Use of visual and olfactory cues of flowers of two brassicaceous species by insect pollinators

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Abstract. 1. Pollinating insects exploit visual and olfactory cues associated with flower traits indicative of flower location and reward quality. Pollination can induce changes in these flower-associated cues, thereby influencing the behaviour of flower visitors.

2. This study investigated the main cues exploited by the syrphid fly Episyrphus balteatus and the butterfly Pieris brassicae when visiting flowers of Brassica nigra and Raphanus sativus plants. Whether pollen is used as a cue and whether pollination-induced changes affect flower volatile emission and the behavioural responses of the two pollinator species were also studied.

3. Pollinator preference was investigated by offering visual and olfactory cues individually as well as simultaneously in two-choice bioassays. Plant treatments included emasculation, hand-pollination and untreated control plants. The composition of flower volatiles from pollinated and unpollinated control plants was analysed by gas chromatography-mass spectrometry.

4. Both pollinators exhibited a strong bias for visual cues over olfactory cues. Neither pollinator used pollen as a cue. However, E. balteatus discriminated between newly opened and long-open flowers at short distance only when pollen was available. Flower visits by pollinators were influenced by pollination-induced changes in B. nigra but not R. sativus flowers. Pieris brassicae only responded to pollination-induced changes when visual and olfactory cues were offered simultaneously. The blend of volatiles emitted by B. nigra, but not R. sativus inflorescences was affected by pollination.

5. Collectively, the findings of this study show that different pollinators exploit different visual and olfactory traits when searching for flowers of two brassicaceous plant species.

Key words. Odour cues, plant volatiles, pollinators, post-pollination changes, visual cues.

Introduction

In nature, animal–plant interactions are mediated by various cues (Kevan & Lane, 1985; von Helversen & von Helversen, 1999; Raguso, 2008; Balamurali et al., 2015; Lucas-Barbosa et al., 2016). Flowering plants have evolved shapes, colours, structures, textures, and fragrances in flowers that are exploited by different animals which act as vectors of pollen, leading to reproductive events such as cross-fertilisation and seed formation (Schoonhoven et al., 2005; Harder & Barrett, 2006; Junker & Parachnowitsch, 2015; Akter et al., 2017). Flower cues are used by pollinators to locate flowers and often indicate the quality of nectar or pollen offered by flowers as reward for the pollination service (Raguso & Willis, 2002; Galizia et al., 2005; Goyret et al., 2007; Schiestl, 2015; Lucas-Barbosa, 2016). Visual and olfactory cues specifically influence the behaviour of different pollinator species and mediate pollination of flowers by insects (Giurfa et al., 1994; Honda et al., 1998; Omura & Honda, 2005; Chittka & Raine, 2006; Ohashi et al., 2015).

Flower colour is one of the most important visual traits exploited by pollinating insects (Renoult et al., 2014; Larue et al., 2016; van der Kooi et al., 2018). The display of colourful...
flowers triggers behavioural responses in various flower visitors (Lunau, 1995; Renoul et al., 2014). In many angiosperm species, flower traits undergo changes that may inform pollinators about the presence and quality of the reward (Weiss, 1991; Oberrath & Bohning-Gaese, 1999; Zhang et al., 2017). Discrimination between floral colour transitions can be very accurate, enhancing efficiency of the visitor, and can increase plant reproductive success by guiding pollinators to unpollinated flowers that offer a better reward (Eisikowitch & Lazar, 1987; Weiss, 1991; Lucas-Barbosa et al., 2016).

In addition to visual cues, olfactory cues also play an important role in animal–plant interactions (Aartsma et al., 2017; Turlings & Erb, 2018). Flower odour may change upon pollination and may depend on flower age (Rodriguez-Saona et al., 2011; Ruiz-Ramón et al., 2014; Lucas-Barbosa et al., 2016). Flowers emitted lower amounts of volatiles after pollination compared with unpollinated flowers and these changes could influence pollinator behaviour with potential effects on plant fitness (Rodriguez-Saona et al., 2011; Lucas-Barbosa et al., 2016).

The olfactory cues released by pollen can provide specific information to pollinator species (Dobson et al., 1996). Flower visitors that feed on pollen may use chemical cues to assess the pollen reward of individual flowers. For example, the volatile blend emitted by pollen of flowers that offer pollen as the only food reward to pollinators can help the insect to discriminate between plant species and reward availability (Dobson et al., 1996). Insect pollinators can also use visual cues from pollen. Syrphid flies, for instance, exhibit behavioural responses to yellow colour from pollen (Lunau & Wacht, 1994; Lunau, 1995; Lunau et al., 2018).

