

SHORT COMMUNICATION

The insect ovipositor as a volatile sensor within a closed microcosm

Pratibha Yadav and Renee M. Borges*

ABSTRACT

We show that the insect ovipositor is an olfactory organ that responds to volatiles and CO₂ in gaseous form. We demonstrate this phenomenon in parasitic wasps associated with *Ficus racemosa* where ovipositors, as slender as a human hair, drill through the syconium (enclosed inflorescences) and act as a guiding probe to locate highly specific egg-laying sites hidden inside. We hypothesize that olfaction will occur in the ovipositors of insects such as parasitic fig wasps where the hosts are concealed and volatile concentrations can build up locally. Relevant stimuli such as herbivore-induced fig volatiles and CO₂ elicited electrophysiological responses from the ovipositors. Silver nitrate staining also revealed pores in ovipositor sensilla, indicating their olfactory nature. Insects could use volatile sensors on their ovipositors to evaluate ecologically relevant stimuli for oviposition. Further investigations on the sensory nature of ovipositors can provide designs for development of ovipositor-inspired micro-chemosensors.

KEY WORDS: Ovipositor, Olfaction, Sensilla, Carbon dioxide, Electrophysiology

INTRODUCTION

Olfaction is important in an insect's search for food, mates and oviposition sites. While olfaction has been largely investigated in the antennae, maxillary and/or labial palps, only close-range chemical detection via gustation (contact chemoreception) has been found in egg-laying structures such as the ovipositor (but see Klinner et al., 2016). The success and diversity of hymenopteran parasitoids is attributed to their ovipositor being functional not only as an egg-laying organ but also as a drilling and steering tool to reach hosts (Quicke et al., 1999). Host discrimination is believed to be achieved by exploiting cues using mechanosensory and chemosensory sensilla present on the ovipositor (Van Lenteren et al., 2007). However, ovipositors have far fewer and smaller sensilla compared with antennae; this may explain an almost complete absence of studies on electrophysiology of ovipositors; the few studies that have been conducted have mostly recorded from gustatory sensilla (Rice, 1977; Crnjar et al., 1989; Van Lenteren et al., 2007; but see Klinner et al., 2016). We expect olfactory sensilla to be present on the ovipositor of hymenopteran parasitoids that exploit certain types of concealed hosts, to facilitate perception of volatile gradients within a chemically crowded matrix contained

within a chamber, and to enhance the likelihood of finding suitable but hidden oviposition sites.

Figs, whose globose inflorescences can be considered as microcosms (Borges, 2015), provide an excellent system to test this hypothesis. All 800+ species of figs have a specific community of pollinators and parasitic wasps associated with them. Pollinating wasps enter the syconium (globular enclosed inflorescences) to pollinate flowers and lay their eggs, whereas parasitic wasps lay their eggs from outside fig syconia using their ovipositor, which acts as the only guiding probe that can provide information about oviposition sites hidden within the syconium (Fig. 1A). Because wasp larvae are immobile (Ghara et al., 2011), it is vital for the ovipositor to find the appropriate oviposition site. The volatile profile of fig syconia changes quantitatively and qualitatively to include herbivore-induced plant volatiles (HIPVs) after oviposition by a sequential series of parasitic non-pollinating fig wasp (NPFW) species (Borges et al., 2013); these oviposit into individual flowers which are subsequently modified as galls (gallers) or into developing wasp larvae within the galls (parasitoids). Using the ovipositor, NPFWs drill through the syconium wall, whose thickness and presence of few stomata, if any, can result in resistance to external diffusion (Niinemets and Reichstein, 2003), and therefore an increase in volatile concentrations within the syconium. The drilling ovipositor navigates between flowers and galls containing different wasp species and could use volatile cues to find egg-laying sites (Fig. 1A) with the diverse sensilla present at its tip (Ghara et al., 2011); after host location, the ovipositor enters the flower when gustation or contact chemoreception might become more important.

