocal microscope TCS SP8 (Leica).

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 imaged with the confocal microscope TCS SP8 (Leica).

RNA extraction and sequencing

RNA was extracted from the abdominal distal tip of 3- to 10-day old mated females. Dissection was done trying to enrich samples with ovipositor valves, however, the anal plate was also sampled (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 Bowtie (Langmead et al., 2009). 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).

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

Expression of chemosensory receptor genes in the *D. suzukii* abdominal distal tip 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 (Atallah et al., 2014). These modified bristles were defined lateral or marginal according to their location on the ovipositor plate and their number did not differ significantly between *D. suzukii* and *D. subpulchrella* (Atallah et al., 2014). Our observations reveal the presence of four different types of cuticular elements: conical pegs type 1 (CP1) (which were also defined lateral marginal bristles by Atallah et al., 2014), conical pegs type 2 (CP2) (which were 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). Such organization resembles that of *D. melanogaster*, whose ovipositor plates are bordered by 11-16 thorn bristles, one long bristle, and three sensilla trichodea (Chen and Baker, 1997).

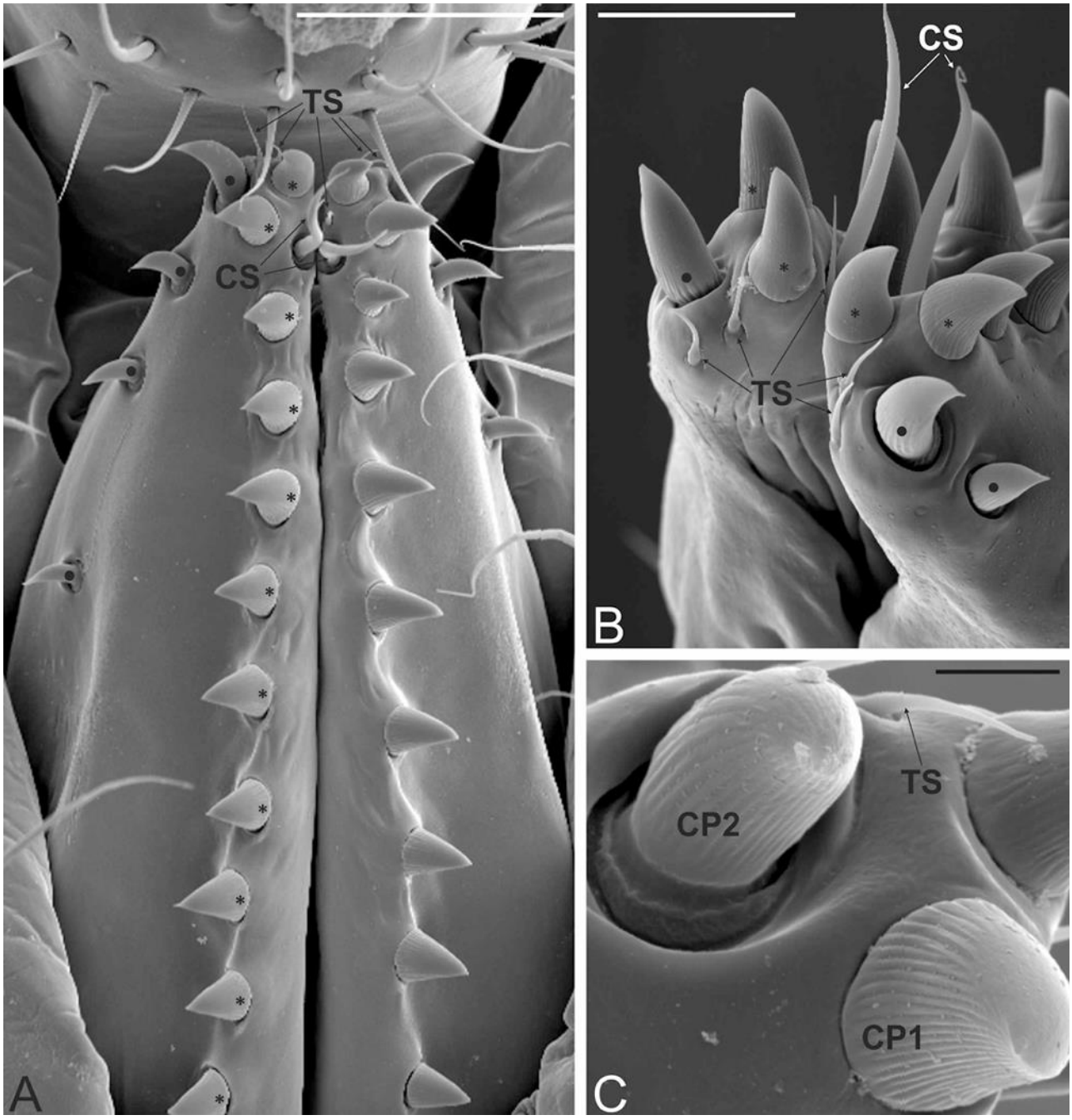
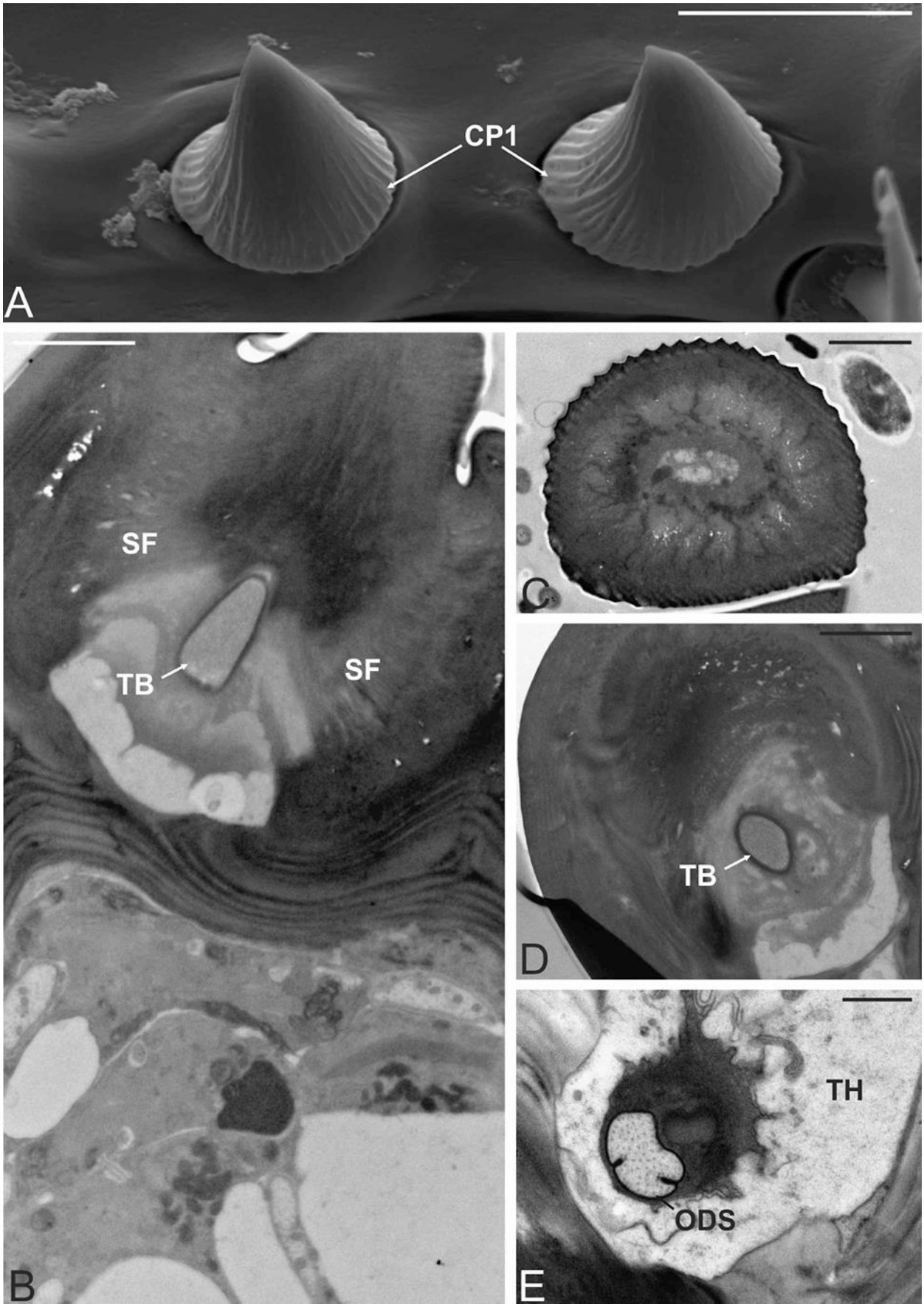


Figure 2. ***Drosophila suzukii*** **ovipositor pegs and sensilla**

(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.

