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ORIGINAL ARTICLE

Mechanisms in mutualisms: a chemically mediated thrips pollination strategy in common elder

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Abstract

Main conclusion **This study provides first evidence of a thrips species pollinating *Sambucus nigra* and describes how interactions are driven by plant biochemical signalling and moderated by temporal changes in floral chemistry.**

Abstract The concept of flower-feeding thrips as pollinating insects in temperate regions is rarely considered as they are more frequently regarded to be destructive florivores feeding on pollen and surrounding plant tissue. Combining laboratory and field-based studies we examined interactions between *Sambucus nigra* (elderflower) and *Thrips major* within their native range to ascertain the role of thrips in the pollination of this species and to determine if floral chemicals mediated flower visits. If thrips provide a pollination service to *S. nigra*, then this will likely manifest in traits that attract the pollinating taxa at temporally critical points in floral development. *T. major* were highly abundant in inflorescences of *S. nigra,* entering flowers when stigmas were pollen-receptive and anthers were immature. When thrips were excluded from the inflorescences, fruit-set failed. Linalool was the major component of the inflorescence headspace with peak abundance coinciding with the highest number of adult thrips visiting flowers. Thrips were absent in buds and their numbers declined again in senescing flowers inversely correlating with the concentration of cyanogenic glycosides recorded in the floral tissue. Our data show that *S. nigra* floral chemistry mediates the behaviour of pollen-feeding thrips by attracting adults in high numbers to the flowers at pre-anthesis stage, while producing deterrent compounds prior to fruit development. Taking an integrative approach to studying thrips behaviour and floral biology we provide a new insight into the previously ambiguously defined pollination strategies of *S. nigra* and provide evidence suggesting that the relationship between *T. major* and *S. nigra* is mutualistic.

Keywords Cyanogenic glycosides · Elderflower · Floral chemistry · Floral traits · Non-bee pollinator · Prunasin · *Sambucus nigra* · Sambunigrin · Thysanoptera · Volatile organic compounds

Abbreviation

CNglcs Cyanogenic glycosides

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Introduction

Mutualisms between insects and plants drive functional-trait diversification in plants (Boucher et al. 1982; Bronstein et al. 2006). Floral traits mapped against pollinator data and pollination syndromes have informed the debate (Johnson and Steiner 2000; Ollerton et al. 2007; Mitchell et al. 2009), although generalist systems where plants are pollinated by many insect species, or by wind (Waser et al. 1996; Wragg and Johnson 2011; Yamasaki and Sakai 2013), are harder to define. Furthermore, pollinator efficiency (Gross 2005; Waser and Ollerton 2006), size and cryptic behaviour may obscure associations between floral traits and specific insect visitors capable of increasing pollination success (Vogel 1978).

Flowers provide many thrips (Thysanoptera) with a food source and a site for reproduction (Teulon and Penman 1990) and in return thrips can contribute to the pollination of these species (e.g., Velayudhan and Annadurai 1987; Ashton et al. 1988; Mound and Terry 2001; Moog et al. 2002; Infante et al. 2017). The capacity of thrips to transfer pollen within-species (Darwin 1876; Ananthakrishnan 1984; Proctor et al. 1996) and their flight agility (Kirk 1997) support growing evidence that they are brood-site pollinators (Sakai 2002), effective in transferring pollen of species that possess adaptations associated with wind pollination, such as many flowers, abundant powdery pollen and large stigmas (Tal 2009).

Most thrips pollination systems recorded are tropical and sub-tropical, with only a few studies in temperate species (Hagerup and Hagerup 1953; Tal 2009; Fukuhara and Tokumaru 2014). While the pollination ecology of *Sambucus* spp. (Adoxaceae) remains poorly understood (Grime et al. 1988; Bolli 1994; Charlebois et al. 2010) the floral morphology of European Elder, *Sambucus nigra* L., corresponds with a thrips pollination syndrome (Kirk 1988; Endress 1994; Williams and Adam 1994). Several authors have observed *Thrips* spp. present in flowers of *S. nigra* (Morison 1968; Mound et al. 1976; Raspudić et al. 2009), most notably Ward (1973) recorded *S. nigra* as a breeding host of the polyphagous species *Thrips major* Uzel (Thripidae) during a survey of native thrips species in flowers of a chalk grassland in southern England.

Here we examine whether thrips pollinate *S. nigra* and if floral chemistry mediates thrips-elder interactions. Our objectives were to (i) examine if thrips in flowers contribute to fruit-set, (ii) determine if floral volatiles fluctuate over 24 h and during flower development and if emission of key scent constituents correlates with thrips numbers foraging in flowers, (iii) identify and map the temporal distribution of cyanogenic glycosides (CNglcs) in floral tissue as flowers develop in relation to numbers and life stages of thrips present in flowers. Further laboratory assays were performed to test the volatile complexes and isolated CNglcs on the behaviour of thrips, thus examining the hypothesis that floral chemical traits of *S. nigra* are attracting and influencing the duration of visitation of native pollen-feeding thrips and potentially benefiting from a pollination service provided by these insects.

Materials and methods

Flower morphology of study species

The small, hermaphrodite flowers of *Sambucus nigra* L. are white, highly scented and arranged in inflorescences of up to 1000 pedicellate flowers (Bolli 1994). Flowers of this species are protogynous (Knuth 1906) and lack floral nectaries, although extrafloral nectaries are present at the base of leaves and leaflets (Dammer 1890; Atkinson and Atkinson 2002). Development stages of the flowers are shown in Fig. 1a–d.

Thrips sampling

Sampling of *S. nigra* inflorescences and thrips was undertaken in May and June (2016), from bud-break to fruit development, from ten plants 200–400 m apart, bordering open pasture on a conventional lowland dairy farm (Henfield, West Sussex, UK). Sampling occurred between 08:00 and 10:00, except where diurnal foraging activity was assessed, when collections were repeated at 06:00, 12:00, and 18:00. For each collection one inflorescence per plant was cut and submerged into 100 mL of 80% ethanol and stored (4 °C). After 24 h plant material was discarded and the immature and adult thrips counted under a Leica M165 FC light microscope (Leica Microsystems Ltd., Milton Keynes, UK). From these, 36 adults (16 females, 20 males) were removed from a sub-set of 221 examined closely to exclude species variability, slide mounted, examined under high-power magnification (x100–x400) and identified to species by reference to published keys (e.g., Mound et al. 2018). Similarly, 20 second-instar larvae were slide-mounted (sub-sample of 477) and characterised (Vierbergen et al. 2010).