The low number of sensilla in a fig wasp ovipositor [just three sensilla in the pollinator *Ceratosolen fusciceps* of *Ficus racemosa* or 17 sensilla in the non-pollinating galler *Sycophaga fusca* in the same fig species (Ghara et al., 2011)] compared with the antennae [~210 sensilla in the pollinator *Eupristina* sp. of *Ficus curtipes* (Li et al., 2014)] makes it a highly challenging task to record an electrophysiological response using a conventional electroantennogram setup (EAG) because most of these sensilla are concentrated near the tip of the ovipositor (Ghara et al., 2011). We modified a gas chromatograph-electroantennogram detector (GC-EAD) setup to function as a GC-electro-ovipositogram detector (EOD) (Fig. 1B) to investigate the response to volatile stimuli by the ovipositor in NPFWs.

MATERIALS AND METHODS**Electrophysiology**

The ovipositors of the galler *Sycophaga fusca* Girault and the parasitoid *Apocrypta westwoodi* Grandi 1916, members of the NPFW community of the fig *Ficus racemosa*, were used in this investigation. The ovipositor was excised from the wasp abdomen in ice-cold physiological saline, cut at its base and its sheath removed. The exposed ovipositor valves were used for recordings with the

Centre for Ecological Sciences, Indian Institute of Science, Bangalore, Karnataka 560012, India.

*Author for correspondence (renee@ces.iisc.ernet.in)