CP1s are found on the outer margin of each ovipositor plate arranged in a single row of around 15 stout, conical pegs sitting on narrow sockets in the cuticle. They 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).



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259 **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.

Four CP2 are arranged in line starting from the tip of each ovipositor plate (Figure 2A). They 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). The cuticular shaft is slightly grooved along the longitudinal axis for most of its length, although the 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 4A). 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 4A), 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 4B-D). The peg itself is attached flexibly to the cuticle through a large socket with an abundance of suspension fibres (Figure 4B).

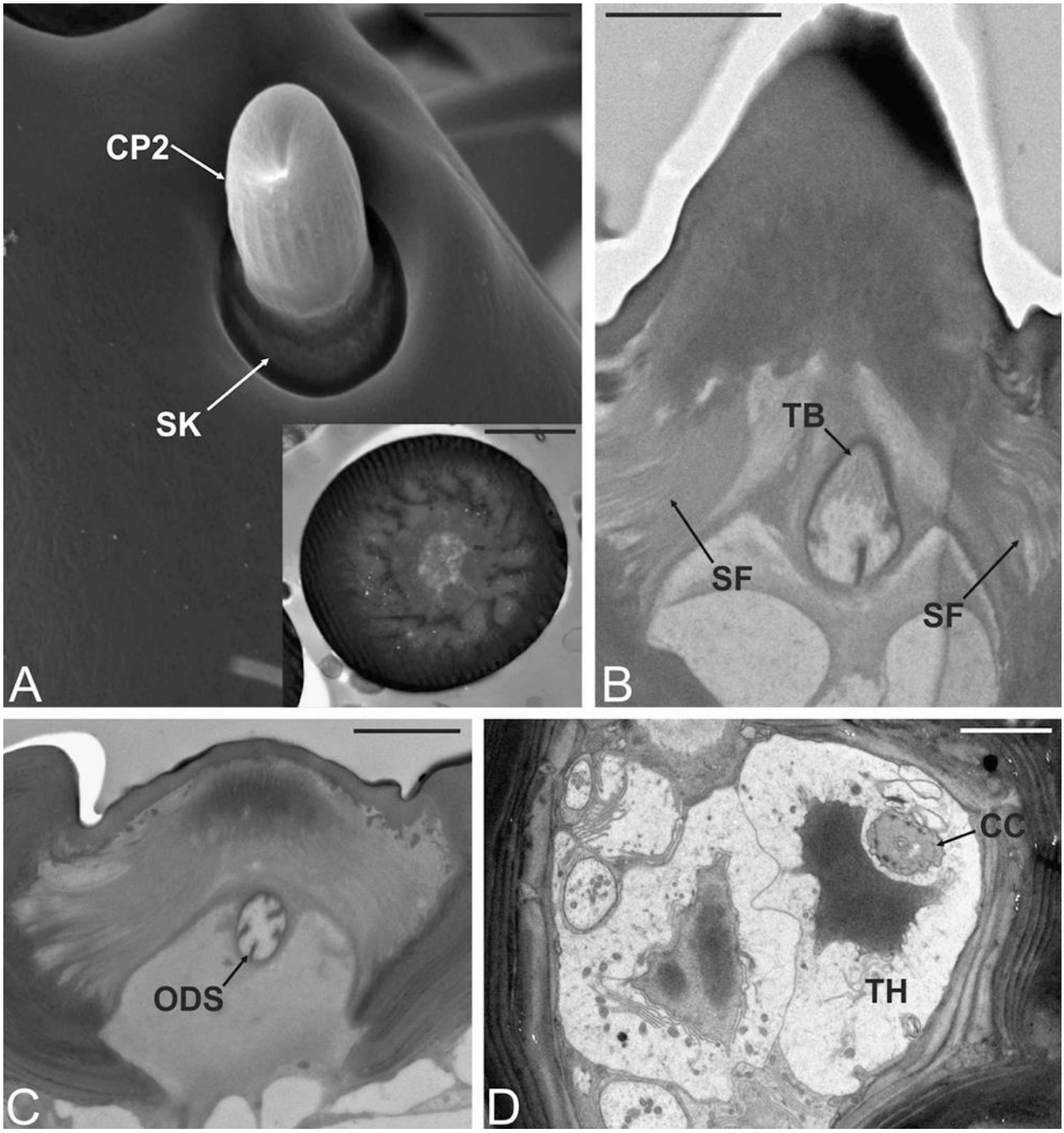

Figure 4. Micrographs showing details of the conical pegs type 1 of the *Drosophila suzukii* ovipositor **(A)** SEM ventral view of the ovipositor plate, showing one of the conical pegs type 2 (CP2), with a slightly grooved cuticle and a sharp tip. Noticeable is also the large socket (SK) on which the 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 very tip region of each ovipositor plate, we observed three small TS (Figure 2B). Two are located on the lower side of the plate, whereas the third one is located apically (Figure 5A). Trichoid sensilla 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, under an angle that makes them run almost parallel to the plate cuticular wall itself. TEM images revealed that TS are made of solid cuticle, there are no pores on the cuticle and no sensory neurons entering the peg lumen (Figure 5B). A single sensory neuron was found to be associated with TS sensilla, reaching the sensillum base through a distal tubular body (Figure 5C and Supplementary Figure S2).

Each ovipositor plate shows the presence of a single CS. This sensillum is positioned apically. It is long (38 µm) and slender (2.5 µm of base diameter), with a typical curved shape and a very fine tip (Figure 5A). 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 5D). At the base, the shaft is attached to the cuticle and suspended through an elaborated socket with numerous suspension fibres (Figure 5E). A single sensory neuron ends in a tubular body that attaches at the sensillum base (Figure 5F).

A common feature among the four class of cuticular elements present in *D. suzukii* ovipositor and identified in this study was the presence of a single neuron ending in a distal tubular body. In *Drosophila*, only type I sensory neurons (which include bristle mechanoreceptors, chordotonal organs and campaniform sensilla) construct cilia or flagell (Gillespie and Walker, 2009; Kernan, 2007). The tubular body is required for mechanotransduction and its absence causes a los of mechanosensitivity (Erler, 1983; Marshall and Lumpkin, 2012). This finding is typical of mechanosensitive-like organs, and togheter with the absence of any pore on the surface of conical pegs and sensilla clearly points out a likely function in mechanotransduction.

