Neural and behavioural responses of the pollen-specialist bee *Andrena vaga* to *Salix* odours

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Summary statement

Pollen-specialist *Andrena vaga* bees are highly tuned to 4-oxoisophorone, a typical *Salix* odorant component, at a very low concentration.

Abstract

An effective means of finding food is crucial for organisms. Whereas specialized animals select a small number of potentially available food sources, generalists use a broader range. Specialist (oligolectic) bees forage on a small range of flowering plants for pollen and use primarily olfactory and visual cues to locate their host flowers. So far, however, little is known about the specific cues oligoleges use to discriminate between hosts and non-hosts and how floral scent compounds of hosts and non-hosts are processed in the bees' olfactory system. In this study, we recorded physiological responses of the antennae (electroantennographic detection coupled to gas chromatography GC-EAD) and in the brain (optical imaging, GC-imaging), and studied host-finding behaviour of oligolectic *Andrena vaga* bees, a specialist on *Salix* plants. In total, we detected 37 physiologically active compounds in host and non-host scents. 4-Oxoisophorone, a common constituent in the scent

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of many *Salix* species, evoked strong responses in the antennal lobe glomeruli of *A. vaga*, but not the generalist honeybee *Apis mellifera*. The specific glomerular responses to 4-oxoisophorone in natural *Salix* scents reveals a high degree of specialization in *A. vaga* for this typical *Salix* odorant component. In behavioural experiments, we found olfactory cues to be the key attractants for *A. vaga* to *Salix* hosts, which are also used to discriminate between hosts and non-hosts, and demonstrated a behavioural activity for 4-oxoisophorone. A high sensitivity to floral scents enables the specialized bees to effectively find flowers and it appears that *A. vaga* bees are highly tuned to 4-oxoisophorone at a very low concentration.

Key words

GC-imaging, antennal and antennal lobe responses, sensitivity, floral scent, oligolectic bees

Introduction

A majority of insects utilize food from a small subset of host plants (Bernays and Chapman, 1994). These specialist insects must be able to find their specific plants in a complex environment. An effective means of finding food sources is essential for their reproductive fitness, since the availability of food is one of the major factors limiting the development of the insect's offspring and survival of adults (Müller et al., 2006). To find appropriate food sources, insects explore various plant stimuli by using different senses, such as olfaction and vision. In many specialized associations, plant-derived volatiles are most important to guide the insects to food sources (Kessler and Baldwin, 2001; Raguso, 2008). Pollinators, for example, use floral scents to orientate towards flowers from long distance, to discriminate between and recognize plant species, and as landing cue or nectar guide (Raguso, 2008). Although floral volatiles were identified in many plant species (Knudsen et al., 2006), the behaviour-modulating components among them and their interplay with multimodal stimuli (e.g. floral colours) have rarely been identified (Dobson, 2006; Raguso, 2020).

Bees are amongst the most important pollinators. While many bee species, including honeybees, visit several different plants, many other bee species restrict their foraging to a subset of available resources. Depending on the habitat, between 15 and 60 percent of the local bee species are strictly specialist (oligolectic) and collect pollen from only few plant species belonging to one plant family or genus (Michener, 2007; O`Toole and Raw, 1991; Westrich, 2018). The collected pollen is primarily used to provision their offspring with food.

The specialized bees recognize their host plants with olfactory cues alone (Schäffler et al., 2015) or with the combination of olfactory and visual cues (Burger et al., 2010). Host finding and recognition is crucial in oligolectic bees, because their larvae normally either fail to develop or experience high mortality rates on non-host pollen (Praz et al., 2008; Sedivy et al., 2008).

Oligolectic bees are very sensitive to the characteristic host-plant volatiles. At the peripheral level, odorants are detected by olfactory receptors neurons on the bees' antennae. As demonstrated by electroantennographic experiments for a megachilid bee (*Chelostoma rapunculi*) that is specialized on *Campanula* spp., antennal responses were recorded to much lower concentrations of host-specific compounds (spiroacetals) than for generalist bees that also visit *Campanula* flowers (Brandt et al., 2017). In the olfactory system, information from olfactory receptor neurons is transferred to the antennal lobe, the first brain structure to process olfactory information (Galizia and Menzel, 2000). Here, all receptor neurons expressing the same olfactory membrane receptor converge onto a specific glomerulus (Galizia, 2014). Using optical imaging experiments, it was recently shown that antennal lobe glomeruli of the oligolectic bee *A. vaga* responded to lower concentrations of 1,4-dimethoxybenzene than the ones of generalist honeybees. 1,4-Dimethoxybenzene is a common floral volatile of *Salix* host flowers and attractive to both bee species (Dötterl et al., 2005, 2014).

Despite these results, the host finding behaviour of *A. vaga* is not fully understood. Although the floral scent of different *Salix* species is well described (Füssel et al., 2007) and some of the compounds elicited physiological (Burger et al., 2013) or behavioural (Dötterl et al., 2005; Dötterl and Vereecken, 2010) responses in *A. vaga*, *Salix* compounds that are absent in co-flowering non-host species and attractive for *A. vaga* are not yet known. Studies that integrate behavioural and neurophysiological experiments in a naturalistic context are rarely performed (but see Najar-Rodriguez et al., 2010; Piñero et al., 2008; Riffel et al., 2009; Riffell et al., 2013) but can give new insights in the foraging behaviour of *A. vaga* as the neural processing of floral volatiles is a central part to their behavioural responses.

The aim of this study was to determine the essential cues *A. vaga* bees use to discriminate between *Salix* host and non-host plants, and to identify physiologically and behaviourally active floral scent compounds. We tested the attractiveness of floral cues under semi-natural conditions in a flight cage. Next, we performed calcium imaging experiments in the antennal lobes and coupled the physiological setup to a gas chromatograph (GC-imaging). This innovative approach, previously developed in *Drosophila* (Schubert et al., 2014), allowed us

to analyse the elements of multi-component samples to reveal potential behaviourally active compounds for bees. We also compared the results of the GC-imaging to results obtained by electroantennographic detection experiments (GC-EAD) to see whether these approaches produce consistent results or complement each other. Finally, we compared the floral scents and colours of different *Salix* and non-*Salix* species to reveal floral cues that are common for the bees` host plants.

Specifically, we asked: (1) What are the spectral and chemical properties of visual and olfactory displays of host and non-host inflorescences? (2) Which components of host and non-host scent bouquets elicit specific responses in the antennae and the brains of *A. vaga*? (3) How sensitive is *A. vaga* to 4-oxoisophorone, a compound of *Salix* scents identified in the present work as being physiologically highly active in the antennal lobes of *A. vaga*, as compared to the generalist honeybee (*A. mellifera*)? (4) What is the relative importance of olfactory and visual cues of *S. cinerea* hosts in the attraction of *A. vaga* bees, and in the discrimination against dandelion (*Taraxacum officinale*) and wild narcissus (*Narcissus pseudonarcissus*) non-hosts? Is 4-oxoisophorone and a synthetic mixture with further physiologically active *Salix* compounds attractive for *A. vaga*?

Material and methods

Study organisms

Andrena vaga Panzer 1790 (Andrenidae) is a solitary bee species active from March to May. The bees are oligolectic (pollen specialist) on *Salix* spp. (Fig. 1). They hibernate as adults and build nests in aggregations preferably in sandy ground. The geographic range is from middle and north Europe to Central Asia. We collected foraging-naïve *A. vaga* bees at nest aggregations, consisting of hundreds to thousands of individuals in the Botanical Garden of the University of Ulm (N48.418453, E9.967323) and at a site (N47.704696, E9.155909) close to Konstanz. Shortly before bees started to become active in spring, we carefully dug out males and females, placed them in boxes containing sand and stored the boxes in dark at 6°C in a fridge. We used the bees either for physiological or behavioural experiments.

For physiological experiments, we placed the boxes with bees in small flight cages (60 cm x 60 cm x 60 cm) containing a sandy ground, and located them close to a window in the laboratory at room temperature few days before experiments started. For behavioural experiments, we placed the boxes with bees in a bigger flight cage (2 m x 3 m x 2 m)

established at an *A. vaga* nesting site in the Botanical Garden of the University of Ulm. Some further individuals directly hatched within the cage. In total, approx. 50 individuals (females and males) were active when bioassays started. To supply them with food, we offered sponges saturated with inverted sugar water (Apiinvert, Südzucker AG, Mannheim/Ochsenfurt, Germany). All bees were still naïve in respect to flower foraging for our experiments.

Honeybees (*Apis mellifera* Linnaeus, 1758) foraging on sugar water baits were collected from hives at the University of Konstanz for physiological experiments. *Salix* is an important food source for honeybees in early spring when only few other plant species are flowering.

We chose different willow species (*S. cinerea* L., *S. fragilis* L., *S. alba* L., *S. alba* x *babylonica*, *S. caprea* L., *S. udensis* Trautv. & C.A.Mey., and *S. purpurea* L.) as host plants, and widespread dandelion (*Taraxacum officinale* aggr.) and wild narcissus (*Narcissus pseudonarcissus* L.) as non-hosts. We collected flowering stems in the Botanical Garden of the University of Ulm and in the park Hockgraben close to the University of Konstanz. The flowers are normally arranged in catkins whereby female flowers offer only nectar, and male flowers pollen and nectar. We used only male *Salix* flowers for the experiments. The chosen non-hosts have yellow flowers, bloom at the same time and in syntropy with *Salix* hosts. Though *N. pseudonarcissus* has a quite restricted native occurrence (www.floraweb.de), we chose this species because members of this genus are known to emit the same main compound, 1,4-dimethoxybenze, as several *Salix* species (Füssel et al., 2007; Knudsen et al., 2006).