 R.M.B., 0000-0001-8586-7380

Received 3 November 2016; Accepted 20 February 2017

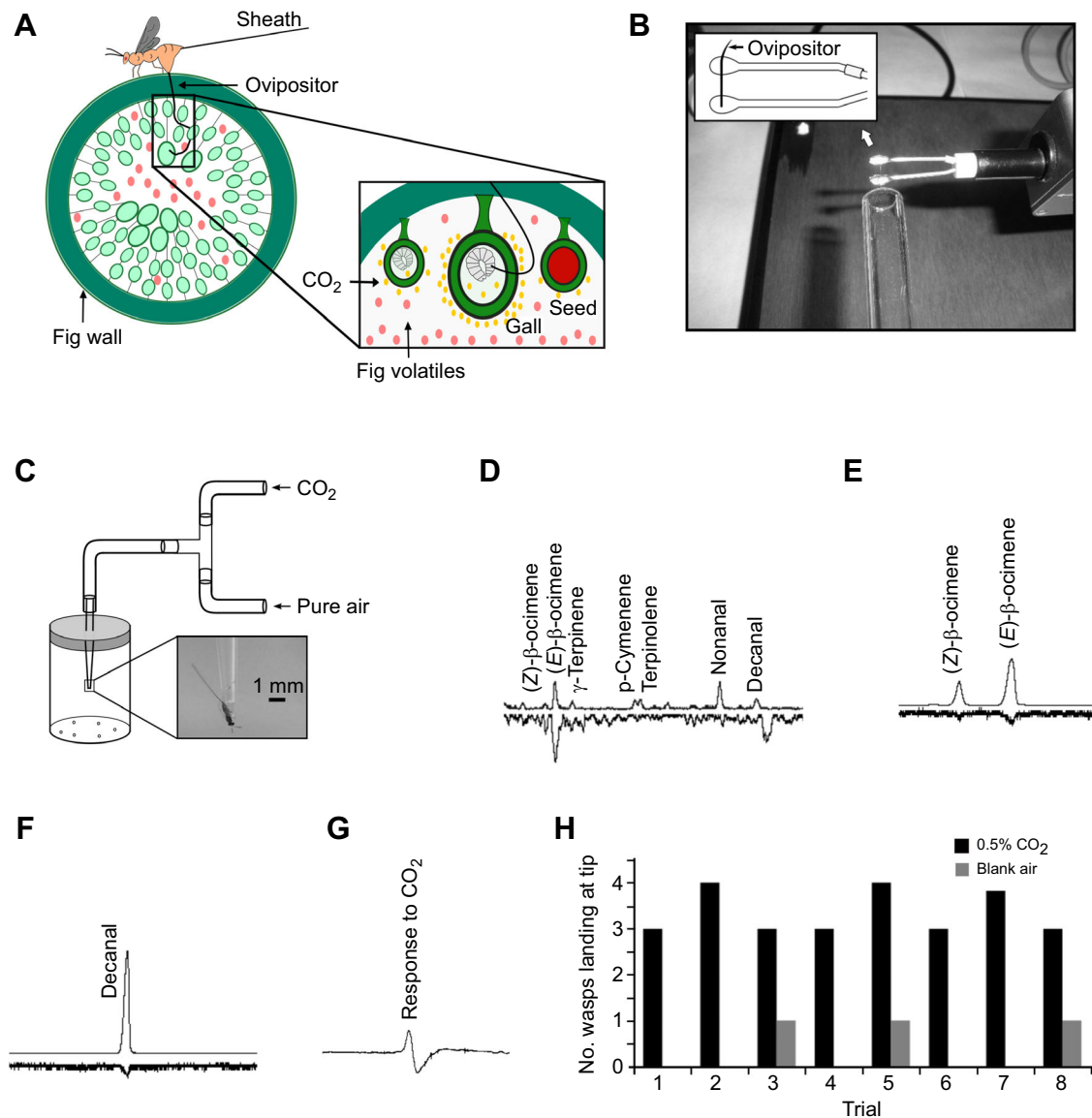


Fig. 1. Electrophysiological and behavioral response. (A) A schematic showing the different stimuli that an ovipositor can encounter inside a syconium. (B) Ovipositor mounted on the electrode. (C) Behavioral assay setup with wasp landing at the tip of CO₂-releasing source. (D–F) GC-EOD recordings of galler *Sycophaga fusca* response to (D) receptive phase volatiles, (E) β -ocimene and (F) decanal. (G) GC-EOD recordings of parasitoid *Apocrypta westwoodi* response to 0.5% CO₂; (H) Behavioral response to CO₂ (Wilcoxon matched pairs signed rank test, $\nu=0$, $P=0.013$); wasps landed significantly more often on the tip when CO₂ was present.

base inserted into the conducting gel on the neutral arm of the metal electrode. The ovipositor was mounted by anchoring it at three-fourths of its length from the base on the recording electrode (Fig. 1B) such that the tip, which harbors all the sensilla (Ghara et al., 2011), remains free. The tip of the ovipositor was excited with stimulus delivery in a GC-EAD setup [GC: Agilent 7890 with an HP-5 column (30 m \times 0.32 mm \times 0.25 μ m); EAG instrument: Syntech, Hilversum, The Netherlands].

We tested volatiles from pollen-receptive fig syconia exposed to oviposition by pollinators as a stimulus for (1) the wasp galler *S. fusca* that arrives for oviposition into the syconium concurrently with the pollinator, and (2) the wasp parasitoid *A. westwoodi* that oviposits into the syconium after this brief stage. Volatiles were collected by external headspace adsorption *in situ* following procedures in Borges et al. (2013). Because we did not have an MS detector connected to the GC-EAD machine, a fraction of the collected sample was run in another GC-MS machine with the same

columns; peaks in the GC-EAD were identified by matching retention times of the compounds. Subsequently, individual pure compounds that were easily available were also tested to confirm the response. We also tested the response of the parasitoid's ovipositor to 0.5% CO₂ (see justification for this concentration in Results and Discussion).

Behavioral assay: response to CO₂

Two behavioral assays were performed to investigate the behavioral relevance of the ovipositor's response to CO₂. In the first assay, five parasitoid wasps at a time (each with silicone-occluded antennae) were enclosed in a vial into which 0.5% CO₂ was released via a micropipette tip at regular intervals (Fig. 1C). Silicone occlusion has been used in several studies on Hymenoptera to prevent antennal detection of volatiles (e.g. Letzkus et al., 2006). The number of wasps landing at the tip were noted. This assay was repeated eight times. In another proof-of-concept assay, CO₂ was directed at the

ovipositor of a tethered wasp (Movie 1). A 5 s CO₂ stimulus was sent at intervals through one arm of a Y-tube apparatus carrying a continuous stream of blank air in its other arm at a flow rate of 60 ml min⁻¹ (Movie 1). The ovipositor deflection towards the source of CO₂, if any, was video-recorded.