Insect exclusion

Pollinator exclusion bags were constructed from two sheets (20 cm × 20 cm) of nylon mesh machine-stitched together with a central aperture enabling the bag to be placed over a developing inflorescence in bud (< 1 cm diam.) and secured by hand-stitching closely around stem. Fifteen inflorescences were selected on each of ten trees. To determine the contributions of thrips to fruit-set, three levels of vector exclusion were used: (1) open to all insect- and wind-transmitted (cross and self) pollen; (2) mesh aperture 1 mm diam. to prevent insects larger than *T. major* (1 mm) from access and (3) mesh aperture 0.125 mm diam. to exclude thrips but large enough to allow pollen through (average diam. 13–18 µm, Bolli 1994) ($n = 5$ per treatment). After 6 weeks the number of developing fruits was recorded. Pollination resulting from pollen transfer between flowers within inflorescence due to a combination of wind (cross) and mechanical disturbance (self) was determined by comparing (1) and (3), where open inflorescences (1) facilitated 100% pollination. Thrips pollination was determined comparing (2) with (3) − (1). *Forficula auricularia* L. (Dermaptera: Forficulidae) destroyed several inflorescences and these were not included in the final analyses.

Fig. 1 Five key flower development stages (DS) in *S. nigra*, observed during the flowering period in 2016. **a** Unopened buds on inflorescence (DS0). **b** Flowers open on inflorescence, pre-anthesis stage with visible lobed stigma (Donoghue et al. 2003): (DS1 ≤ 50% flowers of inflorescence open, DS2 ≥ 90%). **c** Flowers at anthesis with divergent stamens (DS3). Adult female *T. major* feeding on pollen. **d** Flowers post-anthesis stage (DS4). Adult male *T. major* in flower

Chemical analysis of *S. nigra* flowers and insect bioassays

Volatile organic compound (VOC) emissions during floral development

VOCs were collected in situ from inflorescences to identify main constituents of floral emissions. For the headspace sampling we selected inflorescences with unopened buds (Fig. 1; DS0), up to 50% flowers open (DS1), > 90% open (DS2 and DS3) and senescing flowers (DS4) and used leaf material as the background control. A clear oven bag (280 × 300 mm, Sainsbury's Ltd., London, UK) was placed over floral tissue and tied securely with wire fasteners around the stem. Air flow (100 cm^3 min^{-1}) was introduced via air-in, through a filter of activated charcoal (20 × 2 cm; 10–18 mesh, Fisher Chemicals), and air-out silicon tubing, inflating the bag around the inflorescence (air pump system, Barry Pye, Harpenden, UK). A glass pipette (150 mm) packed with Porapak-Q resin (200 mg, 50/80 mesh; Waters Corporation) held between plugs of silanized glass wool was inserted into the air-out tubing, ensuring that air leaving the sampling bag was forced out through the pipette and resin first before entering the air-out tubing. Each collection occurred over 24 h (10–10 am) after which the pipettes were removed, sealed in foil and returned to laboratory for analysis. During a 24 h sampling period flower development progressed to the next development stage so for analysis DS0 and DS1 data and DS2 and DS3 data were combined to compare VOC emissions from inflorescences predominantly in bud against inflorescences containing young flower stages, inflorescences with senescing flowers (DS4) and vegetative material only.

Circadian abundance of VOCs

VOCs from inflorescences (DS2) were analysed over four time periods, each for a duration of 6 h (12:00–18:00, 18:00–00:00, 00:00–06:00, 06:00–12:00).

Resin-captured VOCs were eluted with 1000 µL dichloromethane (99.5% chromatographically pure). Samples were analysed using a Varian 3800 gas chromatograph (GC) equipped with DB-Wax column (30 m long, 0.25 mm inner diameter, 0.25 mm film thickness), and linked to a Varian Saturn 2200 mass spectrometer (MS). Helium was used as a carrier gas at a flow rate of 1 mL min^{-1}. Column temperature was programmed to rise from 40 °C (5 min hold) to 250 °C (20 min hold) at 3 °C min^{-1}. The mass spectra were recorded at 70 eV (in EI mode) with speed of one scan s^{-1}, from 40 to 400 *m/z*. Compounds were identified by comparing mass spectra and relative retention times with standard compounds (Sigma-Aldrich) and with the NIST 05 mass spectral library. Proportional abundance of component VOCs in total scent emissions were compared (total ion mode) using peak area measurements to identify any

variation in representation in floral development stages or circadian rhythm emissions.

Olfactory tests with floral VOCs

Adult female *T. major* were collected from wild populations on *S. nigra* inflorescences. Individuals were deprived of food prior to experiments for 1 h and used in a single trial only.

Y-tube olfactometer experiments were conducted between 9:00 and 16:00 at 25 °C and ambient humidity. A pump (FB65540, Fisher Scientific) drew air through a charcoal filter to remove volatile contaminants, then air was split and passed down silicone tubing to two gas-wash bottles, one containing the test stimuli (VOCs eluents from Porapak-Q filters suspended in dichloromethane, 50% dilution, see Suppl. Fig. S1) and the other the solvent control (100% dichloromethane) and then into each of the two arms of the glass Y-tube olfactometer at a rate of 100 cm^3 min^{-1}. Odour stimuli were replaced and stimuli and control bottle positions switched after every fifth trial. The Y-tube apparatus was regularly flushed through with industrial methylated spirits (IMS) and allowed to air-dry to remove odour cues from previous thrips.

Individual insects were introduced into the approach arm of the olfactometer. A decision was recorded when an insect entered one or the other arm of the olfactometer and the decision time was recorded. Decisions taken after 3 min were excluded from analysis.

Florivore defence

CNglcs and floral development

Buds, flowers and fruit were collected from ten *S. nigra* individuals and stored at − 20 °C. Extracts were made by boiling 100 mg of finely ground freeze-dried plant material in 80% methanol (1 mL 100 mg^{-1}) for 5 min, followed by rapid cooling on ice. Extracts were centrifuged (11,000*g*; 5 min) and the supernatant analysed directly by LC–MS using an LTQ Orbitrap XL system (Thermo Scientific). Chromatography was performed with a 150 mm × 3 mm (i.d.), 3 µm, Luna C18(2) column (Phenomenex) using a 400 µL min^{-1} mobile-phase gradient mixed from H_2O/MeOH/CH_3CN + 1% HCOOH of 90:0:10–50:40:10 (by vol.) in 20 min. Concentrated aqueous ammonia was infused post column at 0.1 µL min^{-1} to augment ionisation. Quantification was performed on the ammoniated molecule at *m/z* 313.1394 (± 5 ppm) extracted from a positive ion MS1 scan of *m/z* 125–600 at 30 k resolution; low-resolution ion trap MS2 data on *m/z* 313 were acquired simultaneously for confirmation. Standard curves were obtained from prunasin (Sigma-Aldrich) and sambunigrin (Toronto Research Chemicals Inc.) which eluted at 10.1 min and 10.6 min, respectively, under the conditions used.