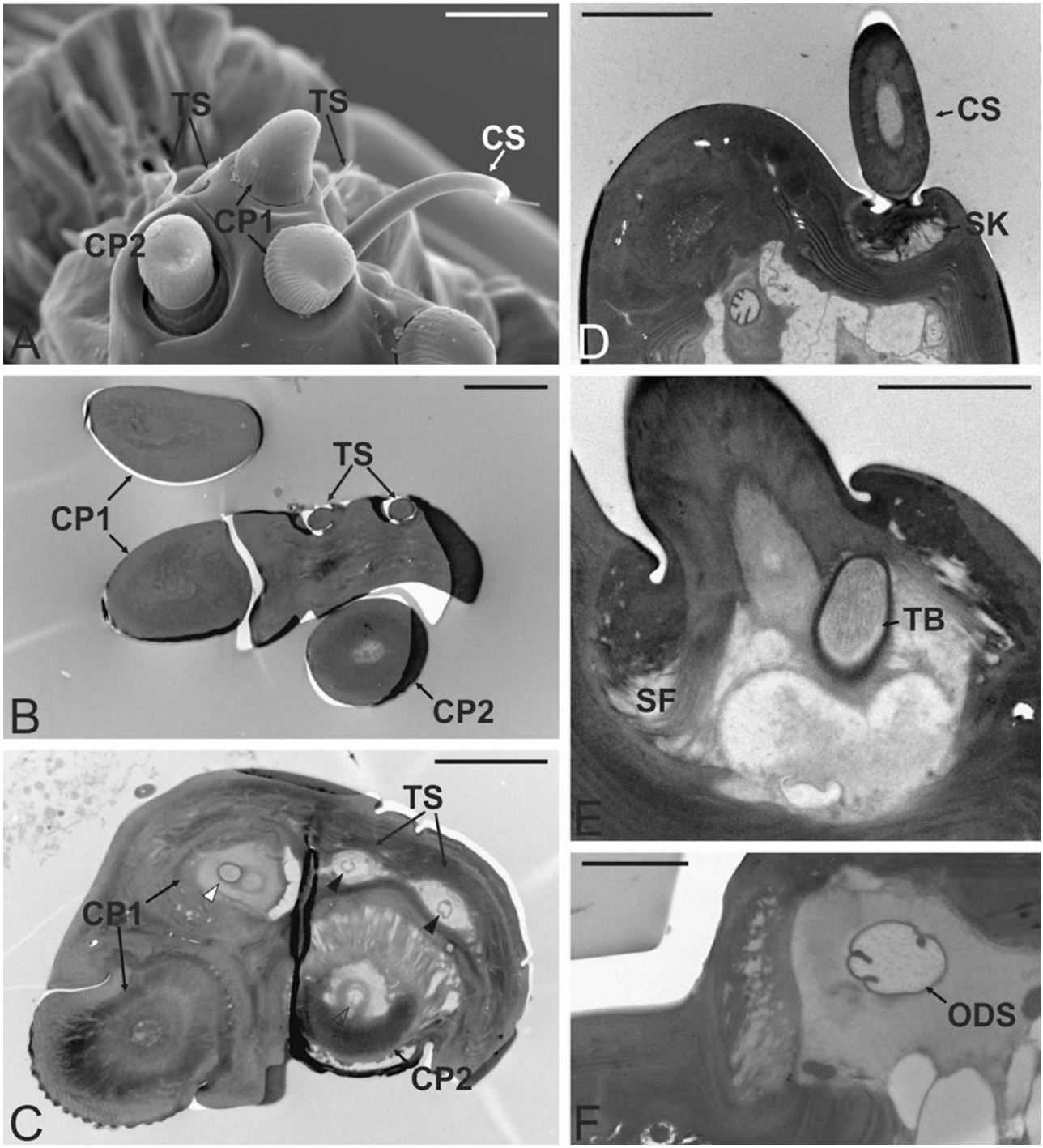

Figure 5. Micrographs showing details of the trichoid sensilla and chaetic sensilla.

(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 cuticle 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. Bar scale: A, 10 µm; B-C-D, 5 µm; E-F, 2 µm.

Neuronal staining of D. suzukii *ovipositor conical pegs is similar to that of* D. melanogaster *and supports their role in mechanotransduction*

Anti-HRP is widely used as a marker to stain the neuronal membrane of the peripheral nervous system in insects (Paschinger et al., 2009). In *D. suzukii*, HRP-labelling of ovipositor tip shows the presence of a single sensory neuron that stops at the base of each CP (Figure 6). These data support ultrastructure observations showing that each CP shaft, as well as TS and CS shafts, is innervated at its base by a single neuron, hence providing further support to their resembling to tactile bristles, which are the most visible and abundant mechanosensitive structures in the adult insect (Kernan, 2007; Tuthill and Wilson, 2016; Walker et al., 2000). Moreover, the pan-neuronal marker *n-syb* labelled a single neuron ending at the base of a single ovipositor bristle also in *D. melanogaster*, whose organization of conical pegs and sensilla in the ovipositor plates is similar to that of *D. suzukii* (Chen and Baker, 1997). This anatomical similarity between *D. melanogaster* and *D.suzukii* suggests the presence of homologous mechanosensitive-like structures in the ovipositor of *Drosophila* species independently of the ovipositor shape.

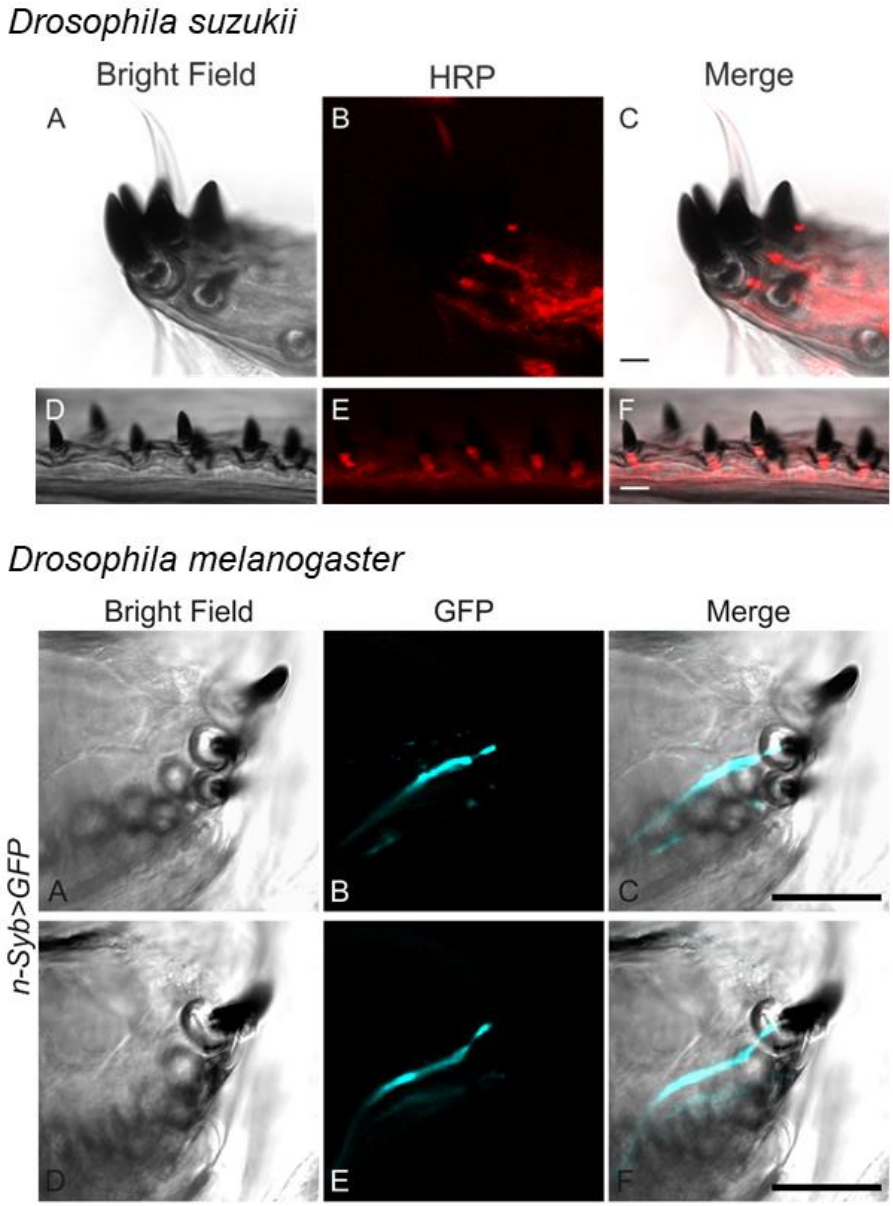

Figure 6 Conical pegs are innervated by single neurons in both *Drosophila suzukii* and *D. melanogaster* ovipositors

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.