Colour measurements

We measured the spectral reflection of floral parts of six different *Salix* host (*S. cinerea*, *S. caprea*, *S. alba*, *S. alba* x *babylonica*, *S. purpurea*, *S. fragilis*) and of the two non-hosts (*T. officinale*, *N. pseudonarcissus*). The measured wavelengths ranged from 300 to 700 nm, which corresponds to the colour spectrum perceived by bees (Peitsch et al., 1992). We recorded the floral colours using an Ocean Optics Jaz Spectrometer (Ocean Optics, Inc., Dunedin , Florida, USA). A deuterium-halogen-lamp floated light over a glass cable at an angle of 45°. We used a black adhesive tape and barium sulphate (Merck KGaA, Darmstadt, Germany) as black and white standard, respectively. We stuck the samples on the black tape for the analyses.

In *T. officinale*, we measured the outer parts (corollas of ray flowers) and the centre of the inflorescences where the pollen is located. The corolla, the pseudocorolla, and the pollen were analysed in *N. pseudonarcissus*. In *Salix*, we measured only the pollen colours because pollen is the only colourful part of male willow flowers. In each species, we used the

flowers/inflorescences from three different individuals for the analyses and calculated an average per species.

We used the spectral data to plot the colour loci in the colour hexagon (Chittka, 1992). Bees are UV-blue-green trichromats (Peitsch et al., 1992), and this colour diagram visualizes how they perceive the colours. We used the reflectance function of a typical green leaf as background colour (Chittka et al., 1994).

Preparation of natural and synthetic floral scent samples

Natural odour samples for optical imaging experiments

We cut *Salix* catkins and *T. officinale* blossoms, immediately transferred them to 20 ml headspace glass vials (Klaus Trott, Kriftel, Germany) filled with gaseous nitrogen (to avoid chemical oxidation), and sealed the vials with aluminium ring caps fitted with a silicon/Teflon septum as described and detailed elsewhere (Pelz et al., 2006). We pooled eight individual male *Salix* catkins and five *Taraxacum* blossoms to obtain one sample for each experimental day. Empty vials filled with nitrogen served as controls.

Synthetic odorants for optical imaging experiments (dose-response assay)

We used synthetic 4-oxoisophorone and 2-phenylethanol (both > 99%, Sigma-Aldrich, St. Louis, Missouri, USA) to stimulate neural responses in antennal lobes of *A. vaga* and *A. mellifera* using optical imaging. We chose 4-oxoisophorone because it is present in small amounts in the bouquets of different *Salix* species and consistently elicited a response in the antennal lobes of *A. vaga*, but was absent in the two other tested non-host species. 2-Phenylethanol is a widely distributed floral scent compound that is found in more than 50 % of all flowering plant species (Knudsen et al., 2006), which is also true for *T. officinale*, and is often selected as a test substance representing commonly occurring floral volatiles (Brandt et al., 2017). Furthermore, the vapour pressure of both compounds is in a similar range, 0.1580 mmHg for 4-oxoisophorone and 0.0868 mmHg for 2-phenylethanol. We prepared nine different dilutions, from 10^{-2} to 10^{-10} (vol./vol.) in mineral oil (> 99%, Fluka). Linalool (> 97%, racemic mixture, Sigma-Aldrich), a widely distributed floral volatile (Knudsen et al., 2006), was included as a reference odorant at a dilution of 10^{-4}, allowing normalization of the magnitude of the calcium responses across tested females. We kept 5 ml of the diluted odorants in 20 ml headspace glass vials as described above. As a control stimulus, we used a vial containing 5 ml mineral oil only.

Natural odour samples for electroantennographic experiments and chemical analyses

As the GC-EAD-system was not equipped with a headspace unit, we trapped the odours from inflorescences/flowers of the various *Salix* hosts (*S. cinerea* N=13, *S. caprea* N=4, *S. aurita* N=5, *S. udensis* N=7, *S. purpurea* N = 4) and the two non-hosts (*T.officinale* N=14, *N.pseudonarcissus* N=7) using dynamic headspace methods. We cut the flowering/inflorescence stems and placed them in water after cutting. Samples were collected from four flowering stems (approx. 50 flowering catkins) of each *Salix* species and approx. 30 blossoms and flowers of *T. officinale* and *N. pseudonarcissus*, respectively. The cut flowering stems were enclosed within polyester oven bags (Toppits®, Minden, Germany; size: 40 cm x 30 cm). We trapped the volatiles for 4 h during daytime in an adsorbent tube using a membrane pump (G12/01 EB, Rietschle Thomas, Puchheim, Germany) at a flow rate of 100 ml min^{-1}. The adsorbent tubes were filled with 10 mg SuperQ (mesh 80/100, Alltech Associates Inc., Deerfield, I, USA), fixed in the tubes with glass wool. We eluted trapped volatiles with 100 µl of dichloromethane (99.9 %, Merck, Darmstadt, Germany) and stored the samples at -20°C. Samples without plant material were taken as blank controls and contaminants were excluded from the analyses.

Synthetic odour mixtures for behavioural experiments

For behavioural experiments, we used a synthetic mixture of physiologically active compounds that resembled the composition of *S. cinerea* scent samples in quantitative and proportional parameters, and 4-oxoisophorone alone. The compounds of the synthetic mixture were diluted in dichloromethane (99.9%, Merck) to obtain stock solutions and then added to the synthetic mixture according to Table S1. In a next step, this solution was further diluted 1:100 and 50 µl of the final mixture were used in bioassays. The amount of each compound was in the range found in natural *S. cinerea* scent samples (N = 7 headspace samples) and verified in GC-MS analyses. The 4-oxoisophorone test solution contained 50 µg of 4-oxoisophorone (> 98%, Sigma) solved in 50 µl dichloromethane.

Optical imaging experiments

Neuronal responses in the antennal lobes of *A. vaga* females and *A. mellifera* foragers were recorded using calcium imaging experiments (Galizia and Vetter, 2005; Najar-Rodriguez et al., 2010). Calcium imaging allows quantifying changes in intracellular calcium concentrations as a measure of odorant-evoked neuronal activity (Galizia and Menzel, 2001).

We recorded responses of *A. vaga* and *A. mellifera* to dilutions of both 4-oxoisophorone (*A. vaga* N = 13, *A. mellifera* N = 11) and 2-phenylethanol (N = 11 in both species). In *A. vaga*, we additionally recorded responses to natural occurring volatiles of host and non-host inflorescences/flowers, either to the unseparated headspace of the samples (*S. cinerea* N=8 recordings, *S. fragilis* N=3, *S. alba* x *babylonica* N=4, *T. officinale* N=7) or to the components of the headspace separated by a gas chromatograph (*S. cinerea* N=11, *S. fragilis* N=4, *S. alba* x *babylonica* N=3, *T. officinale* N=5).

Insect preparation and staining

We mounted individual *A. vaga* or *A. mellifera* bees on a custom-made Plexiglass stage, fixed them with wax and dissected the brains using the technique as described by Galizia and Vetter (2005) and adapted for *A. vaga* by Burger et al. (2013). We stained the ALs for 60 min by bath-applying Calcium Green 5N AM (Molecular Probes, Invitrogen, Carlsbad, CA, USA) dissolved in saline with Pluronic and DMSO (dimethylsulfoxide). This procedure leads to signals that combine the activities of several different types of neurons and possibly also glial cells. In honeybees, it has been shown that the prevailing signal magnitude is a good estimate of sensory neuron responses (Galizia and Vetter, 2005). After staining, we removed the excess dye and washed the brain in saline (in mM: 130 NaCl, 6 KCl, 4 $MgCl_2$, 5 $CaCl_2$, 160 sucrose, 25 D-glucose, 10 Hepes free acid, pH 6.7, 500 mOsmol). We carefully removed the mouthparts of *A. vaga* to prevent movement artefacts, which was not necessary in *A. mellifera*. We covered the exposed ALs with a thin layer of transparent two-component silicon (KwikSil, World Precision Instruments, Sarasota, FL, USA). Within 10-30 min, we placed the bees under an upright microscope (Olympus BX50WI, Hamburg, Germany) with a 20x water-immersion objective (NA = 0.95, Olympus XLUM Plan FI).

Optical recordings and stimulus delivery

Images were acquired with a CCD camera (Imago QE, T.I.L.L. Photonics, Gräfelfing, Germany). An 8 x 8 binning on chip was applied to a spatial sampling rate of 1.57 µm x 1.57 µm $pixel^{-1}$. Excitation light was 470 nm (Polychrome V, intensity 10%) and emission was filtered by a LP505 (Carl Zeiss GmbH, Hamburg, Germany). The exposure time was between 30 and 100 ms per image, depending on the basal fluorescence values of the individual females. Optical recordings consisted of 100 frames for each stimulus at a frequency of 4 Hz.