SEM imaging and silver nitrate staining

The ovipositor was excised from the wasp abdomen in physiological saline, cut at its base, its sheath removed and dehydrated. Ovipositors were then gold sputter-coated at 10 nm and viewed in an FEI Quanta 200 ESEM at the Advanced Facility for Microscopy and Microanalysis (AFMM), Indian Institute of Science (IISc).

Ovipositors were dipped in 1% aqueous silver nitrate solution for 20 min followed by serial dehydration in ethanol, cleared with xylene and fixed in DPX mountant. For X-ray tomography, the stained ovipositors were mounted on entomological pins and examined using XRADIA (Xradia versa XRM500) at AFMM, IISc.

RESULTS AND DISCUSSION

This is the first study to demonstrate an electrophysiological and behavioral response to ecologically relevant volatile compounds and CO₂ by olfactory sensilla present on an ovipositor. We amplified the signal-to-noise ratio by anchoring the ovipositor at three-fourths its

length from the base on the recording electrode such that the sensory tip remains free to respond to stimuli. Another method of arranging small antennae in series during an EAG improves the signal-to-noise ratio (Park and Baker, 2002) but such a method fails with sensory organs such as ovipositors, where the tip is of critical importance as the tip is immersed in conducting gel in such an arrangement and the sensilla are not exposed to the stimulus.

The ovipositor of *S. fusca* responded strongly to pollen-receptive phase volatiles (12–18 mV) (Fig. 1D). Electrophysiologically active compounds of this volatile profile, (*E*)-β-ocimene and decanal, were also tested individually, to which the ovipositor also responded [(*E*)-β-ocimene: 0.4±0.34 mV (mean±s.d., *n*=3; decanal: 0.183±0.076 mV, *n*=3; Fig. 1E,F]. (*E*)-β-ocimene is a well-known HIPV (Dicke and Baldwin, 2010) and is a good candidate for a context-dependent function when combined with CO₂. The ovipositor of the parasitoid *A. westwoodi* that arrives post-pollination did not respond to volatiles of the pollen-receptive phase because this stimulus was ecologically inappropriate as the volatile profile of the syconium changes dynamically with the ontogeny of the syconium (Borges et al., 2013). The parasitoid's ovipositor, however, responded strongly (16.2±4.02 mV, *n*=14) to 0.5% CO₂ (Fig. 1G); this response disappeared when the tip was excised indicative of the presence of the CO₂ sensillum at the tip.

To characterize the ovipositor's electrophysiological response to CO₂ in an organismal context, we performed behavioral assays. In the first assay, 27 out of 40 experimental wasps that had silicone-occluded antennae landed on the tip within 2 min of CO₂ release. With control air flows, these wasps were not attracted to the tip, although a few chance landings did occur. The number of wasps landing at the micropipette tip with blank air and with CO₂ were taken as paired data in each trial; we found that wasps landed significantly more often on the micropipette tip when CO₂ was present (Wilcoxon matched pairs signed rank test, *v*=0, *n*=8 trials, *P*=0.013; Fig. 1H). Because CO₂ sensors may be present elsewhere on the insect body and these may have been also involved in the above response, to demonstrate the ability of the ovipositor to react to CO₂, we performed another assay. In this assay, only the ovipositor of a tethered wasp was exposed to a 5 s stimulus of 0.5% CO₂ injected into the background flow of blank air, in response to which the ovipositor deflected towards the CO₂ source until the CO₂ puff terminated (Movie 2).

CO₂ plays an important role in insect–plant and host–insect interactions when perceived at particular concentrations against the background (Guerenstein and Hildebrand, 2008). Olfactory receptor neurons located on the antennae and maxillary palp mediate the response to CO₂ in *Drosophila* and *Anopheles gambiae* (Suh et al., 2004; Wasserman et al., 2013; Lu, et al., 2007). Context specificity may explain the effectiveness and reliability of this non-specific cue as a short-range oviposition attractant or repellent (Stange, 1999; Goyret et al., 2008). For insect larvae that do not exhibit host-seeking behavior (Brodeur and Boivin, 2004), such as fig wasp larvae that are restricted to their individual galls, it is crucial for ovipositing females to precisely find oviposition sites with the help of their ovipositors. A spatially variable concentration of CO₂ around different galls, owing to the respiration of developing host wasp larvae at different developmental stages and limited permeability of gall tissue, could possibly be used to assess the suitability of the oviposition site. Given that the respiration rate is high in figs (Galil et al., 1973) and that *A. westwoodi* parasitizes prepupal stages of the early-arriving gallers *Sycophaga testacea* and *Sycophaga stratheni* (P.Y. and R.M.B., unpublished data) that develop within large galls, clustered towards the cavity of the