Different insect pollinators may exploit different flower traits depending on the reward they are foraging for (Galizia et al., 2005; Goyret et al., 2007). Some insects may rely on a single cue or a combination of cues provided by flowers. Male Manduca sexta L. (Lepidoptera: Sphingidae) hawkmoths, for example, use olfactory and visual cues from flowers of Oenothera neomexicana Munz, but only the combination of traits elicited proboscis extension and feeding behaviour (Raguso & Willis, 2002). Bees are also more attracted to the combination of visual and olfactory cues than they are to the single cues of some plant species (Kunze & Gumbert, 2001; Milet-Pinheiro et al., 2012; Yan et al., 2016).

Behavioural responses of pollinators to floral characteristics provide an important force in the evolution of floral traits (Schiestl & Johnson, 2013; Van der Niet et al., 2014; Gervasi & Schiestl, 2017). Investigation of the cues exploited by pollinating insects is fundamental to understanding how inducible plant responses may influence pollinator behaviour, affecting plant fitness (Strauss, 1997; Goncalves-Souza et al., 2008; Bruno et al., 2014; Rusman et al., 2018) and flower evolution (Balamurali et al., 2015). Through experimental manipulation, the effects of different cues on flower–visitor interactions can be investigated (Hambäck, 2016; Larue et al., 2016). Here, we experimentally decoupled visual and olfactory cues from the inflorescence of two brassicaceous species. Several plant species in the Brassicaceae family depend on insects for pollination and rank among the most important agricultural crops. Brassica nigra L., commonly known as black mustard, and radish, Raphanus sativus L., are outcrossing species that depend on insect pollination for reproduction (Conner & Neumeier, 1995). Syrphid flies have been reported as efficient pollinators of brassicaceous plants (Conner & Neumeier, 1995; Honda et al., 1998), but it remains poorly understood which cues are used by E. balteatus to select flowers from which they mainly collect pollen (Primante & Dötterl, 2010). Pieris brassicae is a specialist herbivore on Brassicaceae, and uses visual, olfactory and gustatory plant cues when searching for an oviposition site (van Loon et al., 1992; Fatouros et al., 2012; Paschalidou et al., 2013). Pieris brassicae butterflies feed on the nectar of flowering B. nigra (Lucas-Barbosa et al., 2016); however, the main cues that they use when searching for flowers remain unknown. In this study, we focused on plant responses to pollination and sought to determine which cues are exploited by the syrphid fly E. balteatus L. (Diptera: Syrphidae) and the large cabbage white butterfly P. brassicae L. (Lepidoptera: Pieridae) when searching for flowers of B. nigra L. and R. sativus L. plants.

Materials and methods

Plants

Brassica nigra and R. sativus are self-incompatible and have hermaphroditic flowers that occur in racemose inflorescences (Conner & Neumeier, 1995). Flowers of B. nigra are yellow and flowers of R. sativus are white-pink. Seeds of B. nigra were obtained from the Centre of Genetic Resources (CGN, Wageningen, the Netherlands; accession CGN06619) and multiplied by exposing plants to open pollination in the surroundings of Wageningen. Seeds of R. sativus were obtained from De Bolster (Epe, the Netherlands; cultivar Gaundry 3). Plants from both species were transplanted and cultivated in pots (diameter 17 cm, volume 2 litres) filled with potting soil (Lentse potgrond, Lent, the Netherlands) and sand (1:1, v/v). Plants grew in a greenhouse compartment (23 ± 2 °C, 50–70% RH, LD 16:8 h) and were watered daily. Plants in flowering stage 3-1, based on the classification for B. napus (Harper & Berkenkamp, 1975), were used for the experiments.

Insects

Caterpillars of P. brassicae were reared in a greenhouse compartment (21 ± 2 °C, 60 ± 10% RH, LD 16:8 h) on Brussels sprout plants (Brassicaoleracea L. var. gemmifera cv. Cyrus). Adults were fed with a solution of 10% honey in tap water (organic honey, Melvita, Weide & Veldbloemen) provided on cotton wool. Adult insects, both males and females, were tested 2 or 3 days after mating and were starved for 15–18 h before the start of behavioural assays. Females were provided with Brussels sprout plants to allow for oviposition prior to the bioassays.