RNA-seq characterization of the abdominal distal tip of four **Drosophila** *species*

Illumina RNA-seq libraries from the abdominal distal tip of four *Drosophila* species generated an average of 60M (±2.4M SD) 100 bp paired-end reads. This resulted in four *de novo* assembled transcriptomes with contig counts ranging from 31'315 (*D. subpulchrella*) to 40'162 (*D. suzukii*). On average, approximately 80% of reads successfully aligned to the assemblies across species (Supplementary Table S1). 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). Since we did not use replicates, we could not study the species-specific expression profile; our data however allow us to explore in detail the presence/absence of transcripts in the different species. Of 10'774 *D. melanogaster* genes hit by at least one contig in one species, 7'294 (almost the 70%) were common among the four *Drosophila* species, thus representing the conserved transcriptional core for the abdominal distal tip (Figure 7A). GO analysis showed that these common genes were mostly involved in cellular processes (32%), metabolic processes (29%), and biological regulation (15%) (Figure 7B). Species-specific hits were few, namely 163 for *D. suzukii*, 131 for *D. subpulchrella*, 187 for *D. biarmipes*, and 682 for *D. melanogaster*, respectively. We found 811 unique GO terms corresponding to unique *D. suzukii* contigs (Figure 7C, and Supplementary Dataset S2). Among them we found 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 were 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 were 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 gene expression pattern may reflect the cost of their maintenance.

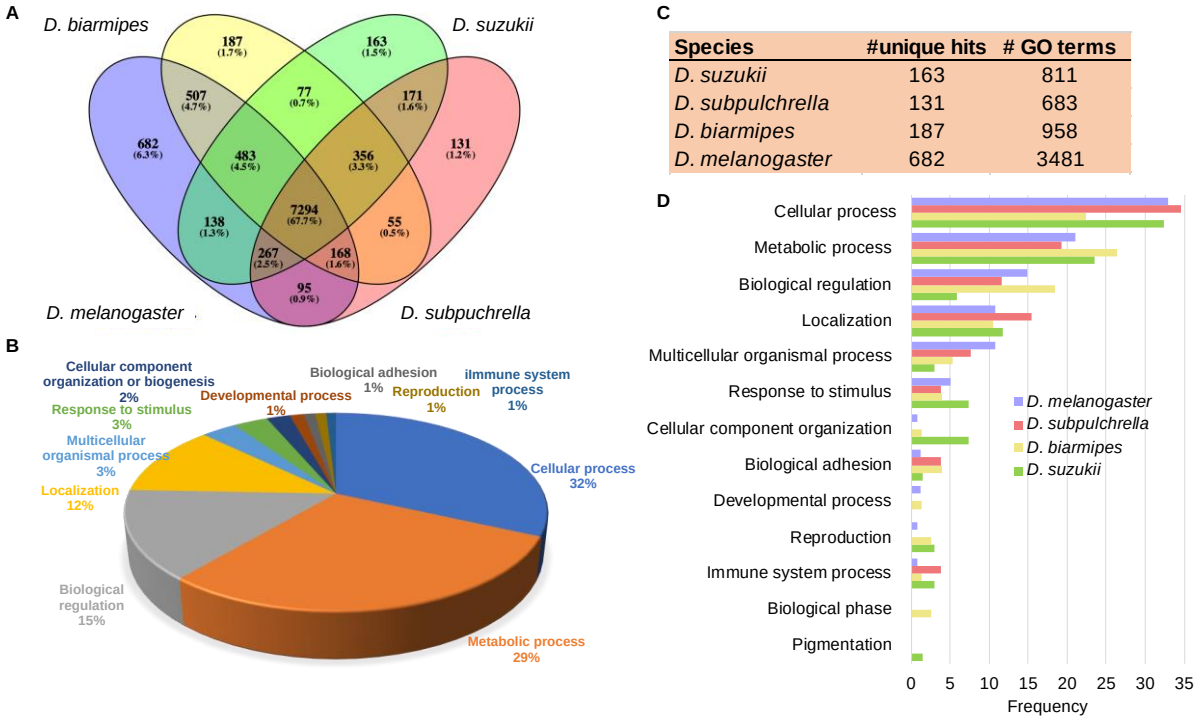

Figure 7: Annotation of transcriptomes from the abdominal distal tip of four *Drosophila* species. (**A**) Venn diagram representing the unique *D. melanogaster* gene hits retrieved by blastx searches using contigs from each assembly as query. **(B)** Gene Ontology (GO) classification for the 7294 gene 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 Process (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 *abdominal distal tip

Accordingly with the results of the ultrastructural analysis of *D. suzukii* ovipositor that revealed the presence of mechanosensitive-like sensilla, we found a conspicuous number of mechanosensitive-related transcrips in the *D. suzukii* assembly (Table 1). We detected molecules orthologous to many 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), the transient receptor potential (TRP) channels, the two-pore domain K+ channel

proteins (K2P), as well as *piezo*, *piezo-like* (*pzl*) and *transmembrane channel-like* (*tmc*) genes (Adams et al., 1998; Cheng et al., 2010; Gong et al., 2004; Göpfert et al., 2006; Gorczyca et al., 2014; Guo et al., 2016, 2014; Hu et al., 2019; Jang et al., 2019; Kim et al., 2012; Tabarean and Morris, 2002; Tracey et al., 2003; Tsubouchi et al., 2012; Walker et al., 2000; Zhong et al., 2010). These mechanosensory-related contigs were never found uniquely in *D. suzukii* ovipositor transcriptome but they were also expressed in at least one of the other *Drosophila* species RNA-seq (Table 1).

Among genes whose expression was completely shared among the four species there were three DeG/eNaC proteins (which in *Drosophila* are commonly referred to pickpocket protein): *ppk*, *rpk*, and *ppk26* (Adams et al., 1998; Gorczyca et al., 2014; Tsubouchi et al., 2012; Zhong et al., 2010). Only transcripts enconding a fourth pickpocket protein (ppk30), which has been recently related to mechanotransduction in *D. melanogaster* (Jang et al., 2019), were not detected in any sample. Likewise pickpocket proteins, *piezo*, its paralog *pzl*, *shaker* and *painless* (*pain*) had a similar expression pattern conserved across species. The four assemblies contained contigs homologous to *piezo*, that is a transmembrane protein involved in mechanosensory 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), were present in the four assemblies as well as contigs homologous to *pain*, which code for a TRP channel required for both thermal and mechanical nociception (Tracey et al., 2003).

Transripts that were expressed in a subset of assemblies were *tmc* and the other TRP channels involved in mechanotransduction. *tmc* is expressed in *D. melanogaster* larval peripheral sensory neurons and it is involved in proprioception and the sensory control of larval locomotion (Guo et al., 2016). We found contigs homologs to this gene 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, we found that *no*

mechanoreceptor potential C (*nompC*), which is the *bona fide* mechanotransduction channel in *Drosophila* (Walker et al., 2000) was present only in *D. suzukii* and *D. melanogaster* assemblies. Contigs homologous to *inactive* (*iav*) were absent in *D. subpulchrella* assembly, and contigs homologous to *nanchung* (*nan*) were present only in the *D. biarmipes* assembly.

Table 1: List of candidate mechanosensitive genes found to be expressed in RNA-seq assemblies from *D. suzukii* ovipositor and other three related *Drosophila* species characterized by gradual differences in ovipositor shapes.