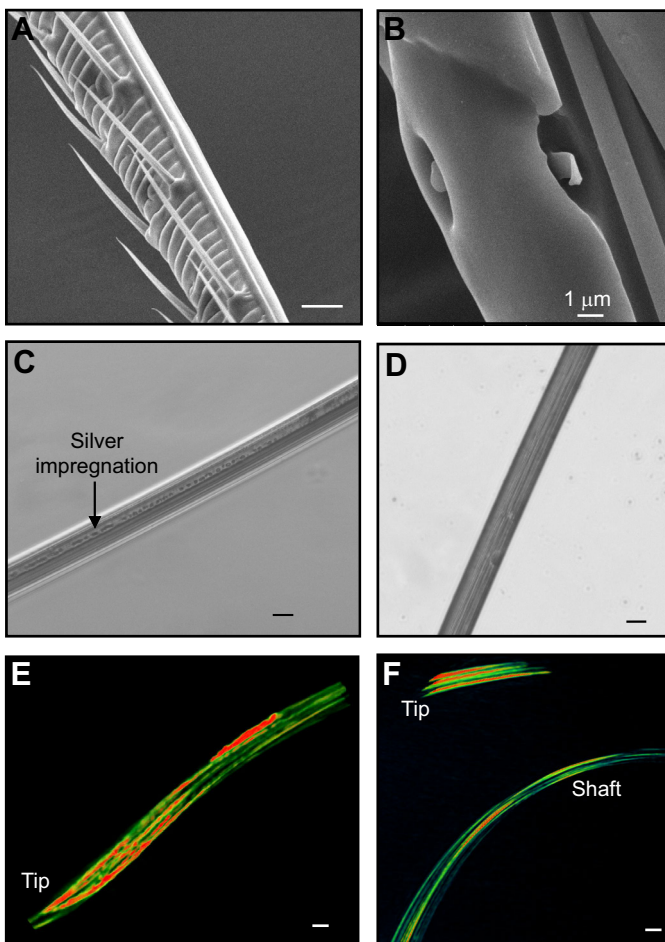


Fig. 2. Demonstration of the porous olfactory nature of sensilla on the ovipositor. SEM images of (A) ovipositor sheath and (B) putative CO₂ sensillum. Silver nitrate staining: (C) silver impregnation inside the ovipositor, (D) unstained control. X-ray tomography images of (E) silver nitrate-stained ovipositor and (F) control. Scale bars (unless specified), 10 μm.

syconium (Fig. 1A), we expected its ovipositor to encounter a relatively high CO₂ concentration deep inside the syconium. We therefore tested the ovipositor response to 0.5% CO₂, which is 1/20th of the highest concentration recorded from figs (Galil et al., 1973). Wasps also frequently expose the sensilla-rich ovipositor tip to chemical cues by an in–out scanning motion (Movie 3) in which the tip is exerted beyond the sheath and then retracted; this motion could help the sensilla-rich tip to perceive volatiles even when the ovipositor continues to remain covered by the sheath prior to oviposition [during oviposition, the sheath remains outside the fig (Fig. 1A); furthermore, the ovipositor sheath has only mechanosensory structures and no chemosensilla (Fig. 2A)]. The previously reported (Ghara et al., 2011) unidentified and sparsely occurring sensillum in the ovipositor of parasitoids (Fig. 2B) resembles the auricillic CO₂-sensillum reported in the antennae of other insects (Stange and Stowe, 1999).