Effect of CNglcs on feeding preference

Choice tests were set up to assess if extracts of bud (DS0), flower (DS3) and green fruit (DS5) tissue of *S. nigra* influenced thrips behaviour compared to leaf tissue. Fresh tissue was ground with 70% ethanol (20 mg mL^{-1}), filtered and freeze-dried, prior to applying as treatments in bioassays at 100 mg mL^{-1}. Pure prunasin and sambunigrin were diluted in distilled water to 0.1, 1.0, 10, 100 mg mL^{-1} and each treatment applied (100 µL) as a residue to 500 mg of excised *S. nigra* flowers and air dried for 20 min. Distilled water (100 µL) only was applied to flowers for the control. Treated and untreated flowers were positioned on opposite sides of compartmentalised Petri-dishes (9 cm) on moistened filter paper (200 µL distilled water). An adult female thrips (collected from wild *S. nigra* source, food deprived 1 h) were placed centrally in Petri-dish and lid sealed in place with parafilm (reduced light, ambient temperature 25 °C). Thrips foraging among treated or control flowers were recorded after 6 h. Thrips on sides of Petri-dishes were excluded from selection preference data.

Statistical analysis

The distribution of thrips' life stages in inflorescences was examined spatially (floral development) using analysis of variance transformation (ANOVA, log+1) to determine overall differences (median ± interquartile range), and in all analysis included a post hoc Tukey's multiple comparison test (Tukey HSD) to identify differences (R Core Team 2016). Similarly, thrips exclusion experiments were examined with ANOVA log(thrips+1) followed by Tukey HSD to identify differences between treatments. Abundance and temporal variation of VOCs were compared using ANOVA without transformation, and with Tukey HSD test to indicate where differences lay. A binomial test was performed for each odour stimulus, additionally applying the Bonferroni correction adjustment to account for multiple comparisons (SPSS Version 23, IBM Corperation). CNglc concentrations were analysed using Kruskal–Wallis one-way analysis of variance (KW) applying Shapiro–Wilk test first to check distribution range, applying binomial tests for dual-choice tests using 95% confidence interval for differences between choice-selection (treatment or control) proportions GENSTAT 14.2 (VSN International Ltd.).

Results

Thrips sampling

A total of 7450 thrips were collected from 127 inflorescences from ten *S. nigra* individuals during flowering. All slide-mounted adults and larvae were *T. major*. This was frequently the only insect species present with occasional pollen beetles (Nitidulidae: *Meligethes* spp.), a single individual of *Frankliniella intonsa* (Trybom) (Thripidae) and a few facultative predatory species *Aeolothrips intermedius* Bagnall (Aeolothripidae). Observation under a microscope revealed that adult and immature thrips moved rapidly over and between stamens and gynoecia with multiple pollen grains adhered to the wings and abdominal setae of adult male and female *T. major* in flowers at anthesis stage (Suppl. Fig. S2).

Thrips were absent from *S. nigra* inflorescences in bud (DS0; Figs. 2 and 3). Overall, flower stage influenced thrips numbers in inflorescences (Fig. 2: ANOVA, $F = 70.5$, $P < 0.0001$) although there was no difference between the sex ratio (ANOVA, $F = 0.17$, $P > 0.05$). Adult male and female thrips appeared in inflorescences as flower buds opened (DS0–DS1), and there was a significant increase when 100% flowers on inflorescence were at pre-anthesis stage (DS1–DS2: male and female, Tukey HSD $P < 0.0001$). Female numbers then fell as flowers reached anthesis (DS2–DS3: Tukey HSD female, $P < 0.05$) suggesting females leave flowers at this point and continue to do so through to senescence (DS2–DS4, female, Tukey HSD $P < 0.01$). Male numbers were more stable from pre-anthesis to senescence (DS2–DS4: male, Tukey HSD $P > 0.05$). All adult thrips were absent from inflorescences once petals had dropped and fruit developed (DS5).

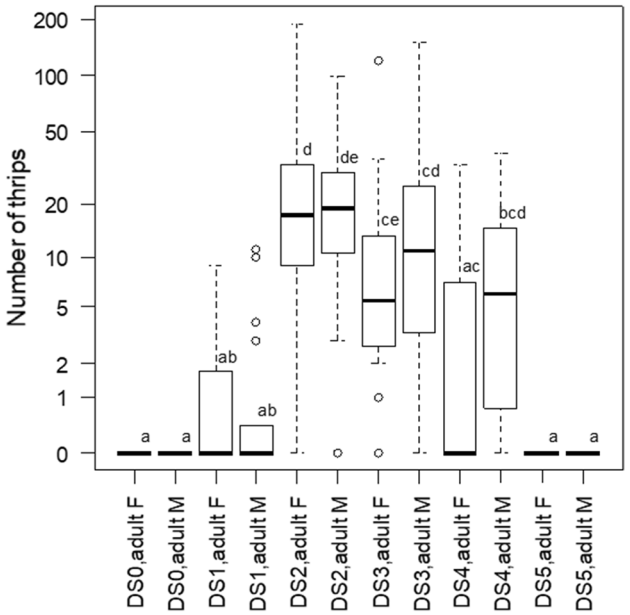

Fig. 2 Number of adult thrips (median ± interquartile range) in inflorescences of *S. nigra* at six stages of floral development (DS0–DS5), sampled June and July 2016. DS0 ($n = 20$), DS1 ($n = 20$), DS2 ($n = 38$), DS3 ($n = 24$), DS4 ($n = 5$), DS5 ($n = 20$), where DS0 = inflorescences comprised of closed buds, DS1 ≤ 50% flowers open, DS2 ≥ 90% flowers open (pre-anthesis), DS3 = 100% flowers open (at anthesis), DS4 = flowers senescing, DS5 = early fruit development. Significant difference between flower development stages (ANOVA, $F = 70.45$, $P < 0.0001$), Tukey's multiple comparison test indicates where differences lie (values with same letters are not significantly different)