Gene symbol	**References**	**Expression in *D. suzukii***	**Expression in other *Drosophila***
Degenerin/epithelial Na+ channel C (DeG/eNaC)			
ppk	Adams et al., 1998; Zhong et al., 2010	Expressed	All species
rpk	Adams et al., 1998; Tsubouchi et al., 2012	Expressed	All species
ppk26	Gorczyca et al., 2014; Guo et al., 2014	Expressed	All species
ppk30	Jang et al., 2019	Absent	Absent
Transient receptor potential (TRP) channels			
pain	Tracey et al., 2003	Expressed	All species
nompC	Walker et al., 2000	Expressed	*D. melanogaster*
iav	Gong et al., 2004	Expressed	*D. biarmipes, D. melanogaster*
nan	Gong et al., 2004	Absent	*D. biarmipes*
Two-pore domain K+ channel proteins (K2P)			
Sh	Tabarean and Morris, 2002	Expressed	All species
Piezo proteins			
Piezo	Kim et al., 2012	Expressed	All species
Pzl	Hu et al., 2019	Absent	Absent
Transmembrane channel-like (TMC) proteins			
tmc	Guo et al., 2016	Absent	*D. biarmipes, D. melanogaster*

Unexpected expression of ***chemosensory-related genes***

We also found contigs homologous to genes involved in chemosensory perception, *i.e.* several odorant receptors (ORs), gustatory receptors (GRs), and ionotropic receptors (IRs) expressed in at least one species, in particular contigs homologous to *ir47a* and *ir62a* were present in all four assemblies. In *D. suzukii*, RT-PCR confirmed the transcription of one OR (*Or43b*), two GRs (*Gr94a* and *Gr66a*), and five IRs (*Ir47a1*, *Ir60d*, *Ir62a*, *Ir75d*, and *Ir87a*) in the abdominal distal tip, and the absence of transcripts encoding the olfactory receptor co-receptor (*orco*) (Supplementary Figure S3). These observations are in contrast with the study of the ultrastructure of sensilla and pegs present on *D. suzukii* ovipositor valves, which found features compatible only with mechanosensitive-like organs. Chemosensory structures may be present in other tissues in the abdominal distal tip, such as the anal plate (Supplementary Figure S1). Morevoer, 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, *Gr43a* is involved in fructose detection in the *D. melanogaster* brain, and it is also expressed in the female uterus, possibly to sense fructose present in the seminal fluid (Sato et al., 2011). We do not detect expression of *Gr43a* in any of the four assemblies generated in this study, but the other taste receptors found may have similar physiological roles in internal sensing. 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 another location where chemoreceptor transcripts are expressed.

CONCLUSIONS

Our results represent the first step towards a full molecular, anatomical, and physiological characterization of sensory perception in the ovipositor of *D. suzukii* and closely related species. In particular, these findings have (i) shown that pegs and sensilla present in the *D. suzukii* ovipositor

tip have a mechanosensilla-like structure, (ii) shown that this feature may be shared with another species with blunt-end ovipositor, *D. melanogaster*, (iii) provided a qualitative overview of genes expressed in the abdominal distal tip 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, providing the information when the ovipositor has penetrated the substrate and peristaltic waves can start to push 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). Hence, in this wider context, our work build 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 mechanosensory-based control strategies. We also suggest that ovipositor mechanosensitive-like organs are likely to be of evolutionary importance across *Drosophila* species indipendently of the ovipositor shape. However, 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 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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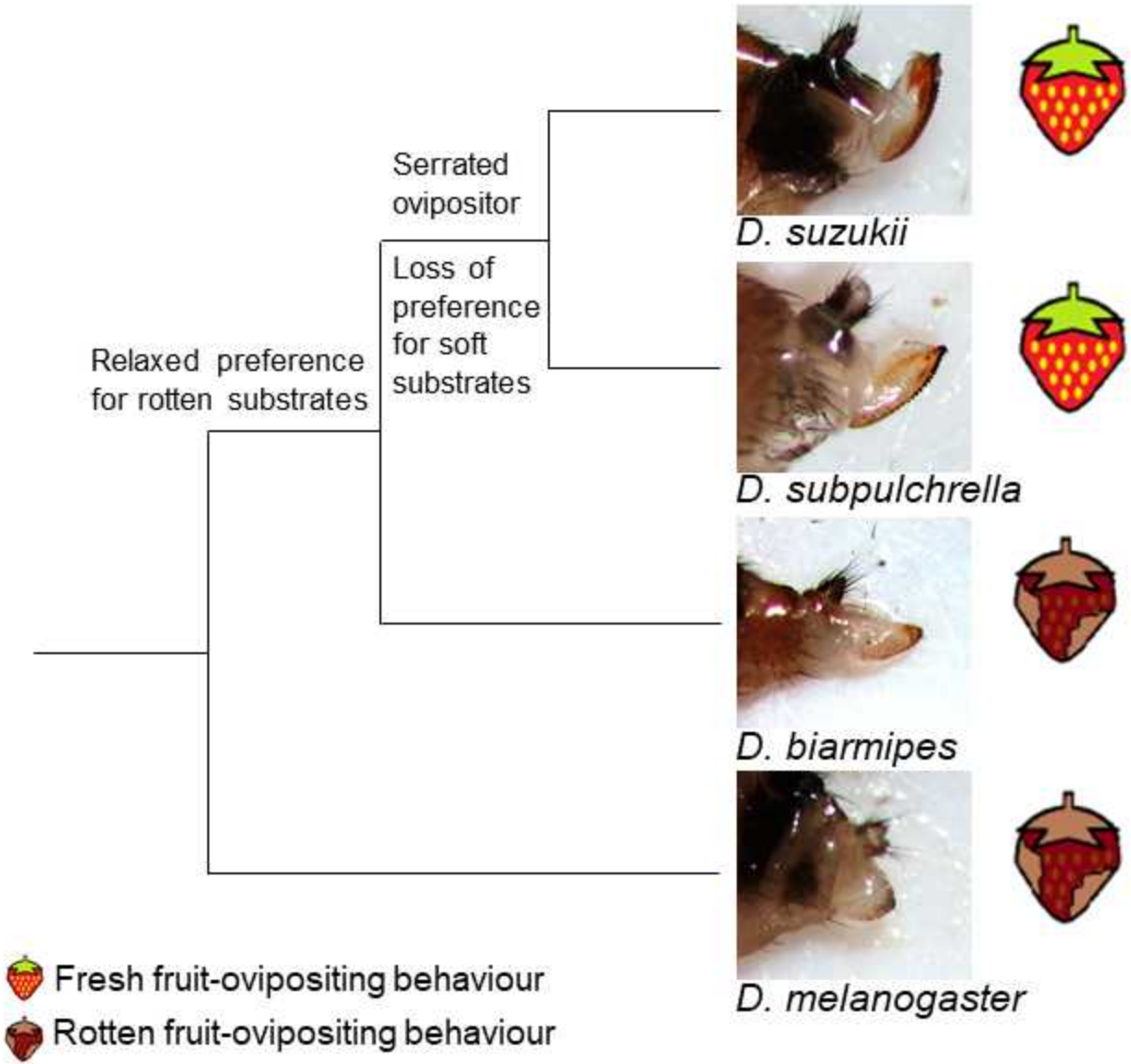
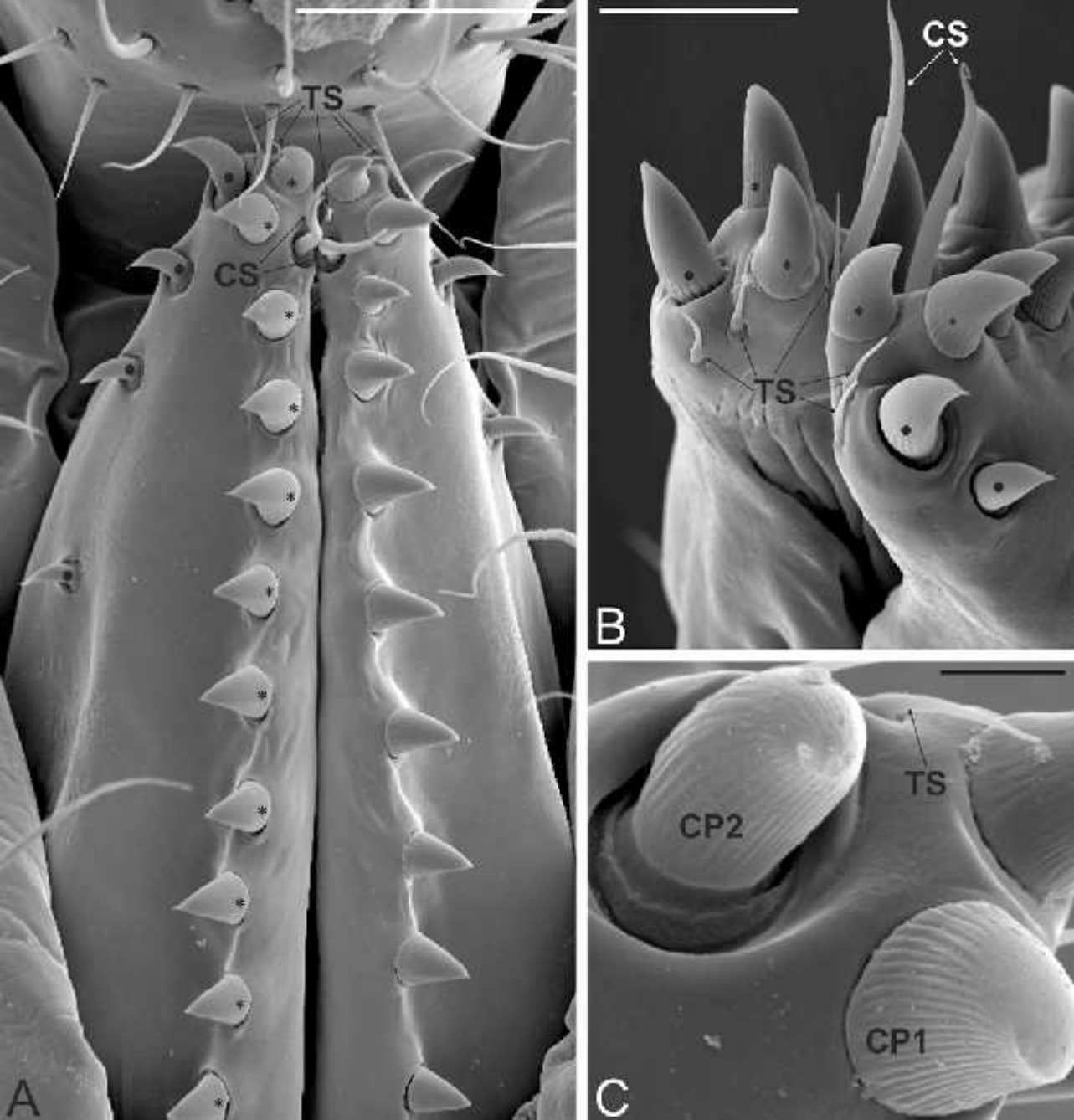