Pupae of the syrphid fly E. balteatus were obtained from Koppert Biological Systems (Berkel en Rodenrijs, the Netherlands). Flies were reared in a cage in a greenhouse (22 ± 2 °C, 60 ± 10% RH, LD 16:8 h) and had free access to organic raw sugar, apple pollen and tap water. The cage contained a Brussels sprout plant.
were performed in a flight chamber consisting of a gauze tent to compensate for unforeseen asymmetry in the setup. Bioassays the two plants were changed (right–left) after testing 10 insects 80 cm distance from the experimental plants. The positions of the two plants were changed (right–left) after testing 10 insects to compensate for unforeseen asymmetry in the setup. Bioassays were performed in a flight chamber consisting of a gauze tent (length × width × height, 293 × 200 × 230 cm) in a greenhouse compartment (25 ± 2 °C, 50–70% RH, LD 16:8 h) with glass walls and a glass ceiling. Each individual insect was used only once in the experiments.

Cues exploited by flower-visiting insects

To investigate whether flower-visiting insects prefer visual or olfactory cues of *B. nigra*, visual and olfactory cues were decoupled, thus facilitating the exposure of the insects to either visual or olfactory cues. Olfactory cues were blocked by enclosing the inflorescence in a glass container. Visual cues were removed by covering the inflorescence with a cylinder made of double-layer grey high-density polyethylene gauze material (diameter 28 cm).

To investigate the use of pollen as a cue, insects were given a choice between an emasculated plant (anthers removed) and an intact plant as control. Anthers were gently removed by manual emasculation using forceps just before the assay. In addition, to determine whether insects prefer either flowers that had been open for more than 1 day or newly opened flowers, plants were marked with a black cotton thread 24 h before the start of the bioassay. To mark the transition from open flowers to flower buds, a cotton thread was placed just above the flowers and below the buds; on the next day open flowers above the thread could be distinguished as newly opened flowers. One insect was released at a time and observed for 5 min. We considered that the insect made a choice when it approached one of the inflorescences up to a distance of 5 cm or less or landed on one of the two choices presented. When an insect did not respond within 5 min, it was scored as ‘non-responding’. Each experiment was conducted when 30 responses had been obtained for each sex of each insect species.

Effects of pollination on floral traits and pollinator behaviour

To investigate whether changes in flower traits in response to pollination influence the behaviour of the flower-visiting insects, *E. balteatus* and *P. brassicae* were offered a choice between a pollinated plant and an unpollinated plant. Flowers of both species were hand-pollinated 6 days after the first flower opened with pollen from a different individual plant (cross-pollination). Hand-pollination treatments were performed 24 h before the bioassays. In the first experiment, we offered a pollinated versus an unpollinated plant with both visual and olfactory cues available. For subsequent experiments, either visual or olfactory cues were presented to investigate the choice between a pollinated and an unpollinated plant. Five vertical yellow paper bands (width 2 cm) were placed on the gauze cylinder, to render the setup visually more attractive to the tested insects. Butterflies (*P. brassicae*) were released one by one, each individual butterfly was observed for 5 min. Syrphid flies (*E. balteatus*) were released in groups of three individuals, and observed for 5 min. An insect was considered to have made a first choice when it approached one of the inflorescences from a distance of maximally 5 cm, or a landing choice when the insect landed on the plant, glass or gauze covering a pollinated or unpollinated plant. When an insect did not approach a plant within 5 min, it was scored as non-responding. For these bioassays, female butterflies and female syrphid flies were tested. Each experiment was concluded when 50 responses had been obtained for each insect species and choice combination.

Collection of plant volatiles

To investigate whether pollination affects volatile emission of *B. nigra* and *R. sativus* inflorescences, volatiles of inflorescences including small leaves on them were collected both from pollinated plants and from unpollinated control plants. Pollinated plants had been hand-pollinated 24 h before the start of the headspace collection. Collections were performed in a greenhouse compartment (22 ± 2 °C, 60 ± 10% RH, LD 16:8 h). The inflorescence of the plant was enclosed in an oven bag (32 × 32 × 70 cm; Toppits® Brat-Schlauch, polyester, Toppits, Minden, Germany). A strip of bag material wrapped around the stem below the inflorescence was used to close the bag. Plants were watered with 50 ml of water just before the experiments. Synthetic air (nitrogen 80%, oxygen 20%, Linde Group, München, Germany) was flushed through the bag at a flow rate of 300 ml min⁻¹ by inserting a Teflon tube through an opening in the upper part of the bag, and air was sucked out with a pump (224-PCMTX8, Air Sampling Pump Deluxe; Dorset, Blandford Forum, U.K.) equipped with an inlet protection filter at a flow rate of 250 ml min⁻¹ through a second Teflon tube at the opening of each bag. Pressure measurements of in- and outgoing flow were made with a digital flowmeter (Model Defender 510-M; Bios International Corp., Butler, New Jersey). Volatiles were collected during 1.5 h on 90 mg Tenax TA (25/30 mesh; Alltech, Breda, the Netherlands) in a glass tube. Floral volatiles of *B. nigra* and *R. sativus* were separately collected from five pollinated and five unpollinated control plants.
Analysis of plant volatiles by gas chromatography-mass spectrometry