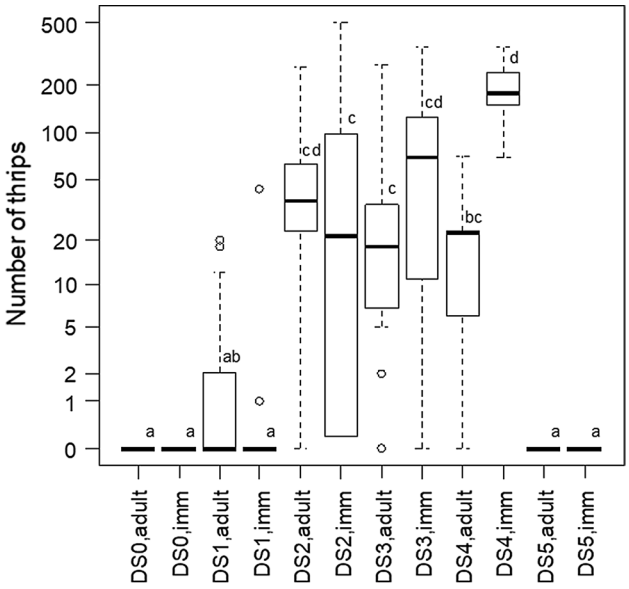

Fig. 3 Number of life stages of thrips (median ± interquartile range) in inflorescences of *S. nigra* at six stages of floral development (DS0–DS5), sampled June and July 2016. DS0 ($n = 20$), DS1 ($n = 20$), DS2 ($n = 38$), DS3 ($n = 24$), DS4 ($n = 5$), DS5 ($n = 20$). Significant difference between flower development stages (ANOVA, $F = 74.61$, $P < 0.0001$), Tukey's multiple comparison test indicates where differences lie (values with same letters are not significantly different)

Overall, there was no difference between relative numbers of adults and larvae in inflorescences at each flower development stage (Fig. 3. ANOVA, $F = 0.07$, $P > 0.05$), but numbers of each life stage varied as flowers developed (ANOVA, $F = 5.01$, $P < 0.001$). As expected, adults appeared in inflorescences before the immatures (DS0–DS1). The number of adults and immatures increased significantly as all flowers opened (DS1–DS2: Tukey HSD, adults and immatures both, $P < 0.0001$). Larvae increased marginally as flowers reached anthesis and continue to increase as flowers begin senescence (DS2–DS3: Tukey HSD, immatures, $P > 0.05$, DS2–DS4: immatures, $P < 0.001$). Larvae were not seen in young developing fruit (DS5) suggesting all thrips had left the inflorescences after senescence and as fruit developed.

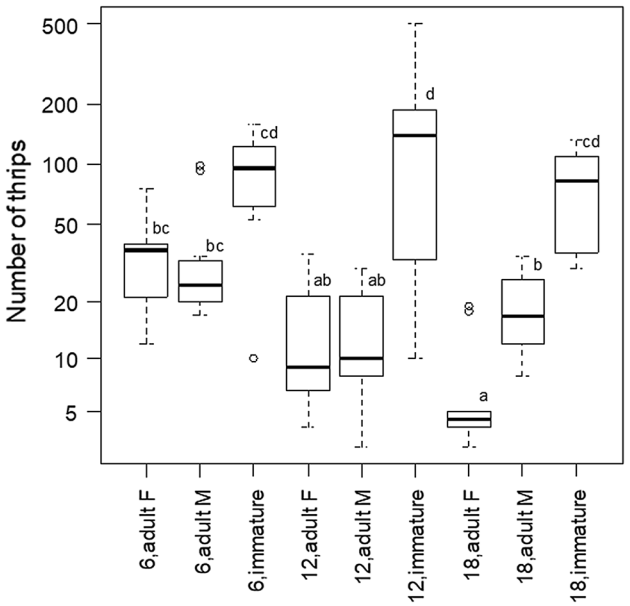

Fig. 4 Diurnal activity of thrips (median ± interquartile range) in inflorescences of *S. nigra*. Collections of single inflorescences taken from 10 individual plant specimens repeated at 6:00, 12:00, and 18:00, showing different life stages of thrips foraging in flowers at specific times of the day. Significant differences between numbers of life stages and time of day (ANOVA, $F = 4.12$, $P < 0.01$), Tukey's multiple comparison test indicates where differences lie (values with same letters are not significantly different)

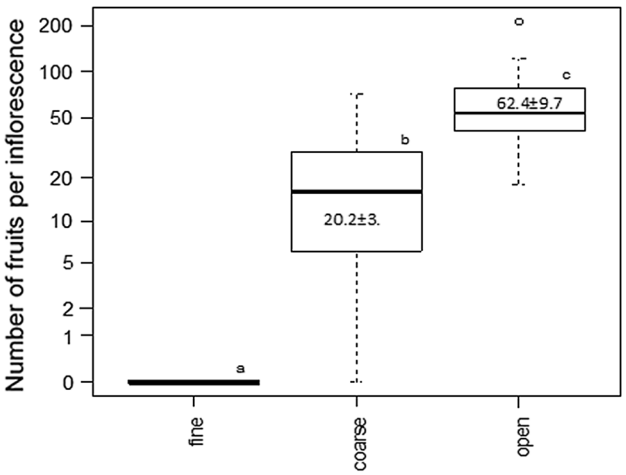

Fig. 5 Number of developing fruit (median ± interquartile range) on inflorescences of *S. nigra*; inflorescences (1) uncovered (open), (2) covered by coarse nylon mesh bag (1 mm diam.), (3) covered by fine mesh bag (0.125 mm diam.) for 6 weeks. Exclusion of thrips and small insects from inflorescences of *S. nigra* (3) prevented any fruit-set occurring, with significantly less fruit developing in coarse mesh (2) than open inflorescences (1). ANOVA, mixed-effect model treating elders as true replicates (ANOVA $F = 276.07$, $P < 0.0001$), Tukey multiple comparison based on mixed-effect model used for compact letter display

There was no significant difference between the proportions of adult male and female thrips present in inflorescences of *S. nigra* at 06:00 and 12:00 (Fig. 4: Tukey HSD, $P > 0.05$ in both cases), however, female numbers were significantly lower than males at 18:00 (Tukey HSD, $P < 0.05$). Numbers of adult females in inflorescences of *S. nigra* decreased from early morning to evening (Tukey HSD, 06:00–12:00: $P < 0.0001$, 12:00–18:00, $P < 0.05$) whereas numbers of male and immature thrips present in inflorescences remained consistent at each three sampling times (Tukey HSD, $P > 0.05$ in all cases). Significantly more larvae than adults were recorded at 12:00 (Tukey HSD, $P < 0.0001$, male and female) and at 18:00 (Tukey HSD, $P < 0.0001$ female, $P < 0.01$ male).

Insect exclusion

Inflorescences from which all insects had been excluded became desiccated after the 6-week exclusion period and no developing fruit were recorded (Fig. 5) whereas 33% fruit-set was recorded in mesh bags that permitted access to flowers by thrips (or thrips-sized insects) compared to open pollinated inflorescences. Bagged inflorescences set significantly less fruit compared with open inflorescences [ANOVA log(fruit+1), $F = 276.07$, $P < 0.0001$], with significantly less fruit developing under fine mesh compared with course (Tukey HSD, $P < 0.0001$) and course mesh compared with open inflorescences (Tukey HSD, $P < 0.0001$). Open inflorescences were accessible to other non-thysanopteran insect visitors and wind-transferred pollen although few other insect classes were observed during our sampling.