Imaging of odorants and unseparated headspace. A computer-controlled autosampler (Combi PAL, CTC Analytics AG, Zwingen, Switzerland) applied 1 ml of odorant-loaded headspace from the 20 ml vials that contained inflorescences/flowers at 1 ml s^{-1} during the recordings of brain activity. The application started at 6 s after the recording had started (frame 24).

GC-imaging. The system consisted of a gas chromatograph (Thermo Finnigan Trace GC Ultra, San Jose, CA, USA) equipped with a flame ionization detector and coupled to the described imaging set-up. The autosampler injected 2 ml of the odour-loaded headspace from the 20 ml vials in splitless mode (injection temperature 200°C) at an oven temperature of 40°C followed by opening the split vent after 1 min and heating the oven at a rate of 7°C min^{-1} to 200°C (held for 1 min). The carrier gas was helium with a constant flow of 1.5 ml min^{-1}. We used an Optima 5 MS column (length 30 m, inner diameter 0.25 mm, film thickness 0.25 µm, Macherey-Nagel, Düren, Germany) with an installed four-arm flow splitter (OSS-2, SGE, UK) that split the column at the end into two pieces of deactivated capillary (length 70 cm, ID 0.32 mm) leading to the detector and to a heated (temperature 200°C) transfer line (length 40 cm, Axel Semrau, Sprockhövel, Germany), respectively. Makeup gas (nitrogen, 30 ml min^{-1}) was introduced through the fourth arm of the splitter. The separated volatiles were transferred via the transfer line to the outside of the gas chromatograph. The column finally ended in a Teflon tube (inner diameter 2 mm, air stream 1.3 m s^{-1}) directed to the antennae of an insect prepared for AL imaging.

In GC-imaging experiments, AL responses were recorded during GC runs using three different temporal sequences. Each sequence consisted of five individual optical recording blocks consisting of 340 frames that were recorded at a frequency of 4 Hz. A break of 35 s between the blocks allowed for recovery from photobleaching. The alternation of relatively short recordings and breaks resulted in an increased longevity of the prepared animal and allowed recordings of clearly recognizable odour responses. The different temporal sequences varied in their offset time (4.0, 4.5, 5.0 min after sample injection) to cover the complete GC run with recordings. The sequences were started one after another in varying orders and the number per animal depended on its longevity.

Signal calculation

We analysed the imaging data using custom-written programs in IDL (Research Systems, Inc., Boulder, CO, USA) and in R (R.CoreTeam, 2015). Raw data were corrected for lateral movement artefacts using anatomical landmarks. Then, data were corrected for fluorescence intensity decay due to bleaching (Galizia and Vetter, 2005) and filtered using a spatial median

filter with a size of 5 pixels to reduce noise. An unsharp mask filter was applied to reduce scattered light produced by strongly activated glomeruli on neighbouring non-responding areas within the ALs (Galizia and Vetter, 2005). The relative calcium change was then calculated for each frame as relative changes in fluorescence ($\Delta F/F$). The background fluorescence (F) was defined as the average fluorescence of frames 4-23 for every pixel.

Imaging of odorants and unseparated headspace. For the false colour images, we used the difference between the fluorescence of frames 29-31 and 59-61. Thus, each pixel was assigned a value that was then translated into a colour. To analyse the responses to the odorant dilutions, time traces for glomeruli were calculated from squares with a side length of 11 pixels (corresponds to 18 µm) and always well within each identified glomerulus. We cannot always exclude some cross-talk of neighbouring glomeruli due to scattered light. Since the anatomical borders of glomeruli were not visible with this staining, identification and localization of glomeruli was based on their physiological responses, and their relative position. In the lack of an anatomical reference atlas for *A. vaga*, we labelled identified glomeruli with letters, allowing for their comparison across individuals. The nomenclature of the analysed glomeruli is the same as in Burger et al. (2013). To ensure that the brain area was comparable across individuals, first, the animal position was adapted in the microscope view using the outline of the brain for orientation (roundish form and tissue of the antennal lobes easily recognizable in contrast to the dark head capsule in the background), and one antennal lobe was positioned in the centre of the recording area. For fine tuning of the position and to check whether the antennal lobe was responding (i.e., not injured during dissection) we run a control sequence including the reference odour and the solvent control. The response pattern of the reference odour, in particular, was useful to determine the location of the target glomeruli. Then, we recorded a variety of odorants in the same animals, e.g. natural headspaces of different plant species, odorants used for the dose-response assay and different synthetic analogues to verify the identity of compounds and response patterns found in GC-imaging.

Dose-response curve analysis: We normalized the data based on the reference odorant measurements within each individual animal (R package GloDataMix). A Friedman's test with post hoc (R package Agricole) was performed to test for differences between odorant responses and responses to the solvent control. The response to 4-oxoisophorone in *A. vaga* was analysed for two glomeruli, the glomerulus with highest sensitivity and the one with the highest maximum response. For all other treatments, only one glomerulus was chosen

(representing the glomerulus with both the highest sensitivity and the highest maximum response)

GC-imaging. For the false colour images of chromatographically separated compounds, measurements were analysed interactively. If a glomerulus responded to a compound, a change in fluorescence was detected that corresponded to an odour peak in the GC run. The response was visualised in false-coded pictures by calculating the $\Delta F/F$ between three frames around the maximum and minimum response detected in the time-traces. Only if a clear glomerular response pattern was seen that was reproducible in at least three runs, the compound was considered as physiologically active. Active compounds were identified in chemical analyses. The identity of the compounds was confirmed by a comparison of retention times and/or response patterns on all used systems with synthetic analogues.

Electroantennographic recordings

We recorded antennal responses in *A. vaga* females to floral scents of the *Salix* hosts (*S. cinerea* N=6 recordings, *S. caprea* N=3, *S. udensis* N=3, *S. purpurea* N=3, *S. purpurea* N=3) and the two non-hosts (*T. officinale* N=6, *N. pseudonarcissus* N=7) using gas chromatography coupled with electroantennographic detection (GC-EAD). In the electroantennographic recordings, a stimulus elicits increased neural activity in olfactory receptor neurons (recorded as voltage drop) located on the insect antennae, the peripheral olfactory organ (Wibe, 2004).

The system consisted of a gas chromatograph (HP 6890, Hewlet-Packard, Agilent Technologies, USA) equipped with a flame ionization detector and coupled to an EAD setup (heated transfer line, 2-channel USB acquisition controller, Syntech, Hilversum, Netherlands). We injected 2 µl of the samples in splitless mode (injection temperature 310°C) at an oven temperature of 50°C followed by opening the split vent after 1 min and heating the oven at a rate of 10°C min^{-1} to 220°C (held for 5 min). The carrier gas was hydrogen with a constant flow of 2 ml min^{-1}. We used a DB-5 ms column (length 30 m, inner diameter 0.25 mm, film thickness 0.25 µm, J&W, USA) with an installed four-arm flow splitter (GRAPHPACK 3D/2, Gerstel, Mühlheim, Germany) that split the column at the end into two pieces of deactivated capillary (length 50 cm, ID 0.32 mm) leading to the detector and to the EAD setup, respectively. Makeup gas (nitrogen, 30 ml min^{-1}) was introduced through the fourth arm of the splitter. The outlet of the EAD ended in a cleaned and humidified airflow (glass tube, inner diameter: 7 mm, air stream 100 ml min^{-1}) directed to a prepared antenna.

For the EAD, we cut an antennae of *A. vaga* females at the base and the tip and mounted it between two glass capillaries filled with insect Ringer solution (in mM: 137 NaCl, 5.4 KCl, 3.6 $CaCl_2$). Gold wires connected the capillaries for a closed electric circuit. We considered a compound to be EAD-active when we recorded a response in at least three runs per species. We identified the physiologically active compounds and determined their absolute and relative amounts in chemical analyses.

Chemical identification and comparisons of floral scents

We identified the physiologically active compounds and determined their absolute and relative amounts using gas chromatography coupled to mass spectrometry (GC-MS). The equipment consisted of a gas chromatograph (7890B GC system, Agilent Technologies, USA) coupled to a mass spectrometer (Agilent 5977A mass selective detector). An autosampler (Gerstel MAS Modular Analytical Systems Controller C506, Gerstel, Mühlheim a. d. Ruhr, Germany) injected 1 µl of the samples in splitless mode into a Gerstel cooled injection system (KAS 4, Gerstel, Mühlheim a. d. Ruhr, Germany). The injection system consisted of a septumless head with an injection temperature of 10°C. The initial temperature increased at a rate of 12°C per second to 300°C. The oven program started at 50 °C followed by opening the split vent after 1 min and heating the oven at a rate of 10°C min^{-1} to 310°C (held for 33 min). The carrier gas was helium with a constant flow of 1.5 ml min^{-1}. We used a DB-5ms column (length 30 m, inner diameter 0.25 mm, film thickness 0.25 µm, J&W, USA). The MS interface had a temperature of 250°C and the ion source 230°C. Mass spectra were taken at 70 eV (in EI mode) from m/z 35 to 450.