Serrated ovipositor
Loss of preference for soft substrates
Relaxed preference for rotten substrates
D. suzukii
D. subpulchrella
D. biarmipes
D. melanogaster
Fresh fruit-ovipositing behaviour
Rotten fruit-ovipositing behaviour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CP1
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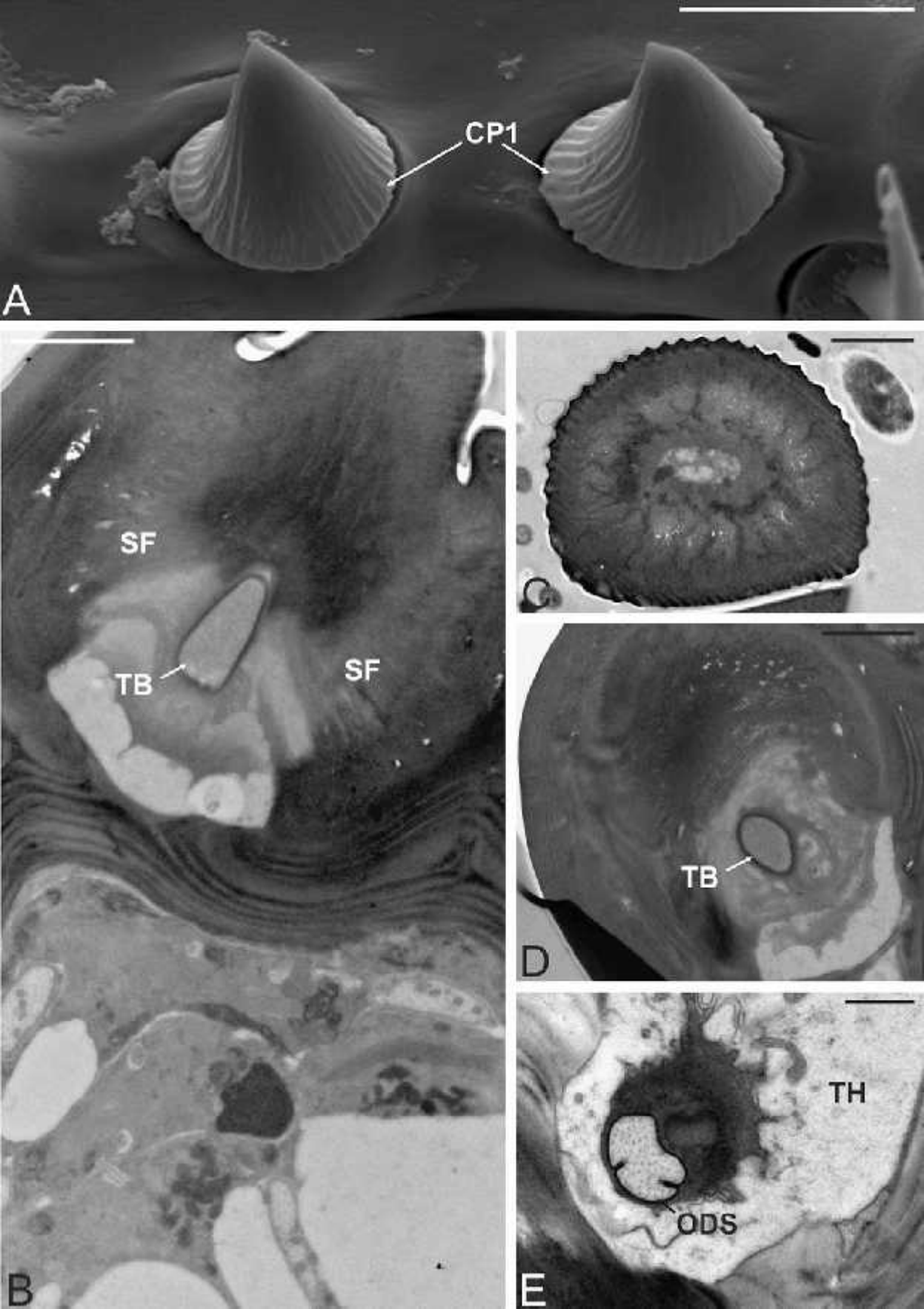