Chemical analysis and insect bioassays

VOC emissions during floral development

Floral scent of *S. nigra* was perceived as being most intense in the morning. Seven VOCs were detected in most odour collections (Table 1) but there were significant differences in the relative and absolute abundance of each compound emitted at inflorescence development stages, DS2–DS4 (ANOVA $F = 2.4$, $P < 0.01$). The most abundant compound emitted from inflorescences with open flowers was linalool, which comprised over 40% of the total VOC collected at DS2–DS3. Linalool was significantly more abundant in odour emissions from inflorescences with open flowers than younger inflorescences with predominantly closed buds (Tukey HSD, $P = 0.0185$). A gradual decline in linalool was evident at floral senescence although the decrease was not significant (Tukey HSD, $P = 0.12$). Similarly, epoxy linalool and

Table 1 Proportional abundance of component VOCs in total scent emissions collected over 24 h from developing inflorescences of *S. nigra*

Floral volatile compound	Retention time (min)	Kovats retention index	Mean peak area (mean % of total volatile emission)			
			DS0–DS1 (%)	DS2–DS3 (%)	DS4 (%)	Control
E-Ocimene	6.09	1229	1921.3 ± 1238.9 (19.3 ± 11.2)	44,291.3 ± 14,792.7 (16.6 ± 4.5)	359.7 ± 359.7 (1.9 ± 1.9)	0 (0)
Linalool oxide	8.57	1416	464.3 ± 287.3 (27.4 ± 24.3)	19,914.3 ± 2728.8 (8.2 ± 1.6)	842.7 ± 595.9 (4.5 ± 4.5)	0 (0)
Linalool	9.88	1523	454.8 ± 454.8 (3.6 ± 3.6)	112,011.0 ± 15,904.8 (44.4 ± 0.7)	1886.3 ± 1886.3 (10 ± 10)	0 (0)
Caryophyllene	10.57	1580	0 (0)	0 (0)	0 (0)	274 ± 274 (13.3 ± 13.3)
Epoxy linalool	12.03	1710	608.5 ± 359.9 (6.5 ± 3.8)	54,882.3 ± 54,882.3 (22.9 ± 5.6)	3221.3 ± 3221.3 (17.0 ± 17.0)	0 (0)
Citronellol	12.34	1739	1745.3 ± 1058.5 (18.2 ± 10.6)	20,432.3 ± 7821.6 (7.9 ± 2.7)	0 (0)	0 (0)
Caryophyllene oxide	14.66	1965	0 (0)	0 (0)	0 (0)	757 ± 757 (36.7 ± 36.7)

Differences were observed in abundance of each of the seven volatile compounds between flower development stages, DS2–DS4 (ANOVA $F = 2.4$, $P < 0.01$)

citronellol were increasingly abundant in scent produced by inflorescences with open flowers (DS2–DS3) compared with senescing flowers (Tukey HSD, $P < 0.05$); however, the abundance of each was similar in emissions retrieved from young inflorescences containing buds and the senescing flower stages (Tukey HSD, $P > 0.05$). In summary, our data suggest that production of linalool increases as flowers in inflorescences open and declines as the flowers senesce. Citronellol and epoxy linalool were present in scent emitted from younger inflorescences still in bud, and levels increased marginally when buds opened, decreasing significantly as flowers senesced, reverting to levels recorded at the earlier floral development stages. All three of these VOCs were absent from leaves.

Circadian abundance of floral VOCs

The seven predominant compounds of the VOCs from inflorescences of *S. nigra* varied significantly (Table 2: ANOVA; $F = 19.29$, $P < 0.001$) and at each collection time (ANOVA; $F = 6.25$, $P < 0.01$). The most abundant compound, linalool, made up > 50% of the total VOC at each time point. Our data suggest that linalool was less abundant in the afternoon but emissions increased during the evening, where absolute levels were greatest between 00:00 and 06:00. Our evidence suggests that the highest levels of linalool, occurring from inflorescences at DS2 peaked between midnight and 06:00, coinciding with the highest abundance of adult female thrips in inflorescences (Figs. 2 and 4).

Olfactory tests with floral VOCs

VOCs from inflorescences at DS2, collected from 00:00 to 06:00 were chosen by thrips significantly more often than by chance, after Bonferroni correction (80.0% of choices; binomial test, $z = 4.52$, $P < 0.0001$, corrected $\alpha = 0.01$) (Fig. 6). While thrips also chose DS3 volatiles collected over 24 h 61.7% of the time, this was not significant after

Table 2 Proportional abundance of component VOCs in total scent emissions collected from an inflorescence of *S. nigra* (DS2 > 90% flowers open) at four time intervals, over 24 h

Floral volatile compound	Retention time (min)	Kovats retention index	Peak area (% of total volatile emission)			
			12:00–18:00	18:00–00:00	00:00–06:00	06:00–12:00
E-Ocimene	6.091	1229	2734 (5)	7449 (5)	21,404 (6)	5117 (4)
Linalool oxide	8.569	1416	5182 (9)	7202 (5)	19,921 (6)	7268 (5)
Linalool	9.876	1523	33,672 (58)	90,866 (61)	190,292 (55)	81,471 (58)
Caryophyllene	10.566	1580	0 (0)	0 (0)	4266 (1)	0 (0)
Epoxy linalool	12.029	1710	13,874 (24)	37,326 (25)	92,056 (26)	40,633 (26)
Citronellol	12.340	1739	2858 (5)	7263 (5)	16,760 (5)	6232 (5)
Caryophyllene oxide	14.664	1965	0 (0)	0 (0)	4056 (1)	0 (0)
Total peak area			58,320 (100)	150,106 (100)	348,755 (100)	140,721 (100)