We identified the compounds based on their mass spectra using multiple references from the NIST11 library and on Kovats retention indices published for *Salix* odours (Füssel et al., 2007). If authentic standards were available, we additionally confirmed the identification of individual components by comparison of both mass spectrum and GC retention data. Active compounds were assigned to GC-MS runs by comparing the elution sequence and Kovats retention indices. Amounts of the compounds were calculated using AMDIS 2.71 (Automated Mass Spectral Deconvolution and Identification System) and determined as absolute amounts based on an internal standard (dodecane, 1 µg per sample). We analysed the differences in the scent bouquets between species using Primer 6.1.15 (Clarke and Gorley, 2006). We calculated the relative amounts of single components with respect to the total amount in a sample and square-root transformed the data. Based on pairwise Bray-Curtis similarities we visualized the similarities and dissimilarities among the samples using non-metric multidimensional scaling

and performed an ANOSIM (9999 permutations). We evaluated the contribution of single substances to the observed dissimilarity between hosts and non-hosts using a SIMPER analysis.

Behavioural experiments

We conducted eleven two-choice bioassays with foraging-naïve bees in a flight cage to test innate behavioural responses to natural visual and natural olfactory floral cues and synthetic odour samples. We performed tests only on sunny days between 9:30 am and 2 pm, when the activity of bees was high. Prior to each behavioural experiment, we removed the sugar water supply.

We performed the experiments in the year 2015 and repeated them in 2016. The recorded responses of both years were summed up. The number of responding bees were similar among years but differed between experiments and was dependent e.g. on the day time, weather conditions, numbers of experiments per day and date of the season. The results of the first year were confirmed in the second year, except one result (combined olfactory and visual cues versus olfactory cues of *S. cinerea*) that changed from a tentative to a significant trend. To assure that an individual bee was counted only once in a specific two-choice test, we caught the responding bees. An individual bee, however, may have participated in different two-choice tests. The responses in each choice experiment were compared using an exact binomial test (www.graphpad.com).

Attractiveness of natural floral cues

We used an experimental set-up analogous to Burger et al. (2010) to test the attractiveness of coupled and decoupled olfactory and visual cues of *S. cinerea* host, and *T. officinale* and *N. pseudonarcissus* non-hosts, for *A. vaga*. We cut flowering branches/inflorescences and placed them into different Plexiglas® cylinders. All cylinders had a height of 39 cm and a diameter of 9.5 cm. For testing of decoupled olfactory cues, we used a grey cylinder with small holes. The plant volatiles were pumped out of this cylinder using a membrane pump with an air flow of 1 l min^{-1} (G12/01 EB, Rietschle Thomas, Puchheim, Germany). For testing of decoupled visual cues, we used a transparent cylinder without holes. For offering a combination of both cues, we used a transparent cylinder with holes. Here again, the volatiles were pumped out with the help of a membrane pump.

In each experiment, we presented two cylinders at a distance of 30 cm and 15 cm above ground. Bees were observed for 30 min, during which we exchanged the position of the cylinders after 15 min to control for a potential side bias. Attractive responses of bees were recorded as approaches and landings. We tested decoupled olfactory and visual cues of *S. cinerea* host flowers each against an empty control and against the corresponding cue of the non-hosts *T. officinale* and *N. pseudonarcissus*. We also tested the combined visual and olfactory cues of *S. cinerea* against an empty control and against the decoupled olfactory cues of *S. cinerea*.

Attractiveness of synthetic compounds and solvent headspace samples

In two-choice experiments, we tested both the synthetic mixture of physiologically active compounds (table S1) and 4-oxoisophorone alone against a solvent control, and the synthetic mixture also against a solvent *S. cinerea* headspace sample. We applied 50 µl of a sample and of a solvent control (dichloromethane) on rectangular sponge strips (35 x 7.5 x 1.5 mm; Kettenbach GmbH & Co. KG, Eschenburg, Germany). We presented the impregnated filter papers at a distance of 30 cm on dissecting needles that were fixed on a horizontal metal bar. This set-up was mounted on a plastic stick that was stuck in the soil and presented at a height of 1.25 m. We exchanged the position of the stick in the flight cage at least four times in each experiment. We reported the behavioural responses of bees for 30 min as approaches and landings.

Results

Host and non-host floral colours differ

The pollen of all studied *Salix* hosts and the two non-hosts was bee green, but the colour loci of host and non-host pollen did not group together (Fig. 2A). The *T. officinale* corollas were bee UV-green and clearly different in the colouration for bees compared to *Salix*. The paracorolla and corolla of *N. pseudonarcissus* were at the transition of bee green and bee blue-green and closely positioned to the *Salix* loci but the flowers can be probably distinguished from *Salix* catkins by bees on the colour pattern formed by the corolla and paracorolla. Taken together, host and non-host colour displays are discernible by the bee visual system.

Physiologically active compounds in host and non-host odorants differ

We chemically analysed host and non-host flower odorants, and tested them for physiological responses in *A. vaga*. We identified a total of 37 active compounds in all physiological experiments among the different *Salix* species, *T. officinale* and *N. pseudonarcissus* (Tab. S2). Two compounds were classified as aliphatic compounds, 16 as terpenes, nine as benzenoids and phenylpropanoids, two as nitrogen containing compounds and seven had an unknown identity. The multidimensional scaling based on the Bray-Curtis index showed species-specific scent bouquets (ANOSIM R = 0.83, $P < 0.001$; Fig. 2B) and a separation between the bouquets of the *Salix* species and the non-hosts *T. officinale* and *N. pseudonarcissus* (ANOSIM pairwise comparisons between host and non-host species: range of R values between 0.89 and 1, $P < 0.01$). The compounds benzaldehyde, (*E*)-β-ocimene and 1,4-dimethoxybenzene were most responsible for the dissimilarity between host and non-hosts (SIMPER, average dissimilarity between host and non-hosts 72%).

In the calcium imaging experiments, the antennal lobes of *A. vaga* females showed clearly distinct response patterns to unseparated natural samples of different *Salix* species and *T. officinale* (Fig. 3). The responding antennal lobe regions were spatially structured and corresponded to patterns consisting of individual glomeruli. If glomeruli were strongly activated, the response of neighbouring glomeruli were sometimes visually overlapping (cluster of glomerulus *a*, *h*, and *b*; Fig. 3). In these cases, the individuality of glomeruli was verified in response patterns to separated odorants in GC-imaging experiments (Fig. 4). Eight different glomeruli responded to the tested samples and had a comparable topology across females (Fig. 3C). Thereof, three to five glomeruli responded strongly to the different *Salix* species (Fig. 3A) and only one glomerulus showed a clear response to *T. officinale* (Fig. 3B). Glomeruli b, e and k responded across all tested *Salix* species but not to *T. officinale* or blank controls.

GC-imaging experiments revealed distinct response patterns to 10 odour components in *S. cinerea* (Fig. 4) and to 13 in all *Salix* species (Tab. 1). The chemical identity of each compound that elicited a response was determined based on the odorant-specific response pattern even though some compounds co-eluted. Common responses among all analysed *Salix* species were recorded to β-myrcene, (*E*)-β-ocimene, linalool, 4-oxoisophorone, veratrole, 1,4-dimethoxybenzene and (*Z*)-3-hexenyl 2-methylbutanoate (the latter probably emitted by green parts of the flowering stems). *S. fragilis* and *S. alba* evoked additional responses to limonene, pinocarvone and verbenone, and *S. cinerea* to benzyl nitrile, octanoic acid and (*E*,*E*)-α-farnesene. The responses to *T. officinale* were mainly to (*E*)-β-ocimene and other

monoterpenes, which all elicited responses in *Salix* species as well. We detected no calcium response to benzaldehyde, the major component in the GC runs.

Based on the GC-imaging results, *Salix*-specific responses of glomeruli *b*, *e* and *k* (Fig. 3) could be attributed to 1,4-dimethoxybenzene (glomerulus *b*), 4-oxoisophorone (glomerulus *k*) and veratrole (glomerulus *e*). Importantly, the responses to 4-oxoisophorone and veratrole were strong (mean ± standard error 4-oxoisophorone 1.29 ± 0.07 % $\Delta F/F$ magnitude in calcium, $N = 10$), although the compounds were emitted in much smaller amounts (1.27 ± 0.14 % of total scent amount of physiologically active compounds) compared to 1,4-dimethoxybenzene (main compound 74.36 ± 2.08 % of total scent, with a mean response of 1.10 ± 0.12 % $\Delta F/F$ in the calcium run, $N = 12$) and (*E*)-*β*-ocimene (17.89 ± 1.57 % of total scent, with 0.92 ± 0.11 % $\Delta F/F$ in the calcium run, $N = 5$). Glomerulus *k* also responded to verbenone in *S. fragilis* and *S. alba* x *babylonica*.

In the GC-EAD analyses, we recorded 13 responses to *S. cinerea* (without responses to contaminants) which corresponded to 20 compounds (Fig. 4). The antennal responses could not always be related to a single substance because some of the compounds co-eluted. In comparison to GC-imaging, we recorded additional responses to (*Z*)-3-hexenol (most probably emitted by green parts), limonene, α-pinene, (*E*)-4,8-dimethyl-1,3,7-nonatriene, 2-phenylethanol, allo-ocimene, lilac aldehyde A, methyl salicylate, lilac alcohol B + C, indole, and germacrene D, but not to β-myrcene. The other responses corresponded to the ones found in GC-imaging.