Quantification and identification of floral volatiles were performed according to the protocol outlined by Bruinsma et al. (2014). Samples were analysed with a gas chromatograph (GC) (6890 series; Agilent, Santa Clara, California) coupled to a mass spectrometer (5973 series; Agilent). Volatiles collected from the inflorescence headspace were desorbed from the Tenax in a thermodesorption trap unit (Gerstel, Mülheim, Germany) by heating from 25 to 250 °C (5 min hold) at a rate of 60 °C min⁻¹ in splitless mode. Released compounds were focused in a cold trap (internal diameter 1.80 mm) filled with glass beads (diameter 0.75–1.00 mm) at a temperature of −50 °C. By flash heating of the cold trap to 250 °C at 12 °C s⁻¹, volatiles were transferred to the analytical column of the GC (internal diameter 60 m × 0.25 mm, film thickness 0.25 μm; DB-5; J&W Scientific, Folsom, California). The oven temperature programme started at 50 °C (1 min hold) and rose at a rate of 20 °C min⁻¹ to 100 °C, subsequently increased at a rate of 4 °C min⁻¹ to 280 °C (1.5 min hold), and finally rose to 300 °C at a rate of 10 °C min⁻¹. Column effluent was ionised by electron impact ionisation at 70 eV. Mass scanning was carried out from m/z 40 to 300 with 5.36 scans s⁻¹. Compounds were identified by comparison of mass spectra with those of NIST, Wiley libraries and the Wageningen Mass Spectral Database of Natural Products. Identity was confirmed by comparison of the retention index described in the literature (Adams, 2007). The emission rates were quantified for compounds that were detected in a minimum of 50% of the samples from one of the treatments to focus on consistently emitted compounds, and peak area of individual compounds was divided by fresh plant biomass (g). Total ion counts were obtained to generate values of peak area of individual insects that responded within 5 min after release were tested for each sex and each insect species. *P < 0.05 [Colour figure can be viewed at wileyonlinelibrary.com].

Data analysis

To test for differences in responses (approaching and landing) between the paired treatments in each of the behavioural assays, linear mixed models based on restricted maximum likelihood (REML) were used with the insect responses and plant pair included in the statistical model. A Wilcoxon signed-rank test was used for comparison of insect responses to flowers of different development stages (newly opened versus long-open flowers) within the same plant. To test the effect of cues on the responses (landing or approaching), a χ² test of independence was used. Projection to latent structures discriminant analysis (PLS-DA) (SIMCA P+ 12.0; Umetrics AB, Umea, Sweden) was used to determine whether the samples subjected to pollination treatments and unpollinated plants could be separated based on the composition of the volatile blend. To determine significant differences for each of the compounds emitted by pollinated and unpollinated flowers, we used a Student’s t-test when the data were normally distributed or a Mann–Whitney U-test when this was not the case. Normal distribution of the data was assessed with the Shapiro–Wilk test. All analyses were performed with the statistical software spss for Windows (Chicago, Illinois), except PLS-DA which was performed with SIMCA P+ 12.0.

Results

Visual versus olfactory cues of Brassica nigra plants

In two-choice tests in which visual cues were offered versus olfactory cues, P. brassicae butterflies and E. balteatus flies visited B. nigra inflorescences significantly more frequently when only visual cues were available than when only olfactory cues were available (REML, all comparisons P = 0.001; Fig. 1). This was similar for females and males (Fig. 1).

Fig. 1. Number of individuals of female and male Pieris brassicae (a) and Episyrphus balteatus (b) responding to visual versus olfactory cues from flowering Brassica nigra plants. Differences in responses in the two-choice bioassays were assessed by generalised mixed models with plant pair included in the model. There was no overall effect of plant pair. In total, seven pairs of plants were tested for each sex and for each insect species; 30 individual insects that responded within 5 min after release were tested for each sex and each insect species. *P < 0.05 [Colour figure can be viewed at wileyonlinelibrary.com].