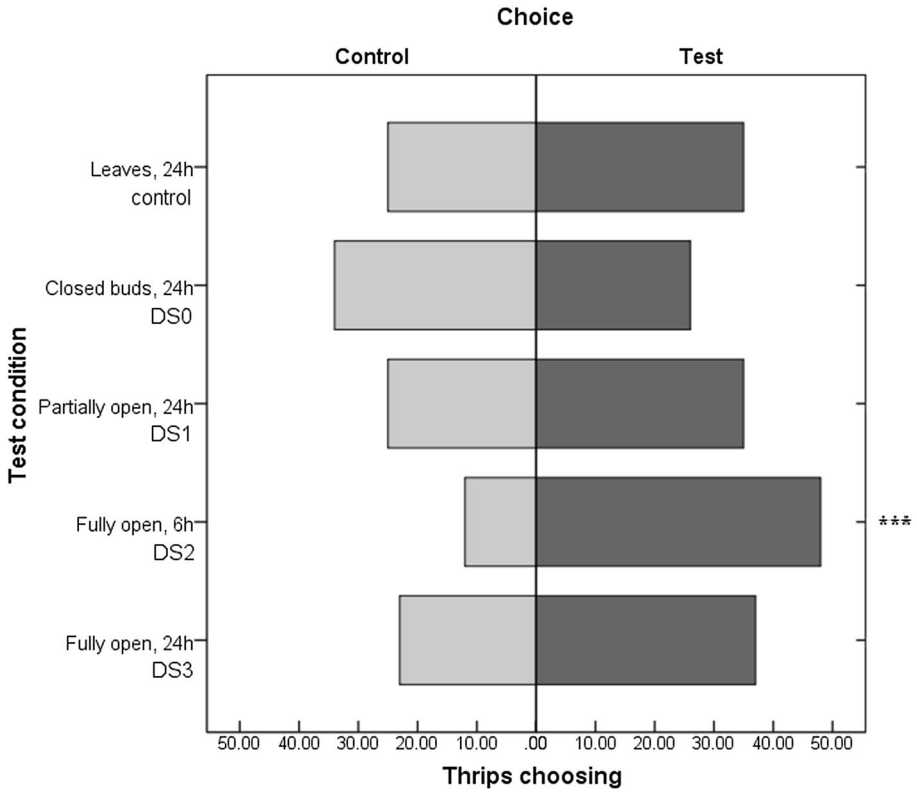

Fig. 6 Thrips selection preference using a y-tube olfactometer for testing volatile extractions of *S. nigra* floral development stages (DS0–DS3) against control. Bars indicate number of individuals choosing each arm. ***$P<0.001$

the Bonferroni correction was applied (binomial test, $z=1.68$, $P=0.0466$, corrected $\alpha=0.01$). A X^2 test revealed no significant differences between responses to different levels of partially open inflorescences ($X^2=4.251$, $P=0.1193$) so these were pooled (DS1) for analysis. Thrips were not attracted to partially open inflorescences (binomial test, $z=1.17$, $P=0.1226$) or those with closed buds ($z=0.90$, $P=0.1891$).

CNglcs and floral development

Defence compounds in floral tissue of *S. nigra*

Sambunigrin and prunasin were detected in reproductive tissue sampled at all development stages examined (Suppl. Fig. S3). Prunasin occurred at very low concentrations ranging from 1.2 to 1.3 µg mg^{-1} (fresh tissue weight) in buds and senescing flowers to 0.8 and 0.3 µg mL^{-1} in open flower tissue and mature red fruit, respectively. Sambunigrin was recorded at significantly higher concentrations than prunasin (KW, $H=6.19$, $P<0.05$) in all *S. nigra* floral tissue; up to 50 times more concentrated when compared at each flower development stage.

The concentration of sambunigrin was highest in the young bud tissue of *S. nigra* and decreased as flower development progressed and flowers opened (Fig. 7a; DS0 vs DS1 and DS2–3: *MW*, $U=0$, $P<0.001$ in both cases). As flowers matured and petal drop initiated the level of sambunigrin increased again to levels similar to those found in the flower buds (DS4 vs DS0; MW, $U=6$, $P>0.05$).

Similarly the concentration of prunasin was significantly higher in young bud tissue, decreasing in larger buds and as flowers open (Fig. 7b, DS0 vs DS1, MW $U=1$, $P<0.01$) with a significant increase as flowers progressed in age (DS1 vs DS2–3, MW, $U=2$, $P<0.01$) increasing further in senescing flowers returning to levels present in young buds (DS2–3 vs DS4, MW, $U=1$, $P<0.05$). As with sambunigrin there was a decreasing trend in the concentration of prunasin, comparing senescing flower tissue to green fruit and mature red fruit but this decrease was not significant (DS4 vs DS5 and mature fruit; MW, $U=0$, $P>0.05$).

Effect of CNglcs on feeding preference (dual-choice test)

Extracts of green fruit, buds and leaves of *S. nigra* deterred adult female thrips from treated flowers (binomial test, $z=0$, $P<0.001$; $z=0.179$, $P<0.01$; $z=0.134$, $P<0.001$, respectively), yet the thrips were selective for 100% open flower-treated extracts over controls (DS3) (binomial test, $z=0.134$, $P<0.01$). Assays undertaken with isolated CNglcs indicated thrips preferred flowers which had not been treated with either prunasin (90% selecting control) or sambunigrin (67% selecting control). Prunasin was highly deterrent to thrips under these conditions even at the lowest concentration applied