Sensitivities for key volatiles differ across bee species

We recorded dose-responses for 4-oxoisophorone and 2-phenylethanol in *A. vaga* and *A. mellifera*, in order to test their sensitivity to characteristic host and non-host compounds. We found that the lowest concentrations of 4-oxoisophorone and 2-phenylethanol that evoked a significantly higher response than the solvent control differed between the two bee species. Glomerulus *k* in *A. vaga* responded to 4-oxoisophorone already at the dilution 10^{-7} (Friedman`s test, critical value = 82.34, $P < 0.01$, $N$ =13, Fig. 5A,C) and glomerulus *c* to the concentrations $10^{-4}$, $10^{-3}$ and $10^{-2}$ (Friedman`s test, critical value = 71.47, $P < 0.001$, $N = 13$, Fig. 5A,D); however, the highest response was stronger in glomerulus *c* as compared to glomerulus *k* (2.45 ± 0.31 % $\Delta F/F$ as compared to 4.78 ± 0.52 % $\Delta F/F$). The magnitude of the response to 4-oxoisophorone in glomerulus *k* in the GC imaging runs (1.29 ± 0.07 % $\Delta F/F$) was comparable to the response of the 10^{-6} dilution (1.24 ± 0.14 % $\Delta F/F$, non-normalized data). 2-Phenylethanol evoked a significant response in glomerulus *m* at the three highest

concentrations of 10^{-4}, 10^{-3} and 10^{-2} only (Friedman`s test, critical value = 63.89, $P < 0.01$, $N$ =11, Fig. 5A) in *A. vaga*. In order to elicit responses, much higher odorant concentrations were needed in the honey bee: A glomerulus responding to 2-phenylethanol was only found at the dilution $10^{-4}$ and higher concentrations (Friedman`s test, critical value = 64.45, $P < 0.05$, N =11, Fig. 5B), and 4-oxoisophorone only at the two highest concentrations 10^{-3} and 10^{-2} (Friedman`s test, critical value = 47.31, $P < 0.05$, N =11, Fig. 5B) in *A. mellifera*.

Olfactory cues of hosts and 4-oxoisophorone are highly attractive

The combination of olfactory and visual floral cues of flowering *S. cinerea* stems was significantly more attractive than an empty control (Fig. 6). In contrast, the bees showed no preference when testing visual cues alone against an empty control or against the visual cues of the non-hosts *T. officinale* and *N. pseudonarcissus*. When testing olfactory floral cues, the bees significantly preferred their host over an empty control and the non-hosts *T. officinale* and *N. pseudonarcissus*. However, the combination of both cues of *S. cinerea* were significantly more attractive than the olfactory cues alone.

A synthetic mixture of the physiologically active compounds of *S. cinerea* was significantly more attractive than a solvent control, and had the same attractiveness as a solvent headspace sample collected from flowering *S. cinerea* stems (Fig. 6). Similar to the synthetic mixture of compounds, the bees preferred 4-oxoisophorone over a solvent control.

Discussion

4-Oxoisophorone is an important scent component in Salix

We analysed the physiological responses to host and non-host odorants and found that many of them elicited physiological responses, indicating that *A. vaga* has the appropriate complement of olfactory receptors to detect these substances. The bees responded to both minor and major compounds of the scent bouquet of *Salix* hosts in our physiological experiments. Among those, we found that 4-oxoisophorone elicited particularly strong responses in the antennal lobes, and was present in all analysed *Salix* specimen. Interestingly, despite of eliciting among the strongest responses of all chemical constituents, it was only a minor compound within the blend. It appears that the olfactory system of *A. vaga* bees is highly tuned to this compound at a very low concentration.

A. vaga *is particularly sensitive to 4-oxoisophorone*

Indeed, our dose-response measurements confirmed this assumption, and showed that, in contrast, generalist honeybees were less sensitive. A high sensitivity in *A. vaga* was previously also shown for another behaviourally active *Salix* component, 1,4-dimethoxybenzene (Burger et al., 2013). Both 4-oxoisophorone and 1,4-dimethoxybenzene are present in the odour of many willow species (Füssel et al., 2007). We therefore hypothesize that oligolectic *A. vaga* bees could use the common presence of these compounds in *Salix* spp. to specifically recognize their hosts and discriminate them from non-hosts. Indeed, in contrast to 1,4-dimethoxybenzene, 4-oxoisophorone was absent in the non-host plants used here, and according to Knudsen et al. (2006) it is not widespread among floral scents. However, there are recent studies that describe 4-oxoisophorone as floral scent component in a broader range of species (e.g. El-Sayed et al., 2018; Lukas et al., 2020; Lukas et al., 2019). Future studies should test how *A. vaga* bees behaviourally respond to 1,4-dimethoxybenzene and 4-oxoisophorone when they are offered simultaneously, and to which extent these two compounds may be synergistically involved in host recognition.

Complementary physiological data from imaging and antennograms

We detected responses to more compounds in the antennal GC-EAD measurements (12 responses/20 compounds in *S. cinerea*) than in the glomerular GC-imaging patterns (9 responses/10 compounds). One reason was that the samples for GC-EADs were collected for several hours from flowering stems and were thereby enriched, in contrast to few flowers enclosed in a headspace glass used for GC-imaging. We also analysed more samples in GC-EADs because more runs can be performed in the same time than for GC-imaging. Thus, at first sight, GC-EAD appears to be more sensitive. However, when compounds co-elute in GC-EADs, the antennal responses cannot be assigned to a specific compound, and responses to minor compounds can be hidden or overseen. Specifically, in previous GC-EAD measurements, 4-oxoisophorone co-eluted with other compounds, and it remained unclear to which compounds the antennae responded (Dötterl et al., 2005; Jürgens et al., 2014). A high sensitivity in *A. vaga* towards this compound was first revealed in this study using optical imaging. Using synthetic analogues, the assigned activity of a compound can be proven in both methods, but imaging provides, besides a quantitative signal (in form of a peak), a qualitative one consisting of the odorant-specific glomerular pattern. Conceptually, the information of glomerular patterns is analogous to a mass spectrum in GC-MS runs used to reveal the identity of a compound, compared to GC-FID runs that only give chromatogram

peaks. Since every glomerulus collects information from a uniform family of olfactory receptors, imaging data also reveal odour-response spectra of these receptors. This indicates, in our current data, that *A. vaga* has at least two separate olfactory receptors for 4-oxoisophorone (Fig. 5): one with very high sensitivity (low-concentration mode, glomerulus *k*), and one with high saturation levels (high-concentration mode, glomerulus *c*). However, imaging experiments do not monitor all glomeruli of the antennal lobe but only the subset in the superficial focal plane – while receptor responses can be separated, not all can be measured. In contrast, antennograms measure all receptors, but they cannot be separated. We conclude that both physiological methods have advantages and limits. When used together, they are complementary tools when searching for potential attractants within complex scent bouquets.

Preference of olfactory over visual cues

Our behavioural experiments revealed that the olfactory cues of *S. cinerea* flowering stems were highly attractive for *A. vaga* bees and that the bees preferred their host plants when those were offered against *T. officinale* or *N. pseudonarcissus* non-hosts. In contrast, decoupled visual cues were not attractive and not preferred over the ones of non-hosts, although the spectral analysis revealed that the bees should be able to distinguish host and non-hosts inflorescences. However, visual cues appear to be relevant for *A. vaga* when present in combination with olfactory cues. This confirms results in other bee species, which also demonstrated that the combination of olfactory and visual cues is more attractive both for specialist (Burger et al., 2010; Dötterl and Vereecken, 2010) and generalist bees (Dötterl et al., 2014; Rachersberger et al., 2019). In line with the physiological experiments, we demonstrated that 4-oxoisophorone was behaviourally active using synthetic analogues and therefor contribute to the attractiveness of *Salix* scent cues.

Conclusion

A. vaga bees rely mainly on olfactory cues while searching for *Salix* host flowers. Physiological experiments combined with bioassays allowed us to describe characteristic *Salix* compounds that enable them to effectively locate food sources. In particular, we could show that 4-oxoisophorone is a low-concentration-high-efficiency compound in the olfactory bouquet of natural *Salix* scents, behaviourally active for *A. vaga* and detected with high

sensitivity. This might represent an evolutionary adaptation in bees that strongly depend on willows to collect pollen and therefore can profit from sensory adaptations to effectively find host flowers.

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Competing interests

No competing interests declared.

Figures

Fig. 1. Study organisms. *Andrena vaga* female (left) and one of its host plants, *Salix cinerea* (right), used for physiological and behavioural experiments in the present work (photos: M. Kaminski).