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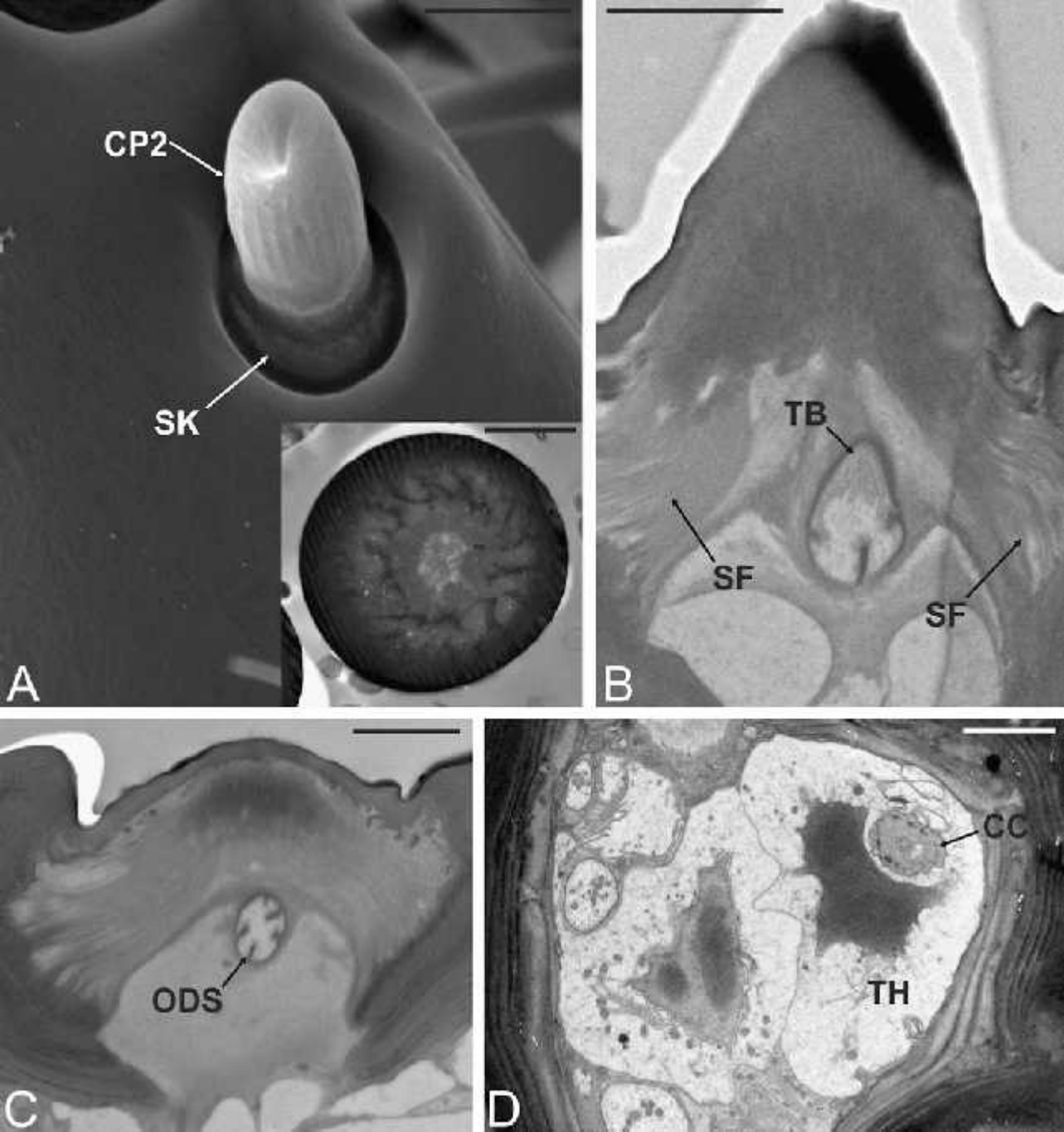
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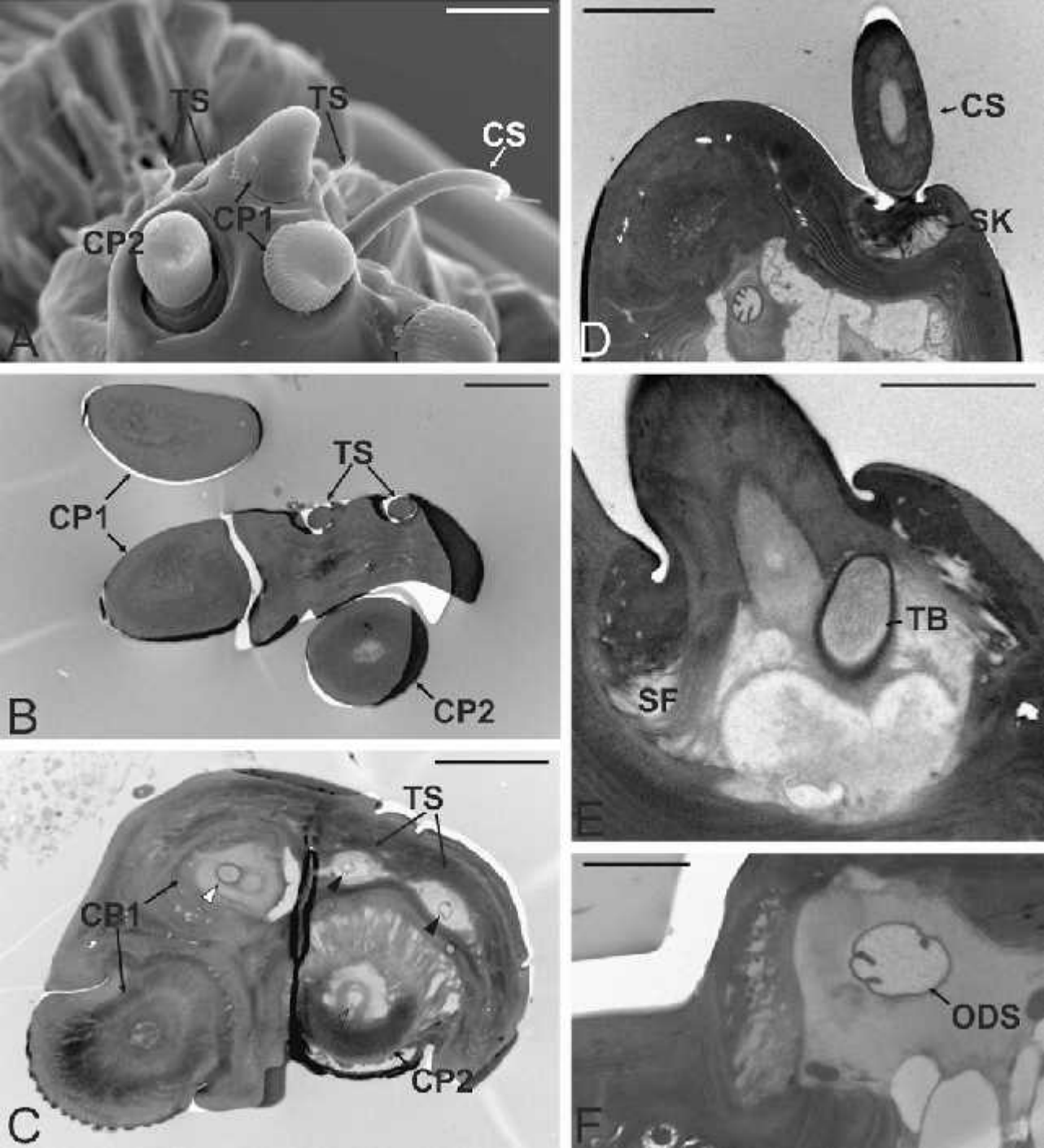
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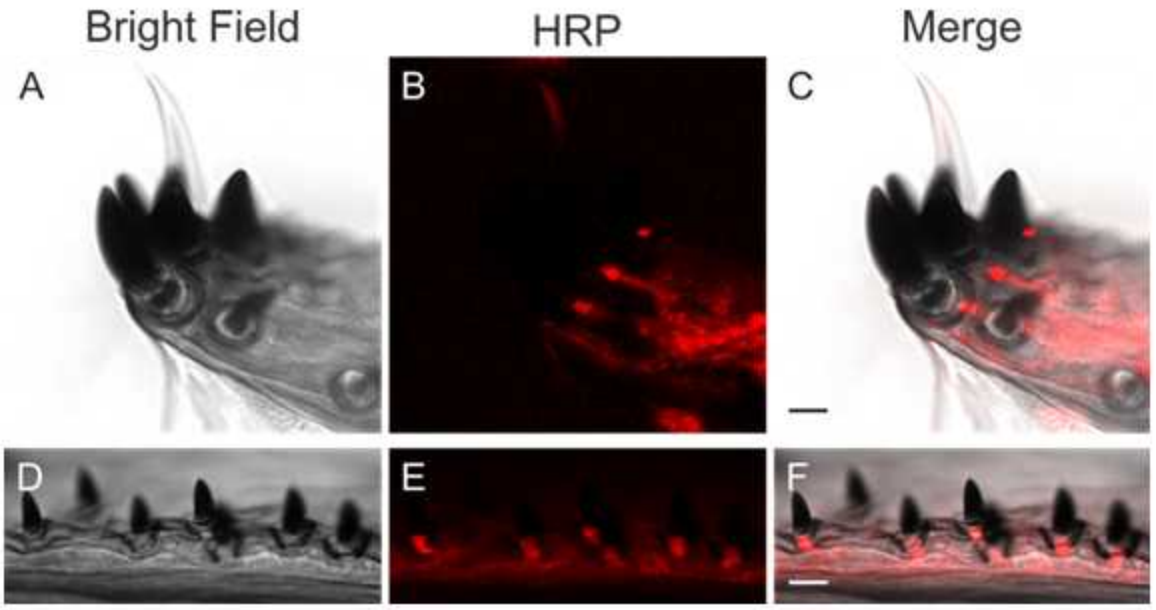
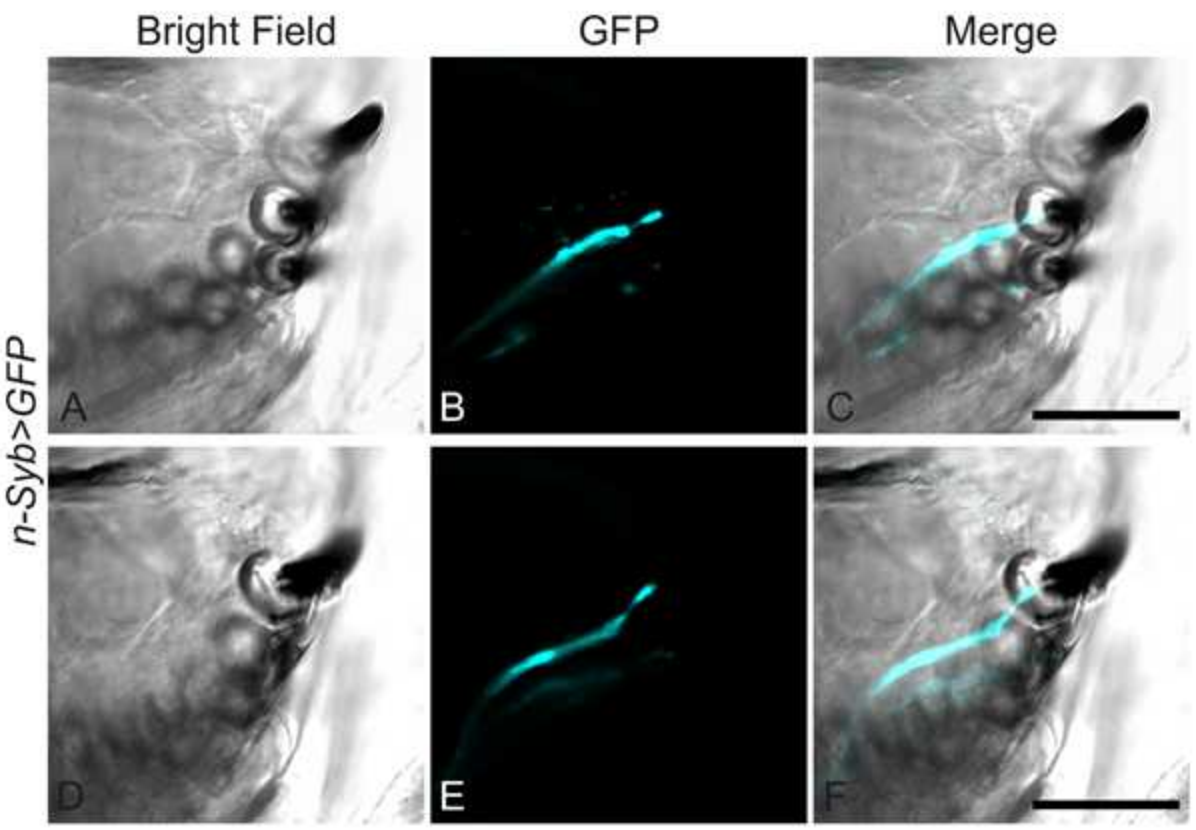
ODS
F