We used silver nitrate staining followed by X-ray tomography (XRT) to investigate the porous, olfactory nature of ovipositor sensilla. Upon penetration through the pores, if any, silver turns black upon exposure to light or organic material. We observed impregnation of silver inside the ovipositor under bright field (Fig. 2C) which was absent in the control (Fig. 2D). XRT indicated the presence of silver along the mid-section of the ovipositor in addition to the tip, reflecting pores in ovipositor sensilla (Fig. 2E). A faint electron-dense patch at the tip and another region in the control (Fig. 2F) is due to zinc in the parasitoid ovipositor (Kundanati and Gundiah, 2014). TEM imaging to explore the internal structure of sensilla was unsuccessful because of the micro-dimensions of the ovipositor (thinner than a human hair) and a strongly sclerotized tip (Ghara et al., 2011), resulting in insurmountable difficulties with fixation and sectioning.

The ability of the antennal olfactory system to evolve rapidly and adapt to changing conditions contributes to the success of insects (Hansson and Stensmyr, 2011). Our study demonstrates olfactory perception of ecologically relevant volatiles in an ovipositor, but the evolution of olfaction in the ovipositor and its role in host-finding across insect taxa remain to be investigated. We predict that olfactory sensilla will occur on ovipositors of insects that are completely dependent on their ovipositor to locate hidden hosts and where a concentration build-up of informative volatiles can occur. For example, the wasp pollinators of *F. racemosa* have only three sensilla on their ovipositors (Ghara et al., 2011) and these are likely to be mechano-chemosensory, but not olfactory, in nature. This is because, unlike NPFWs, pollinating wasps enter into fig syconia, have an abundance of relatively easily accessible oviposition sites, and may not need precise chemosensors that can detect volatile gradients. Further explorations into the sensory nature of ovipositors in such systems and into measuring these volatile gradients can provide designs for ovipositor-inspired micro-chemosensors.

Acknowledgements

The authors are thankful to Avalokiteswar Sen, Yuvaraj Ranganathan and Srinivasan Kasinathan for helping with the GC-EOG setup, and to Frederick Marion Poll and two anonymous reviewers for their valuable comments.

Competing interests

The authors declare no competing or financial interests.

Author contributions

P.Y. and R.M.B. conceived and designed the study, P.Y. conducted the experiments, P.Y. and R.M.B. wrote the paper.

Funding

This work was supported by grants from the Ministry of Environment, Forest and Climate Change; the Department of Biotechnology, Ministry of Science and

Technology; and FIST (Department of Science and Technology, Ministry of Science and Technology).

Supplementary information

Supplementary information available online at <http://jeb.biologists.org/lookup/doi/10.1242/jeb.152777.supplemental>