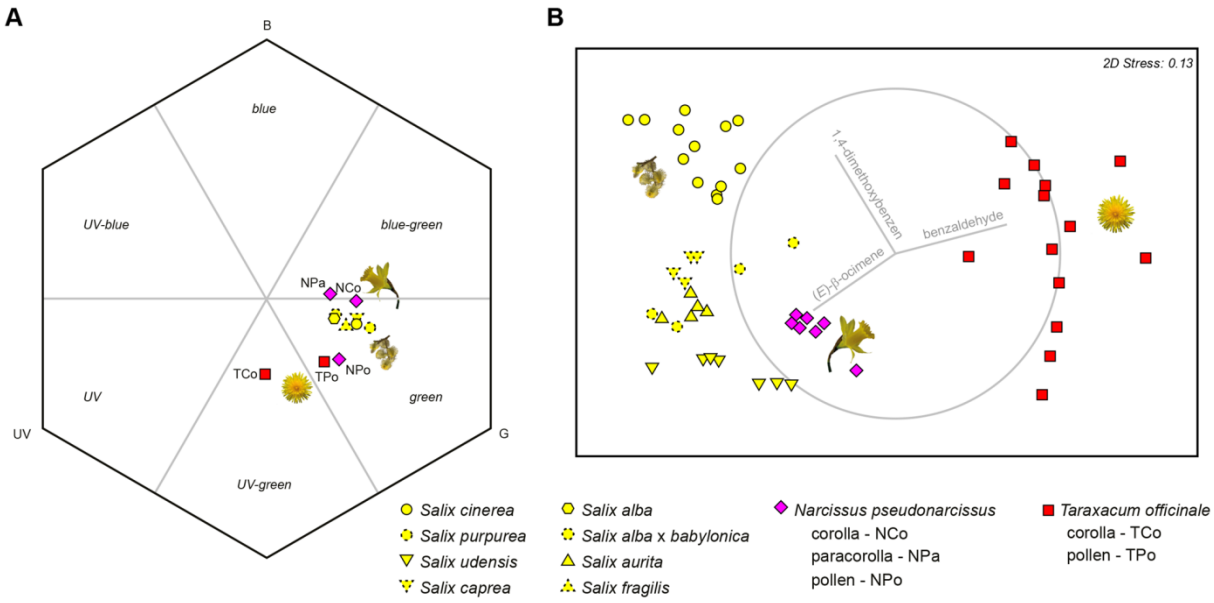

Fig. 2. Host and non-host floral colours and odour bouquets differ. Comparison of floral colours (A) and scents (B) between different *Salix* host species (yellow) and *T. officinale* (red) and *N. pseudonarcissus* non-hosts (purple). A) Spectral reflectance of inflorescences/flowers modelled in the colour hexagon. B) Multi-dimensional scaling of the bouquets of physiologically active compounds based on the Bray-Curtis Index (ANOSIM, R = 0.83, $P < 0.001$). Vectors represent the multiple correlation for compounds most responsible for the dissimilarity between samples (vector length and direction indicate the strength of the correlation of the compound, the circle indicates a perfect correlation with a value of 1).

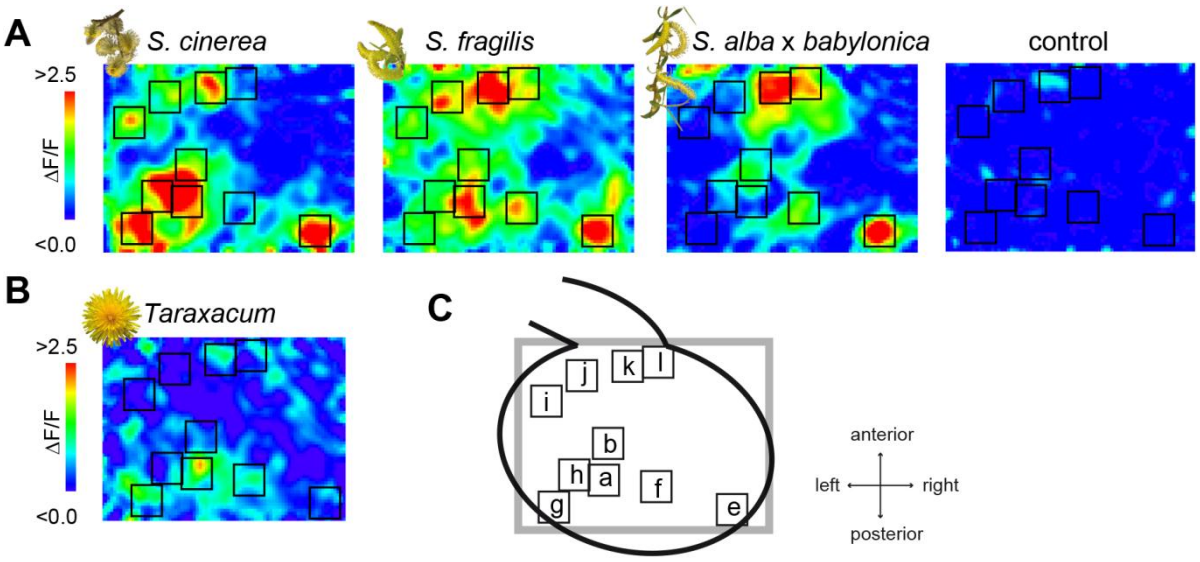

Fig. 3. Glomerular responses to natural samples of different host and a non-host species differ. Response patterns in the right antennal lobe of representative *A. vaga* females using calcium imaging. False-colour coded responses (A) of a specific individual to headspace samples of *S. cinerea*, *S. fragilis*, *S. alba* x *babylonica*, and a control (nitrogen), and (B) of another individual to a headspace sample of *T. officinale*. (C) Squares give the position and size of the regions of interest used in the analyses and the names of the activated glomeruli for the shown individuals.

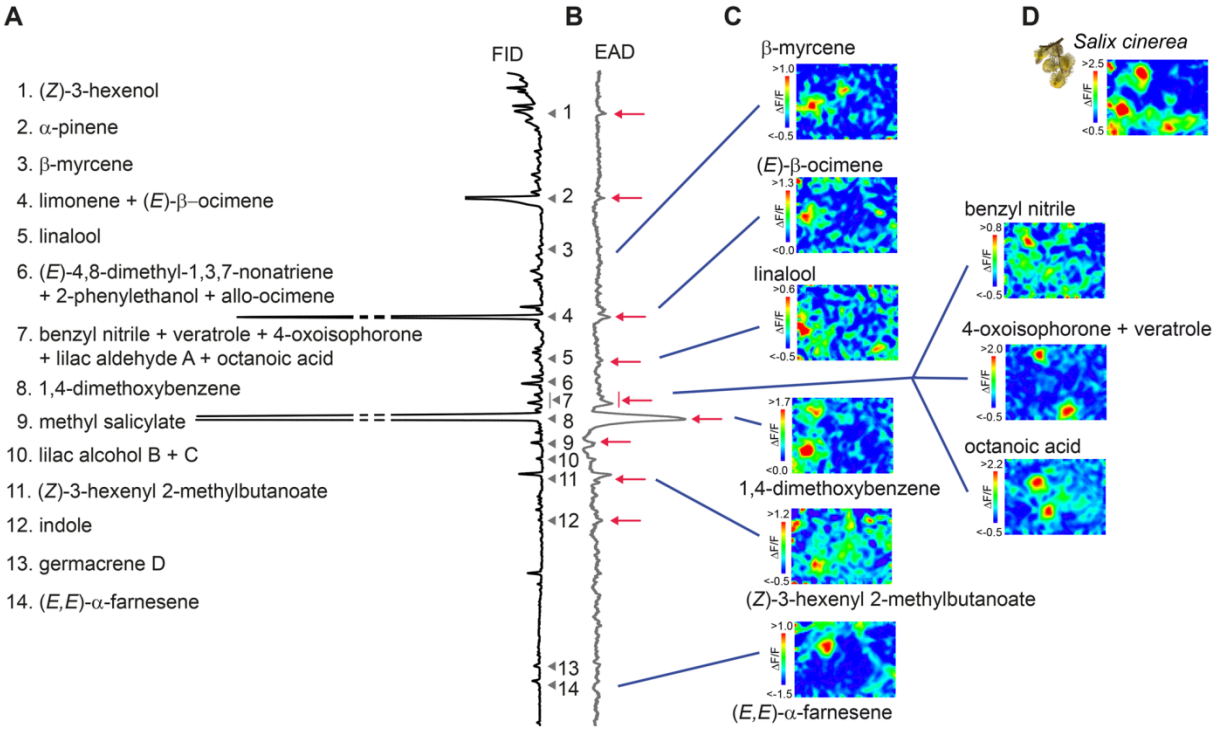

Fig. 4. **Responses to separated odorants in GC-EAD and GC-imaging**. Physiological responses of antennae and antennal lobes of representative *A. vaga* females to odorants of *S. cinerea*. (A) The identified compounds in *S. cinerea* are listed according to their retention time, and are labelled in the FID chromatogram. Co-eluting compounds have the same number. Headspace samples were separated by a gas chromatograph and separated compounds were transferred via a transfer line to (B) a cut antennae (GC-EAD) or to (C) an animal prepared for measuring responses in the antennal lobe of the brain (GC imaging). A solvent headspace sample was injected for GC-EAD runs (shown FID signal) and a direct headspace sample of cut *S. cinerea* catkins for imaging experiments (FID chromatogram not shown). GC-EAD active compounds are indicated by red arrows. Glomerular response patterns in the right antennal lobe are false-colour coded. (D) Imaging response pattern to the unseparated *S. cinerea* headspace, as recorded in the same individual as shown in C.