Drosophila suzukii



Drosophila melanogaster



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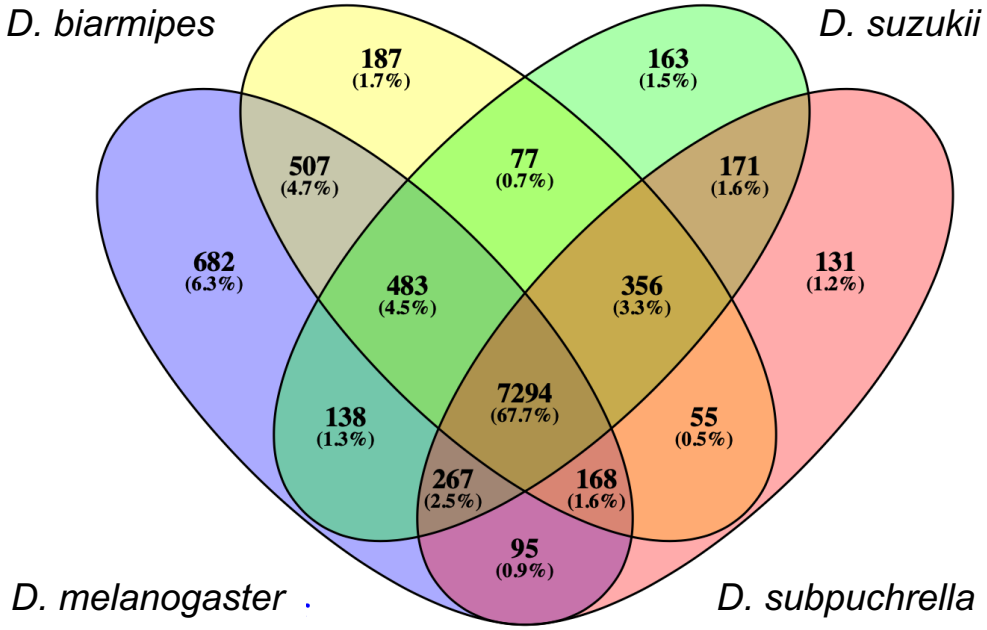

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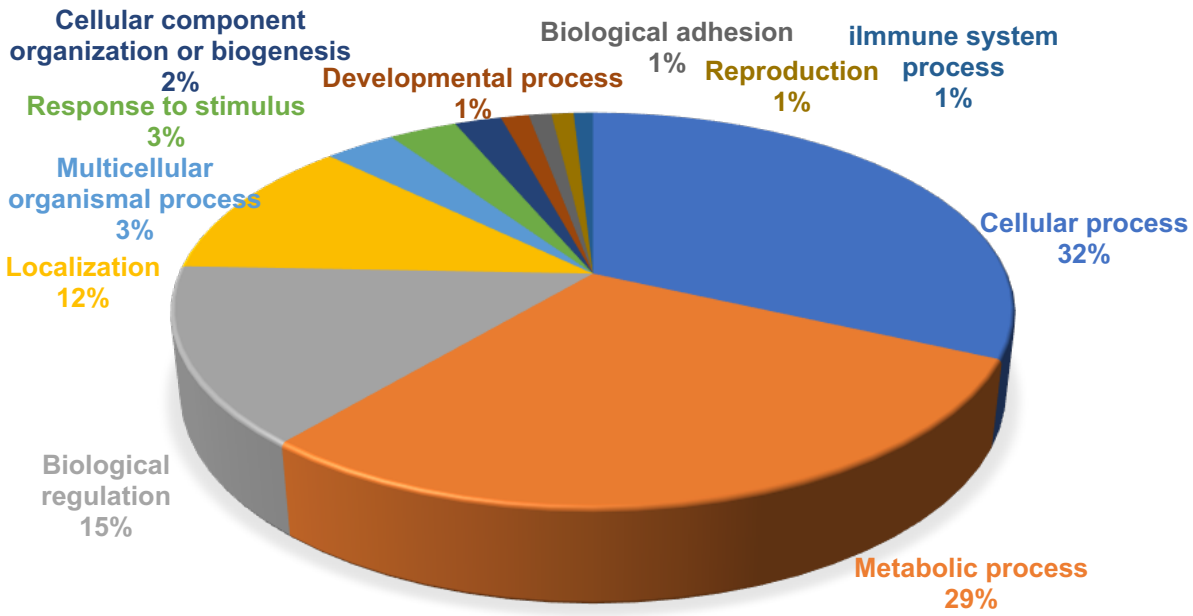

C

Species	#unique hits	# GO terms
D. suzukii	163	811
D. subpulchrella	131	683
D. biarmipes	187	958
D. melanogaster	682	3481

D