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Flower cues used by insect pollinators

0 5 10 15 20
0 5 10 15 20
0 5 10 15 20
0 5 10 15 20

(a) (b)

Fig. 2. Number of female and male individuals landing (yellow, green) on or approaching (grey) emasculated versus intact flowers of Brassica nigra plants. (a) Pieris brassicae; (b) Episyrphus balteatus. Generalised linear mixed models and Wilcoxon signed-rank tests were used to analyse the data. Asterisks underneath bars refer to difference between newly opened (< 24 h) and longer-open (> 24 h) flowers. There was no overall effect of plant pair. In total, seven pairs of plants were tested for each sex and each insect species; 30 individual insects that responded within 5 min after release were tested for each sex and each insect species. *P < 0.05; ns, not significant (P > 0.05) [Colour figure can be viewed at wileyonlinelibrary.com].

Pollen as a cue and discrimination between newly opened and longer-open flowers

Brassica nigra. Both pollinator species responded, independent of sex, as frequently to emasculated as to control inflorescences (P. brassicae, REML: females, P = 0.135; males, P = 0.421; E. balteatus, REML: females, P = 1.000; males, P = 0.769; Fig. 2), indicating that pollen cues do not influence their responses.

We also assessed the insects’ choice between newly opened and longer-open B. nigra flowers. Males of P. brassicae landed more frequently on newly opened flowers than on longer-open flowers, in both emasculated (Wilcoxon signed-rank test, P = 0.023; Fig. 2a) and control plants (P = 0.041; Fig. 2a). Pieris brassicae females visited newly opened and longer-open flowers similarly frequently, in both emasculated (P = 0.06; Fig. 2a) and control plants (P = 0.414; Fig. 2a). Female syrphid flies landed more frequently on newly opened flowers of control inflorescences (P = 0.020; Fig. 2b). Male E. balteatus flies did not discriminate between newly opened and longer-open flowers (P = 0.060; Fig. 2a).

Raphanus sativus. No discrimination between treated and control R. sativus plants was recorded for either P. brassicae butterflies or E. balteatus flies. Nor did both insect species discriminate between emasculated and non-emasculated flowers, or between newly opened and longer-open R. sativus flowers (Fig. S1).

Influence of post-pollination changes in flower traits on pollinator behaviour

Brassica nigra. Females of P. brassicae landed more frequently on flowers of unpollinated plants than on pollinated flowers when visual plus olfactory cues were present. When only visual cues were offered, a marginally significant (P = 0.054) preference was observed (Fig. 3b). When only olfactory cues were present, no significant preference was recorded (Fig. 3c). The proportion of approaching butterflies that landed on the plants was similar for pollinated and unpollinated plants in all treatments (visual plus olfactory cues, P = 0.606; visual cues, P = 0.670; olfactory cues, P = 0.711).

Episyrphus balteatus visited pollinated flowers as frequently as unpollinated flowers when olfactory and visual cues were present (REML, P = 0.314; Fig. 3d), when only visual cues were available (P = 0.314; Fig. 3e), and when only olfactory cues were available (P = 0.284; Fig. 3f). The proportion of approaching hoverflies that landed on the plants was similar for pollinated and unpollinated plants in all treatments (visual + olfactory cues, P = 0.606; visual cues, P = 0.670; olfactory cues, P = 0.711).

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cues,  \( P = 0.908 \); visual cues,  \( P = 0.417 \); olfactory cues,  \( P = 0.191 \).

**Raphanus sativus.** When flowering *R. sativus* plants were investigated, both insect pollinators landed as frequently on unpollinated flowers as on pollinated flowers (Fig. S2).

**Effect of pollination on volatile emission by the inflorescence**

*Brassica nigra.* Pollination of flowers of *B. nigra* did not significantly affect the emission rates of 13 volatile compounds (Table S1). Benzaldehyde was the main compound present in the headspace of both pollinated and unpollinated flowers, followed by (E)-\( \beta \)-ocimene and sylvestrene (Table S1).

A PLS-DA largely separated headspace samples from pollinated flowers and those from unpollinated control flowers of *B. nigra* (Fig. 4a,b). The first and second principal components explained 19% and 25% of the variance, respectively (Fig. 4a,b). The first principal component separated samples of pollinated from those of unpollinated flowers. The benzenoid benzaldehyde, the monoterpene (3E,5E)-2,6-dimethyl-1,3,5,7-octatetraene, and the monoterpenoids verbenone and 2-methyl-6-methylene-1,7-octadiene-3-one contributed most to the separation of the odour blends of pollinated and unpollinated flowers [Variable Importance in Projection (VIP) values > 1; Table S1].

**Raphanus sativus.** Volatile emission from flowers of *R. sativus* did not change in response to pollination. We recorded two aromatic compounds in the headspace of the inflorescences of *R. sativus*: benzaldehyde and the methyl 2-methylbenzoate. These compounds were present in similar amounts in the volatile profile of pollinated and unpollinated flowers of *R. sativus* (Table S2).