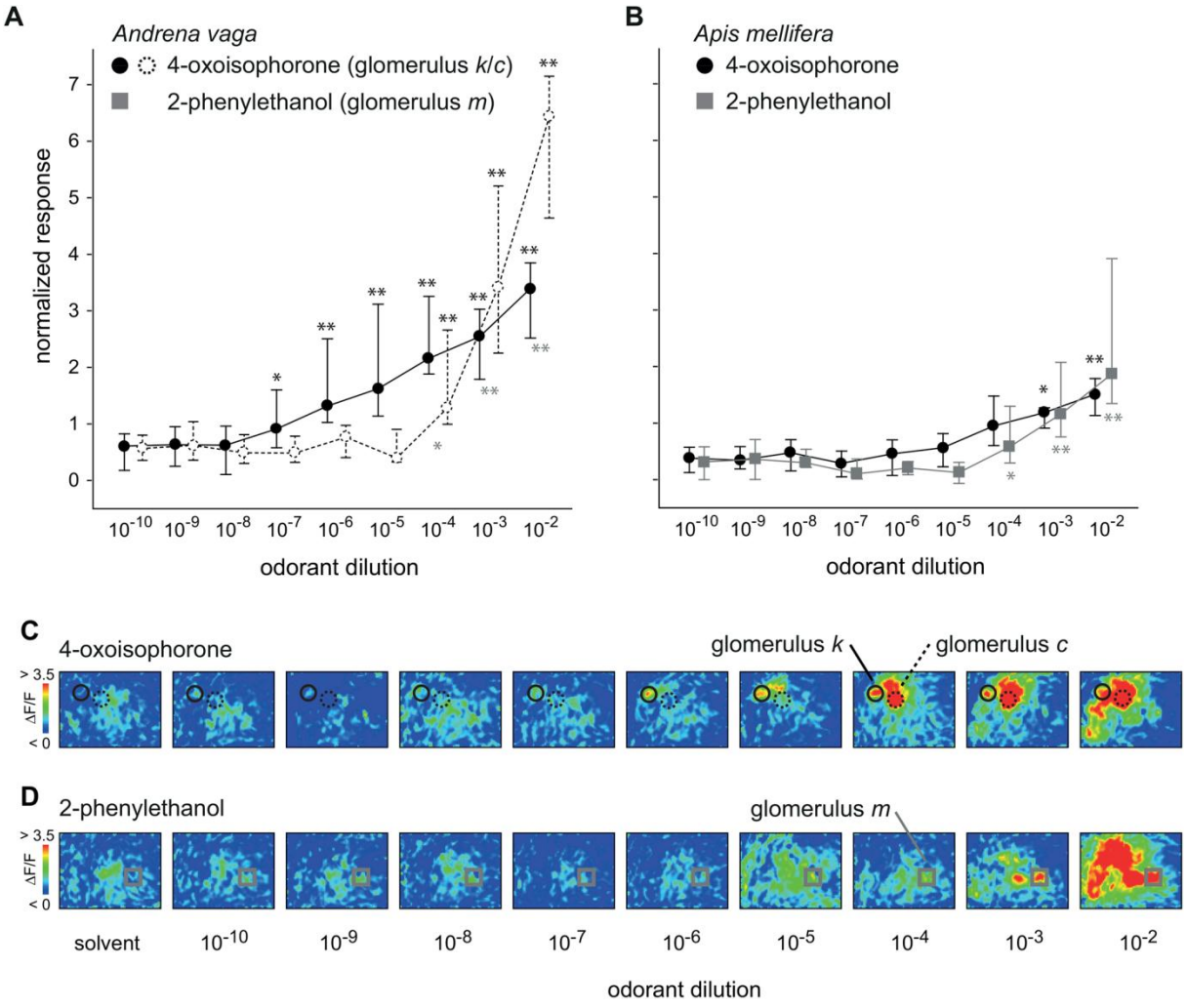

Fig. 5. 4-Oxoisophorone evoked strong responses in the antennal lobes of *Andrena vaga*, but not *Apis mellifera*. Dose-response relationships (median, whiskers 25-75% of normalized data) in (A) *A. vaga* (N=13 individuals) and (B) *A. mellifera* (N=11 individuals) bees responding to 4-oxoisophorone (dots, black lines) and 2-phenylethanol (square, grey lines). Asterisks indicate a significantly higher response compared to stimulations with a solvent control (Friedman`s test with post-hoc, *: $P < 0.05$, **: $P < 0.001$). The glomerular response pattern and the region of selected glomeruli are shown for the different dilutions of (C) 4-oxoisophorone in glomeruli *k* and *c* and (D) 2-phenylethanol in glomerulus *m* of the right antennal lobe of a representative *A. vaga* female coded in false-colour using calcium imaging (symbols of glomeruli correspond to symbols used in the dose-response curves).

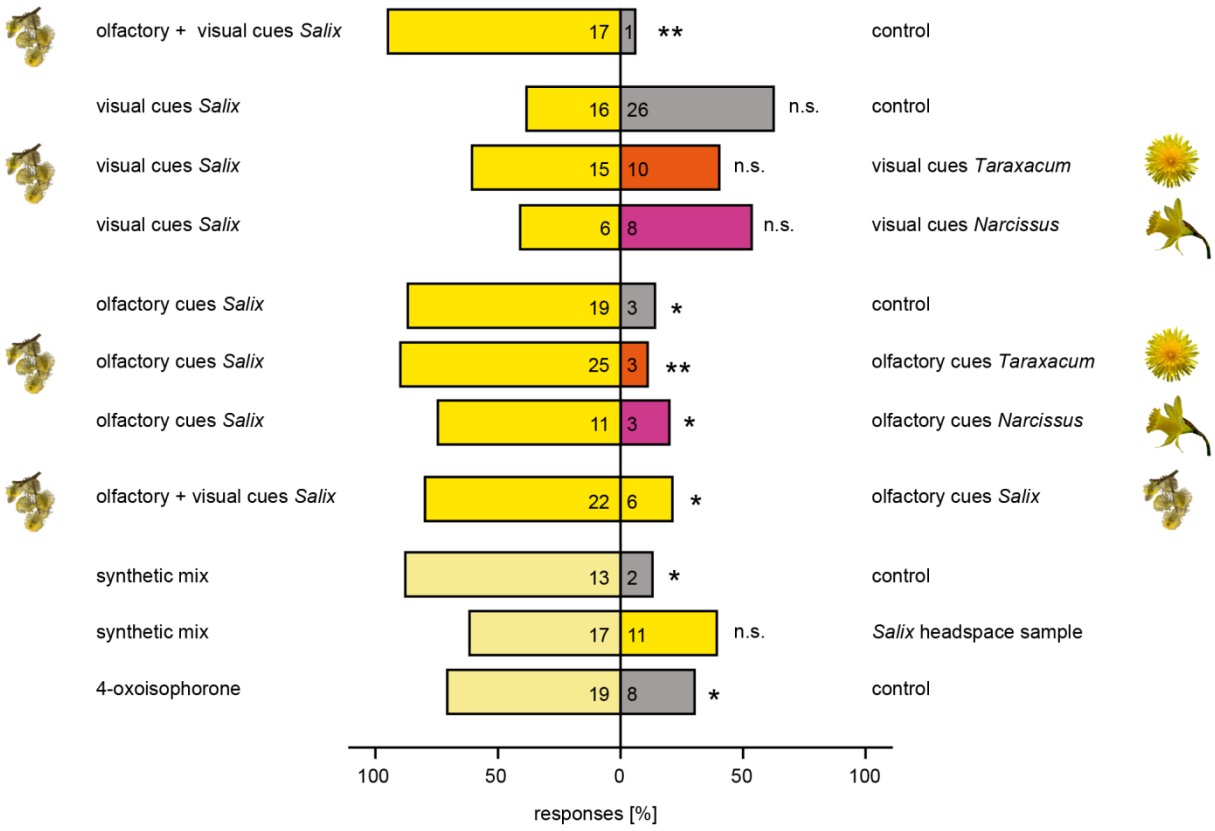

Fig. 6. Olfactory cues of hosts and 4-oxoisophorone are highly attractive. Behavioural responses (absolute number of approached/landed bees are given in the bars) of *A. vaga* bees to olfactory and visual floral cues of *S. cinerea* host (yellow), and the non-hosts *T. officinale* (red) and *N. pseudonarcissus* (purple), as well as to synthetic (light yellow) mixtures of physiologically active compounds, the single compound 4-oxoisophorone, and solvent *S. cinerea* headspace samples (binomial test: n.s.: $P > 0.05$, *: $P < 0.05$, **: $P < 0.001$). Empty cylinders were used for control experiments (grey) with natural floral cues and the solvent dichloromethane when tested against synthetic samples.

Tab. 1. Common *Salix* odorant components of GC-imaging. Physiologically active compounds of antennal lobes to direct headspace samples of different *Salix* species in GC-imaging experiments

Compound name	*S. cinerea*	*S. fragilis*	*S. alba* x *babylonica*
β-Myrcene	x	x	x
Limonene		x	x
(*E*)-β-Ocimene	x	x	x
Linalool	x	x	x
Benzyl nitrile	x		
Veratrole	x	x	x
4-Oxoisophorone	x	x	x
Octanoic acid	x		
Pinocarvone		x	x
1,4-Dimethoxybenzene	x	x	x
Verbenone		x	x
(*Z*)-3-Hexenyl 2-methylbutanoate	x	x	x
(*E,E*)-α-Farnesene	x		

Table S1. Composition of the *S. cinerea* synthetic mixture used in bioassays. Diluted compounds (stock solutions, in dichloromethane) were added to the mixture according to absolute and relative amounts of *S. cinerea* headspace samples. The mixture was further diluted 1:100 and 50 µl of the final solution was used in a bioassay.