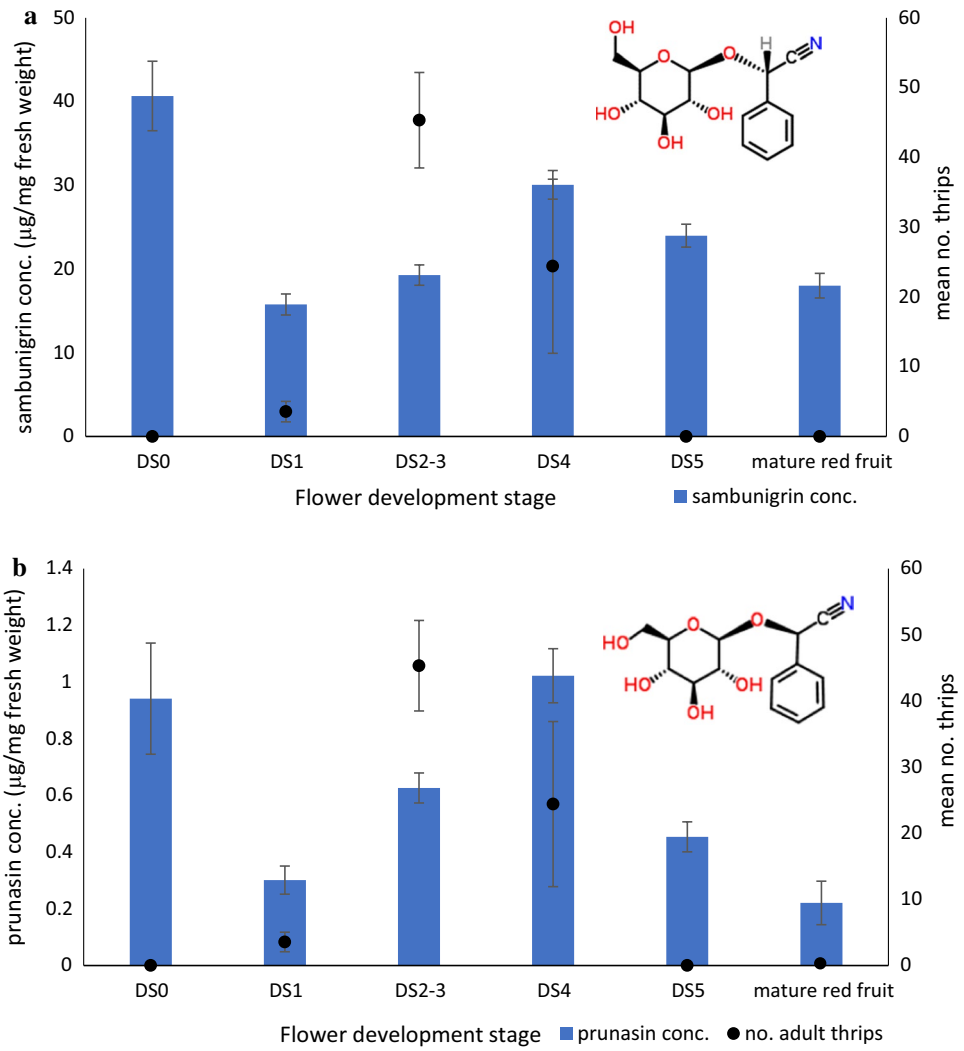

Fig. 7 Concentration (µg mg^{-1} FW) of CNglcs. Sambunigrin (**a**) and prunasin (**b**) (mean ± SE), plotted against mean no. adult thrips (male and female combined, mean ± SE) in developing reproductive tissue of *S. nigra*. Floral development stages DS0–DS5 and mature red fruit, sampled June and July 2016, DS0 ($n=9$), DS1 ($n=6$), DS2 and DS3 combined ($n=9$), DS4 ($n=3$), DS5 ($n=3$) and mature red fruit ($n=3$), where DS0 = closed bud, DS1 = <50% flowers in inflorescence open, DS2 ≥ 90% flowers open (pre-anthesis), DS3 = 100% flowers open (at anthesis), DS4 = senescence, DS5 = early development of green fruit

Table 3 Dual-choice bioassays indicating preference for adult female thrips selecting CNglc-treated (T) and untreated (C) flowers

Treatment (T) (µg mL^{-1})	Conc. (µg mL^{-1})	Number of thrips		*z* value	*p* value
		(*T*)	(*C*)		
Prunasin	0.1	1	12	4.32	0.001***
	1	2	20	5.43	0.001***
	10	1	12	4.32	0.001***
	100	2	15	4.46	<0.0001***
Sambunigrin	0.1	5	11	2.12	0.034
	1	10	17	1.91	0.057
	10	5	11	2.12	0.034
	100	7	16	2.65	0.008**

Total 147 thrips included in analysis. Thrips exposed to residues of prunasin and sambunigrin solutions, 100 µL applied at 0.1, 1, 10, and 100 µg mL^{-1}

**$P<0.01$ and ***$P<0.001$

($z=4.32$, $P<001$). In contrast, sambunigrin was found only to be deterrent at the highest concentration tested (binomial test, $z=2.65$, $P<0.01$, corrected $\alpha=0.01$, Table 3).

Discussion

Thrips are ancient pollen feeders (Penalver et al. 2012) and it is widely acknowledged that pollen evolved as the first reward for early insect mutualists (Labandeira 1998; Grimaldi 1999). *Shorea* spp. (Dipterocarpaceae) (Proctor et al. 1996) provide a well-documented example of a thrips brood-site mutualism, where trees produce high volumes of flowers over a relatively short period coinciding with the rapid build-up of large thrips populations which transfer pollen to new hosts as flowers die. In our study we observed high numbers of adult thrips visiting the inflorescences of *S. nigra*, coinciding with the peak release of VOCs attractive to thrips, when flowers were at

pre-anthesis and were pollen-receptive. Larvae increased in number, exceeding adult numbers as flowers in inflorescences matured. The longevity of the *S. nigra* inflorescences provided an environment for *T. major* to develop from egg to winged-adult stage at summer temperatures (see Lewis 1973), increasing the likelihood of thrips moving in and between host plants. We found that excluding all insect visitors from *S. nigra* inflorescences prevented fruit-set whereas allowing thrips access to flowers significantly increased the chances of fruit developing. Thrips were absent from flowers of *S. nigra* at petal-drop, which coincided with concentrations of CNglcs in mature flowers and developing fruits that were deterrent to adult thrips. Thus, our data suggest that the floral chemistry of *S. nigra* mediates this interaction and also that thrips are contributing to the pollination of this plant. There is precedent for this since multiple interacting factors causing pollinator decline and absence in floral life-cycles have previously been associated with changes in floral chemistry (Raguso and Pichersky 1999; Terry et al. 2007) and diminishing rewards (Schiestl and Ayasse 2001).

Phylogenetic evidence, as well as taxonomic and biogeographical distributions, suggest that all insect–plant mutualisms are not only frequently gained, but also frequently lost; for example, the shift in reliance on alternative abiotic pollination strategies (wind pollination and self-pollination). Mutualisms can establish in habitats where relevant pollinating taxa are abundant, alternative mutualists rare or where the benefit of mutualistic service is high (see Bronstein et al. 2006). Many previous general literature sources on flowering plants and pollination have overlooked the presence of large numbers and association of thrips with flowers and incorrectly assigned pollination function to wind or bees, even in the absence of bees (see Knuth 1906; Hagerup and Hagerup 1953). One example is the pollination of *Acer pseudoplatanus* L. (Sapindaceae) which was attributed in part to wind by Binggeli (1992) and insects such as bees and flies (Binggeli 1992; Proctor et al. 1996) although these insects were rarely observed during a detailed study of this species (Tal 2009). While wind probably contributed to the pollination of *A. pseudoplatanus* (Hesse 1979), Tal (2009) highlighted that wind pollination had been previously inferred on the basis that pollination still occurred after exclusion of insects with nets of 1 mm mesh size (Binggeli 1992), an aperture size that would not exclude thrips. Although pollen deposition by non-bee pollinators is often recorded lower than bees, high visitation frequency as occurs with thrips can reduce this deficit (Rader et al. 2016), with some species capable of carrying > 100 grains per insect (Ananthakrishnan 1982; Velayudhan and Annadurai 1986). Thrips often forage at night (Kirk 1997), leaving flowers as ambient temperature rises by day. In inflorescences of *S. nigra* female thrips numbers were recorded at highest levels at 06:00 and decreased throughout the day, while numbers of males and immatures remained stable. Although this study did not track the direction of movement of female thrips within or between inflorescences of *S. nigra*, such circadian dispersal activity of insects facilitates pollen transfer to conspecific flowers.