**Discussion**

Our data show that *P. brassicae* butterflies and *E. balteatus* flies, which exploit different rewards offered by flowers, utilise visual and olfactory cues in different ways. *Pieris brassicae* butterflies discriminated between unpollinated and pollinated flowers of *B. nigra*. Interestingly, discrimination was only recorded when
visual and olfactory cues of *B. nigra* were offered together. Such discrimination was not recorded for syrphid flies, which landed on pollinated and unpollinated plants with similar frequency. Emasculation of the *B. nigra* flowers had no effect on either of the two pollinator species. Male *P. brassicae* butterflies discriminated between newly opened and longer-open flowers both when pollen was available and when it was absent, whereas females did not. By contrast, *E. balteatus* females discriminated between newly opened and longer-open flowers only when the pollen was available in *B. nigra* flowers, suggesting that cues from the pollen itself played a role at short distances. Neither of the two pollinator species exhibited a preference between treated (pollination or emasculation) and control *R. sativus* plants, or between newly opened and longer-open *R. sativus* flowers.

Cues exploited by pollinators in *Brassica nigra*

We employed behavioural bioassays to assess the effects of visual and olfactory cues separately. We found that, from longer distances, visual cues were more important for *P. brassicae* and *E. balteatus* than were olfactory cues, when these cues were tested against each other (Fig. 1). The importance of visual cues for butterflies and syrphid flies is well known (Sutherland et al., 1999; Andersson & Dobson, 2003; Omura & Honda, 2005). *Pieris* and *Vanessa* butterflies use visual cues to detect nectar sources at long distances, and they discriminate the most suitable reward by olfactory cues at short distances (Honda et al., 1998; Omura & Honda, 2005). The butterfly *Pieris rapae* L. (Lepidoptera: Pieridae) uses mainly colour as its first cue and subsequently uses odour when choosing...
flowers of *Brassica rapa* L. (Oshima et al., 1999). Colour also appears to be more important than scent for *Vanessa indica* (Herbst) (Lepidoptera: Nymphalidae) butterflies which forage for nectar of plants in the Compositae family (Oshima & Honda, 2005). As in butterflies, colour also strongly influences foraging behaviour of *E. balteatus* (Sutherland et al., 1999). *Brassica nigra*'s yellow flowers are very attractive to *Pieris* butterflies and syrphid flies, and the literature suggests that these insects have a strong preference for yellow (Sutherland et al., 1999; Oshima & Honda, 2005). Proboscis extension in *Eristalis tenax* is elicited by yellow colour hues (Lunau et al., 2018).

### Pollen as a cue

*Brassica nigra.* *Pieris brassicae* and *E. balteatus* landed as frequently on emasculated flowers as they did on intact flowers of both *B. nigra*, suggesting that these pollinators do not use cues from pollen. However, *E. balteatus* females did discriminate between newly opened and longer-open flowers, but only for plants that had pollen available. We speculate that at short distances, this pollen feeder can use cues from pollen itself, and chose younger flowers. Other pollen-feeding insects are known to use pollen odour to evaluate reward suitability in individual flowers (Dobson et al., 1996; Dobson & Bergström, 2000; Russell et al., 2018).

Males of the nectar-feeding *P. brassicae* also preferentially landed on newly opened *B. nigra* flowers, as was the case for *E. balteatus* females. However, in contrast to *E. balteatus*, *P. brassicae* males did discriminate between newly opened and longer-open flowers in both emasculated and intact flowers, suggesting that male butterflies do not use pollen cues, but rather other flower cues, to distinguish between newly opened and longer-open flowers. In other studies, the display of the colourful corolla and changes occurring in it have been considered the most important cues exploited by pollinators (Casper & La Pine, 1984; Russell et al., 2018). Cues from the corolla may guide *P. brassicae* butterflies to the nectar offered by freshly open flowers. *Brassica nigra* plants responded to pollination by changing odours and flower pigments that, in turn, influenced the behaviour of *P. brassicae* butterflies, which visited more unpolli-nated than pollinated flowers (Lucas-Barbosa et al., 2016). The distinctive behaviours of both pollinators when they are exploiting flower cues may be explained by their feeding preferences, as *P. brassicae* feeds on nectar of brassicaceous plants, whereas *E. balteatus* collects pollen from such flowers (Goulson & Wright, 1998; Lucas-Barbosa et al., 2016); hence, each pollinator uses the cues that are related to the reward in which it is interested.