Compound	Company	Purity	Stock solution (mg/ml)	Mixture (µl)
(*Z*)-3-Hexenol	Fluka	98%	10	25.4
α-Pinene	Fluka	99%	10	9.5
β-Myrcene	*	90%	10	51.7
D-Limonene	Sigma	97%	100	40.0
(*E*)-β-Ocimene	*	70%	100	433.7
Linalool	Sigma-Aldrich	97%	100	16.0
(*E*)-4,8-Dimethyl-1,3,7-nonatriene	*	85%	10	32.6
2-Phenylethanol	Merck	99%	10	18.5
allo-Ocimene	Sigma-Aldrich	80%	10	57.3
Veratrole	Sigma-Aldrich	99%	10	8.1
Benzylnitrile	Sigma-Aldrich	98%	10	26.7
Lilac aldehyde A	**	25%	10	30.0
4-Oxoisophorone	Sigma	98%	100	17.5
Octanoic acid	*	70%	10	30.0
1,4-Dimethoxybenzene	SAFC	99%	100	967.3
Methyl salicylate	Sigma-Aldrich	99%	100	51.2
Lilac alcohol B + C	**	70%	10	12.0
(*Z*)-3-Hexenyl 2-methylbutanoate	Sigma-Aldrich	97%	10	13.2
Indole	Aldrich	99%	10	46.4
Germacrene D	*	86%	10	18.5
(*E,E*)-α-Farnesene	SAFC	70%	10	43.6
sum				**1949.3**

available in the reference collections of the labs in Ulm (*) and Salzburg (**)

Table S2. Physiologically active compounds in hosts and non-hosts. Absolute (first line) and relative amounts (mean ± standard error, tr: trace amounts < 0.05 %) of physiologically active compounds in *Andrena vaga*. Compounds are listed by compound class and Kovats retention index (KRI). The mass-to-charge ratio (*m/z*) is given for unknown compounds. Compound amounts in bold indicate most abundant compounds. All compounds were verified through authentic standards.

	KRI	*S. cinerea* N = 13	*S. caprea* N = 4	*S. aurita* N = 5	*S. udensis* N = 7	*S. purpurea* N = 4	*T. officinale* N = 14	*N. pseudonarcissus* N = 7
Absolute amount (ng per floral unit* per hour)		62.89 ± 11.41	55.05 ± 16.95	34.01 ± 9.20	38.65 ± 4.57	4.45 ± 1.07	4.68 ± 1.51	207.80 ± 23.99
Aliphatic compounds								
(*Z*)-3-Hexenol	858	0.06 ± 0.03	tr	-	0.12 ± 0.05	1.09 ± 0.43	3.72 ± 2.46	-
(*Z*)-3-hexenyl 2-methylbutanoate	1234	tr	-	-	tr	0.07 ± 0.04	-	-
Terpenes								
α-Pinene	938	0.23 ± 0.02	0.07 ± 0.92	tr	0.38 ± 0.07	0.13 ± 0.05	3.85 ± 1.10	1.12 ± 0.21
β-Myrcene	992	0.21 ± 0.06	0.39 ± 0.62	0.44 ± 0.15	2.36 ± 0.90	4.71 ± 3.34	3.69 ± 0.28	17.11 ± 2.69
Limonene	1043	2.06 ± 0.59	6.48 ± 1.65	9.65 ± 1.84	15.89 ± 3.01	9.82 ± 2.14	0.48 ± 0.33	8.02 ± 1.17
(*E*)-β-Ocimene	1053	**17.89 ± 1.57**	**67.62 ± 17.73**	**83.76 ± 12.75**	**74.25 ± 5.01**	**70.35 ± 15.63**	2.45 ± 1.52	**33.03 ± 5.09**
Linalool	1102	1.24 ± 0.17	0.05 ± 0.09	0.08 ± 0.03	-	0.70 ± 0.32	0.49 ± 0.07	0.48 ± 0.10
(*E*)-4,8-Dimethyl-1,3,7-nonatriene	1119	0.20 ± 0.04	0.08 ± 0.99	0.64 ± 0.25	-	tr	5.05 ± 0.84	0.17 ± 0.09
allo-Ocimene	1133	0.39 ± 0.04	0.75 ± 0.24	1.49 ± 0.33	3.41 ± 1.01	1.43 ± 0.46	0.05 ± 0.06	4.56 ± 0.83
4-Oxoisophorone	1150	1.27 ± 0.14	tr	tr	tr	0.60 ± 0.17	-	-
Lilac aldehyde A	1157	0.07 ± 0.01	tr	tr	-	0.18 ± 0.09	-	-
Pinocarvone	1161	tr	0.20 ± 0.05	0.16 ± 0.02	0.24 ± 0.07	0.10 ± 0.04	-	-
α-Terpineol	1198	-	-	-	-	-	0.32 ± 0.08	-
Lilac alcohol B + C	1218	tr	-	-	-	-	-	-
Verbenone	1219	-	0.05 ± 0.13	0.06 ± 0.04	0.07 ± 0.03	tr	0.70 ± 0.06	1.23 ± 0.22
Citral	1235	-	tr	0.09 ± 0.05	1.00 ± 1.00	-	-	0.46 ± 0.06
Germacrene D	1498	tr	-	-	-	0.62 ± 0.31	0.29 ± 0.18	-
(*E,E*)-α-Farnesene	1503	0.18 ± 0.05	0.18 ± 0.06	0.21 ± 0.04	tr	0.42 ± 0.20	-	1.19 ± 0.29
Benzenoids and phenylpropanoids								
Styrene	898	2.62 ± 1.48	0.05 ± 0.05	0.06 ± 0.02	1.59 ± 0.93	6.30 ± 3.15	0.11 ± 0.08	0.11 ± 0.09

Benzaldehyde	965	0.08 ± 0.04	0.07 ± 12.75	0.05 ± 4.48	0.58 ± 0.15	2.10 ± 0.66	**69.11 ± 3.48**	tr
Benzyl alcohol	1038	-	-		0.05 ± 0.05	0.37 ± 0.17	0.12 ± 0.01	-
Phenylacetaldehyde	1049	-	-	-	-	0.45 ± 0.33	2.78 ± 1.42	-
Methylbenzoate	1103	-	-	-	-	0.37 ± 0.18	0.07 ± 0.00	tr
2-Phenylethanol	1119	0.07 ± 0.02	tr	tr	tr	3.97 ± 1.12	tr	-
Veratrole	1141	tr	0.05 ± 0.13	0.05 ± 0.04	0.79 ± 0.42	tr	0.73 ± 0.03	2.69 ± 0.30
1,4-Dimethoxybenzene	1169	**74.36 ± 2.08**	**23.77 ± 7.41**	3.14 ± 2.02	tr	1.72 ± 0.88	0.10 ± 0.00	**27.92 ± 2.00**
Methyl salicylate	1204	1.27 ± 0.78	0.18 ± 0.05	0.13 ± 0.03	tr	0.32 ± 0.10	0.11 ± 0.07	tr
Nitrogen containing compounds								
Benzyl nitrile	1145	1.10 ± 0.06	-	-	-	0.32 ± 0.17	5.26 ± 1.14	-
Indole	1302	0.11 ± 0.06	-	0.05 ± 0.02	-	tr	-	-
unknowns m/z								
m/z 39, 41, 97, 70, 69	866	tr	0.31 ± 0.38	0.20 ± 0.12	1.51 ± 0.98	-	2.17 ± 0.12	3.69 ± 1.02
m/z 71, 43, 99, 59, 85	974	-	tr	tr	0.24 ± 0.11	tr	2.87 ± 0.15	2.51 ± 0.36
m/z 98, 69, 41, 39, 53	987	tr	-	-	0.06 ± 0.03	-	0.09 ± 0.13	0.85 ± 0.12
m/z 122, 121, 77, 107, 91	1014	0.73 ± 0.87	tr	-	0.29 ± 0.27	tr	1.59 ± 0.36	9.38 ± 1.94
m/z 150, 135, 91, 107, 79	1100	tr	0.30 ± 0.38	0.20 ± 0.10	1.34 ± 0.62	tr	2.24 ± 0.30	4.01 ± 0.39
m/z 91, 92, 107, 135, 79	1124	0.13 ± 0.13	0.14 ± 1.25	0.25 ± 0.40	1.39 ± 0.63	2.01 ± 1.41	2.72 ± 0.83	7.56 ± 0.72
m/z 134, 133, 119, 105, 91	1171	0.13 ± 0.10	-	tr	1.74 ± 0.79	0.07 ± 0.44	0.57 ± 0.22	2.43 ± 0.86
m/z 97, 72, 68, 41, 43	1280	tr	0.23 ± 0.28	0.30 ± 0.13	1.85 ± 0.65	0.13 ± 0.39	1.64 ± 0.13	10.29 ± 1.67

* floral unit: catkin in *Salix*, flower in *Narcissus*, inflorescence in *Taraxacum*