Exclusion of thrips from the developing *S. nigra* inflorescences using fine-mesh nets resulted in an absence of developing fruits, despite the mesh pore-size capable of accommodating wind-transmitted pollen grains (Bolli 1994). In contrast, fruit-set readily occurred when inflorescences were uncovered and accessible to pollen transmission (wind and all insect visitors) and when inflorescences were contained within course mesh nets (1 mm), which allowed access to thrips and wind-blown pollen but excluded larger insects. Slight reduction in pollination occurring with course mesh coverage may be partially attributed to thrips being physically hindered by the mesh bags, with some pollen removal possible during entry. While Wragg and Johnson (2011) noted that seed set in wind- and insect-pollinated *Cyperus* spp. was unaffected by the use of pollination bags, Wassnera and Ravetta (2005) suggest that mesh bags may reduce air movement, increase humidity and light transmission and that chemistry may be affected influencing insect behaviour. However, experimental exclusion of thrips from flowers in pollinator studies is infrequently reported because of technical difficulties in containing such small, highly mobile organisms and thus it is likely that the full significance of thrips in plant reproduction remains considerably underestimated.

Floral scents act as signalling cues for both pollinators and antagonists (Theis and Adler 2012; Kessler et al. 2015). To increase chances of pollen transmission to receptive flowers, floral ontogenical changes in chemistry can also be mechanistic in directing pollinators to unpollinated flowers (Schiestl and Ayasse 2001). Of the comprehensive list of volatile organic compounds (VOCs) previously recorded from flowers of *S. nigra* (Kaack et al. 2006; Salvador et al. 2017), few report VOCs emitted from intact inflorescences (Knudsen et al. 2006; Peñuelas et al. 2014). Here we monitored seven key volatile compounds from inflorescences in situ over 24 h. Linalool was the most abundant of the VOCs, peaking when flowers in inflorescence were open (pre-anthesis), while almost absent when at bud and senescence stages; corresponding with the abundance of thrips in inflorescences. The abundance of linalool in *S. nigra* inflorescence volatile emissions increased between midnight and early morning coinciding with peak visitation by female thrips to flowers. The attraction of *T. major* to *S. nigra* by linalool emitted from inflorescences with open flowers is supported by previous studies reporting the attraction of *Thrips obscuratus* (Crawford) to Japanese honeysuckle flowers, *Lonicera japonica* (Thunberg) (Caprifoliaceae) (El-Sayed et al. 2009), which have been shown to emit high

levels of linalool at night (Miyake et al. 1998). An additional monoterpenoid of interest present was β-ocimene. This ubiquitous VOC emitted from plant reproductive structures and vegetative tissue by numerous species of plants (Knudsen et al. 2006) has several ecological functions including pollination attraction (Filella et al. 2013; Farré-Armengol et al. 2017). Terry et al. (2007) demonstrated and quantified the coordination between the release of VOCs, which included two isomers of β-ocimene from the male cones of *Macrozamia lucida* LAS Johnson, and the behaviour of their host-specific thrips pollinators; *Cycadothrips chadwicki* Mound. Our study suggests that while female thrips responded to varying levels of the key components of the VOCs emitted by *S. nigra* inflorescences, male and immature thrips remained constant in numbers in inflorescences of *S. nigra* throughout the circadian cycle despite the fluctuation in emission of these compounds.

Mutualisms can develop where trade-offs between partners are reduced. Although there are isolated cases of *T. major* causing damage to crop hosts in its native range, when such cases are reported they are usually present in conjunction with invasive species of thrips (OEPP, EPPO 2004). Production of defence compounds to protect valuable reproductive tissue from pollen-feeding flower visitors can be a highly efficient strategy to preserve gametes and developing embryos (Sedivy et al. 2012), particularly when resources are deployed for their production at critical times in development (McCall and Fordyce 2010). CNglcs (α-hydroxynitrile glucosides) and their activating β-glucosidases occur in tissues of *S. nigra* (Velíšek et al. 1981; DellaGreca et al. 2000). These phytoanticipin compounds (VanEtten et al. 1994) are spatially separated at a cellular level until activated and releasing hydrogen cyanide (cyanogenesis) through tissue disruption such as insect feeding (Zagrobelny et al. 2004; Møller 2010). We recorded two CNglcs, prunasin and sambunigrin, in floral tissues of *S. nigra* and showed that these compounds were at the lowest levels when the flower was receptive to fertilisation (pre-anthesis) and adult thrips accumulated in flowers, while levels increased in developing buds and at fruit-set when thrips were absent. CNglcs are established feeding deterrents (Zagrobelny et al. 2004) and our bioassays confirmed that adult thrips were deterred by the presence of sambunigrin, the dominant CNglc, at relatively high concentrations. No behavioural effects were observed when sambunigrin was presented at lower concentrations similar to those recorded in newly opened flowers. Therefore, CNglcs in the floral tissue of *S. nigra* may play a role in protecting unopened flower buds and developing fruit from potential florivores. While we can postulate that defence of valuable tissues is the primary role of CNglcs, the multifunctionality of these compounds as a source for sugars and reduced nitrogen for the physiological changes that occur during the reproductive stages (Selmar et al. 1988; Neilson et al. 2013; Del Cueto et al. 2017) has not been considered here. To ameliorate the costs of chemical defence, secondary metabolites that initially served defence purposes may acquire new functions in host–insect recognition or be recruited as storage compounds that are mobilized when needed to counteract imbalances in primary metabolism (Zagrobelny et al. 2008).

Conclusions

S. nigra floral traits are consistent with it being pollinated by thrips (see Kirk 1997) such as compact inflorescences (De Jong 1976), many flowers per inflorescence, abundant and powdery pollen (Hesse 1979). If thrips provide a pollination service to *S. nigra*, then this will likely manifest in traits that attract the pollinating taxa at temporally critical points in floral development. The combination of field and laboratory experiments with chemical analysis of floral tissue provided an innovative approach to our original research question 'Do thrips provide a pollination service to *Sambucus nigra*'? Here we provide new evidence that the floral chemistry of *S. nigra* attracts a flower-feeding thrips to pollen-receptive flowers in high numbers and then deters them before fruit development. In addition, visitation by thrips increases the chances of fruit-set in this species. In return the plant provides thrips with sites for oviposition and a food source for developing larvae.

Opportunistic mutualisms arising between insects and predominantly wind-pollinated genera within closed habitats have shown that plants can transfer from wind pollination to insect pollination in certain ecosystems (Wallander 2008; Wragg and Johnson 2011), with plant-trait shifts occurring after transition, as an adaptation to enhance efficiency of a new pollination mode. Understanding what drives these shifts in pollination strategies can enable future investigations into studies which examine more complex relationships between land-use change and plant health, particularly as mutualisms are known to become antagonistic if ecological conditions change (Bronstein et al. 2006).

Author contribution statement AS planned the research, undertook field sampling, laboratory experiments and analysis of data. DWC provided taxonomic identification of insects. SA analysed olfactory bioassay data and GK and IF provided LCMS technical assistance undertaken in PS lab. AS wrote manuscript with all authors editing and approving the final manuscript.

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