*Raphanus sativus.* In contrast to the situation for *B. nigra*, butterflies and syrphid flies did not differentiate between emasculated and intact *R. sativus* flowers. *Brassica nigra* has a shorter life span than *R. sativus*. Flower wilting started in the second week in *B. nigra* plants and only in the third week in *R. sativus* (K.Y. Barragán-Fonseca, pers. obs.). Age-related changes may occur more slowly in *R. sativus* flowers than in *B. nigra* flowers. Previous studies revealed that a prolonged floral longevity results in enhanced pollinator attraction to newly opened receptive flowers, because the persistence of flower traits of older flowers contributed to the overall flower display of a plant (Van Doorn, 1997; Teixido et al., 2019).

### Influence of post-pollination changes in traits of *Brassica nigra* and *Raphanus sativus* on pollinator behaviour

*Brassica nigra.* In our experiments, post-pollination changes in *B. nigra* influenced the preference of *P. brassicae*. We, indeed, expected butterflies to be sensitive to post-pollination changes because upon pollination flowers undergo changes in pigmentation, scent, and nectar production, and these changes are associated with ovule fertilisation (Theis & Raguso, 2005; O’Neill, 1997; Lucas-Barbosa et al., 2016). Pollinated flowers probably have less nectar than unpollinated ones, or no nectar at all, because pollination triggers nectar resorption (Luyt & Johnson, 2002). Additionally, in *B. nigra* the phenolic content of flowers was this decreased at 24 h after pollination (Lucas-Barbosa et al., 2016). Insect pollinators may exploit these changes to enhance foraging efficiency and, consequently, increase pollination efficiency (O’Neill, 1997; Weiss & Lamont, 1997; Rodriguez-Saona et al., 2011; Zhang et al., 2017). In this study, butterflies discriminated between pollinated and unpollinated flowers when the full suite of cues was offered, preferring unpollinated to pollinated flowers. However, they did not discriminate between pollinated and unpollinated flowers when visual or odour cues were offered separately. These findings suggest that *P. brassicae* needs both types of cues to recognize post-pollination changes. A combination of olfactory and visual cues synergistically improves foraging behaviour of other pollinators as well, resulting in higher frequency and duration of flower visits (Honda et al., 1998; Kunze & Gumbert, 2001; Raguso & Willis, 2002, 2005; Andersson & Dobson, 2003; Yan et al., 2016; Nordström et al., 2017).

Although pollination did not have an effect on emission rates of individual compounds, multivariate analysis (Fig. 4) revealed that the total volatile profile composition of pollinated flowers is different from that of unpollinated flowers. Pollination-induced variation in flower volatile emission has been documented previously (Tollsten & Bergstrom, 1989; Schiestl & Ayasse, 2001; Negre et al., 2003; Theis & Raguso, 2005; Schiestl, 2015; Lucas-Barbosa et al., 2016). Benzaldehyde comprises 35% of the total volatile blend of unpollinated *B. nigra* flowers (Table S1), and a decrease in its emission rate contributes significantly to the difference in scent produced by pollinated and unpollinated flowers. A reduction in the concentration of benzaldehyde upon pollination has been observed in other flowering plants. For instance, *Petunia* flowers showed a significant decrease in benzaldehyde emission at 36 h after pollination (Negre et al., 2003). *Brassica nigra* plants responded to pollination by drastically down-regulating the levels of some phenolic compounds (Lucas-Barbosa et al., 2016). Previous studies have suggested that the synergistic interaction observed between colours and odours of flowers could be due to a shared biosynthetic pathway for precursors of these floral traits (Rusman et al., 2019). Benzaldehyde is produced downstream of the pathway

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through which flavonoids are biosynthesised (Dormont et al., 2014). Benzenoids constitute a prominent class of compounds in floral scents (Knudsen & Tollsten, 1993; Andersson et al., 2002; Delle-Vedove et al., 2017). Flavonoids are important pigments responsible for the colour of most flowers in nature (Lucas-Barbosa et al., 2016). It has been proposed that when, for instance, flavonoid production is altered, this could influence the production of volatile benzenoids (Dormont et al., 2014).

A lower amount of benzaldehyde in pollinated flowers might result from a decrease in phenolic compounds, as reported by Lucas-Barbosa et al. (2016).

If pollination does not significantly affect the availability or quality of pollen, one may expect that changes in flower traits upon pollination do not affect foraging behaviour of pollen-feeders such as syrphid flies.