References

- Borges, R. M. (2015). How to be a fig wasp parasite on the fig–fig wasp mutualism. *Curr. Opin. Insect Sci.* **8**, 34–40.
- Borges, R. M., Bessi re, J.-M. and Ranganathan, Y. (2013). Diel variation in fig volatiles across syconium development: Making sense of scents. *J. Chem. Ecol.* **39**, 630–642.
- Brodeur, J. and Boivin, G. (2004). Functional ecology of immature parasitoids. *Annu. Rev. Entomol.* **49**, 27–49.
- Crnjar, R., Angioy, A., Pietra, P., Stoffolano, J. G., Jr, Liscia, A. and Barbarossa, I. T. (1989). Electrophysiological studies of gustatory and olfactory responses of sensilla on the ovipositor of the apple maggot fly, *Rhagoletis pomonella* Walsh. *Ital. J. Zool.* **56**, 41–46.
- Dicke, M. and Baldwin, I. T. (2010). The evolutionary context for herbivore-induced plant volatiles: beyond the ‘cry for help’. *Trends Plant Sci.* **15**, 167–175.
- Galil, J., Zeroni, M. and Bar Shalom, D. (1973). Carbon dioxide and ethylene effects in the coordination between the pollinator *Blastophaga quadraticeps* and the syconium in *Ficus religiosa*. *New Phytol.* **72**, 1113–1127.
- Ghara, M., Kundanati, L. and Borges, R. M. (2011). Nature’s Swiss army knives: ovipositor structure mirrors ecology in a multitrophic fig wasp community. *PLoS ONE* **6**, e23642.
- Goyret, J., Markwell, P. M. and Raguso, R. A. (2008). Context- and scale-dependent effects of floral CO₂ on nectar foraging by *Manduca sexta*. *Proc. Natl. Acad. Sci. USA* **105**, 4565–4570.
- Guenerstein, P. G. and Hildebrand, J. G. (2008). Roles and effects of environmental carbon dioxide in insect life. *Annu. Rev. Entomol.* **53**, 161–178.
- Hansson, B. S. and Stensmyr, M. C. (2011). Evolution of insect olfaction. *Neuron* **72**, 698–711.
- Klinner, C. F., K nig, C., Missbach, C., Werckenthin, A., Daly, K. C., Bisch-Knaden, S., Stengl, M., Hansson, B. S. and Gro e-Wilde, E. (2016). Functional olfactory sensory neurons housed in olfactory sensilla on the ovipositor of the hawkmoth *Manduca sexta*. *Front. Ecol. Evol.* **4**, 130.
- Kundanati, L. and Gundiah, N. (2014). Biomechanics of substrate boring by fig wasps. *J. Exp. Biol.* **217**, 1946–1954.
- Letzkus, P., Ribl, W. A., Wood, J. T., Zhu, H., Zhang, S.-W. and Srinivasan, M. V. (2006). Lateralization of olfaction in the honeybee *Apis mellifera*. *Curr. Biol.* **16**, 1471–1476.
- Li, Z., Yang, P., Peng, Y. and Yang, D. (2014). Ultra structure and distribution of sensilla on the antennae of female fig wasp *Eupristina* sp. (Hymenoptera: Agaonidae). *Acta. Zool.* **95**, 73–83.
- Lu, T., Qiu, Y. T., Wang, G., Kwon, J. Y., Rutzler, M., Kwon, H.-W., Pitts, R. J., van Loon, J. J. A., Takken, W., Carlson, J. R. et al. (2007). Odor coding in the maxillary palp of the malaria vector mosquito *Anopheles gambiae*. *Curr. Biol.* **17**, 1533–1544.
- Ninemets, U. and Reichstein, M. (2003). Controls on the emission of plant volatiles through stomata: Differential sensitivity of emission rates to stomatal closure explained. *J. Geophys. Res. Atmos.* **108**, 4208.
- Park, K. C. and Baker, T. C. (2002). Improvement of signal-to-noise ratio in electroantennogram responses using multiple insect antennae. *J. Insect Physiol.* **48**, 1139–1145.
- Quicke, D. L. J., LeRalec, A. and Vilhelmsen, L. (1999). Ovipositor structure and function in the parasitic Hymenoptera with an exploration of new hypotheses. *Rendiconti* **47**, 197–239.
- Rice, M. J. (1977). Blowfly ovipositor receptor neuron sensitive to monovalent cation concentration. *Nature* **268**, 747–749.
- Stange, G. (1999). Carbon dioxide is a close-range oviposition attractant in the Queensland fruit fly *Bactrocera tryoni*. *Naturwissenschaften* **86**, 190–192.
- Stange, G. and Stowe, S. (1999). Carbon-dioxide sensing structures in terrestrial arthropods. *Microsc. Res. Tech.* **47**, 416–427.
- Suh, G. S. B., Wong, A. M., Hergarden, A. C., Wang, J. W., Simon, A. F., Benzer, S., Axel, R. and Anderson, D. J. (2004). A single population of olfactory sensory neurons mediates an innate avoidance behaviour in *Drosophila*. *Nature* **431**, 854–859.
- Van Lenteren, J. C., Ruschioni, S., Romani, R., Van Loon, J. J. A., Qiu, Y. T., Smid, H. M., Isidoro, N. and Bin, F. (2007). Structure and electrophysiological responses of gustatory organs on the ovipositor of the parasitoid *Leptopilina heterotoma*. *Arth. Struct. Dev.* **36**, 271–276.
- Wasserman, S., Salomon, A. and Frye, M. A. (2013). *Drosophila* tracks carbon dioxide in flight. *Curr. Biol.* **23**, 301–306.