**Raphanus sativus.** A second main finding of the present study is that the two pollinator species did not discriminate between unpollinated and pollinated flowering *R. sativus* plants. We also did not record a difference in volatile emission by pollinated and unpollinated *R. sativus* flowers (Table S2). This could be due to the fact that ovules had yet not been fertilised within 24 h after pollination. It has been proposed that fertilisation is a prerequisite for the reduction of volatile emission after pollination (Negre et al., 2003). In previous studies with *R. sativus*, it has been shown that although pollen tubes reached the ovary within 24 h, at this time point only half of the ovules had been fertilised (Marshall & Diggle, 2001). Thus, we expect that more time is needed for differences in volatile emission between pollinated and unpollinated flowers of *R. sativus* to be evident. Responses of *R. sativus* flowers to pollination could proceed more slowly in *R. sativus*. Indeed, we observed that in *B. nigra* plants, many new flowers open daily, whereas in *R. sativus*, much fewer flowers are produced on a daily basis. Not changing flower traits after pollination has been suggested as a strategy to increase the floral display size of a plant to improve attraction of pollinators (Van Doorn, 1997; Teixido et al., 2019). Hence, *R. sativus* may not respond to pollination to remain attractive to pollinating insects that collect and spread pollen, increasing the reproductive success of radish plants.

Volatile compounds emitted differed between the two plant species studied. Previous studies of brassicaceous species showed that *R. sativus* emits different compounds from those observed in other brassicaceous species, including *B. nigra* (Kobayashi et al., 2012). Closely related plant species can produce different floral scents to attract different types of pollinators (Negre et al., 2003). Benzaldehyde was the only compound present in the volatile blend of both plant species. This compound and methyl 2-methylbenzoate were the only two compounds present in more than 50% of the samples of each treatment in *R. sativus*, and both are benzenoids. This class of chemical compounds is known to play a role in attraction of pollinators, including *Pieris* butterflies and syrphid flies (Omura et al., 1999; Shonouda, 2008). Kobayashi et al. (2012) reported that the volatile compounds produced by *R. sativus* flowers are almost exclusively benzenoid compounds. In *B. nigra*, the flower volatile blend is mostly composed of monoterpenoids (59%), followed by benzenoid compounds (39%) (Kobayashi et al., 2012). This is consistent with our data. Kobayashi et al. (2012) recorded more compounds in the headspace of *R. sativus* and *B. nigra* flowers. This may be a result of the different methods used. For instance, we sampled the headspace of intact inflorescences, whereas Kobayashi et al. (2012) detached the inflorescences to collect volatiles. Moreover, they collected older flowers (2–6 weeks after the start of flowering).

**Conclusion**

Our data show that *P. brassicae* and *E. balteatus* exploit flower-related cues differentially. Although both pollinator species rely on visual cues to orient to flowers from longer distances, at short distances *P. brassicae* uses cues from the flower corolla to detect changes in the flowers, and this is probably associated with nectar availability. *Episyrphus balteatus* uses cues related to pollen to select the younger, newly opened flowers, but can detect these cues only at short distances. That butterflies only perceive post-pollination changes when visual and olfactory cues are offered simultaneously confirms the importance of the synergistic effect of these cues (Raguso & Willis, 2002; Nordström et al., 2017; Russell et al., 2018). Furthermore, these results, together with the findings of Lucas-Barbosa et al. (2016) regarding changes in the flower flavonoid content after pollination, suggest that changes in colours might be correlated with changes in odours, as flavonoids and benzenoids share the same biochemical pathway (Dormont et al., 2014). Further studies on *R. sativus*, regarding post-pollination changes occurring later on, are necessary to further understand the cues used by pollinators of this important crop species.

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**Author contributions**

KB-F and DL-B planned and designed the study; KB-F collected the data, KB-F and DL-B analysed the data, and KB-F, DL-B, JvL and MD interpreted the data; and KB-F, DL-B, JvL and MD wrote the manuscript.

**Supporting Information**

Additional supporting information may be found online in the Supporting Information section at the end of the article.

**Fig. S1.** Number of individuals of female and male *Pieris brassicae* and *Episyrphus balteatus* landing on or approaching emasculated and intact flowers of *Raphanus sativus* plants.

**Fig. S2.** Number of individuals of female *Pieris brassicae* and *Episyrphus balteatus* responding to cues from pollinated and unpollinated flowers of *Raphanus sativus* plants.

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Table S1. Volatile compounds identified by GC-MS from flowers of pollinated *Brassica nigra* plants and unpollinated control plants.

Table S2. Volatile compounds identified by GC-MS from flowers of pollinated *Raphanus sativus* plants and unpollinated control plants.

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