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Floral Scent Contributes to Interaction Specificity in Coevolving Plants and Their Insect Pollinators

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Abstract Chemical defenses, repellents, and attractants are important shapers of species interactions. Chemical attractants could contribute to the divergence of coevolving plant-insect interactions, if pollinators are especially responsive to signals from the local plant species. We experimentally investigated patterns of daily floral scent production in three *Lithophragma* species (Saxifragaceae) that are geographically isolated and tested how scent divergence affects attraction of their major pollinator—the floral parasitic moth *Greya politella* (Prodoxidae). These moths oviposit through the corolla while simultaneously pollinating the flower with pollen adhering to the abdomen. The complex and species-specific floral scent profiles were emitted in higher amounts during the day, when these day-flying moths are active. There was minimal divergence found in petal color, which is another potential floral attractant. Female moths responded most strongly to scent from their local host species in olfactometer bioassays, and were more likely to oviposit in, and thereby pollinate, their local host species in no-choice trials. The results suggest that floral scent is an important attractant in this interaction. Local specialization in the pollinator response to a highly specific plant chemistry, thus, has the potential to contribute importantly to patterns of interaction specificity among coevolving plants and highly specialized pollinators.

Keywords Coevolution · Diurnal rhythm · Host specialization · Geographic mosaics · Plant–insect communication · Speciation

Introduction

As coevolving organisms diversify into separate species, they do so through divergence in a combination of morphological, behavioral, life history, and chemical traits. Divergence in morphology has been a dominating theme of coevolutionary studies (for recent examples see *e.g.* Benkman et al. 2012; Pauw et al. 2009; Thompson et al. 2013; Toju et al. 2011), but studies of chemical diversification increasingly have shown that coevolution is just as often about attractants, repellents, toxic compounds, and counter responses to those compounds (Berenbaum and Zangerl 2006; Brodie and Ridenhour 2003; Ehrlich and Raven 1964; Foitzik et al. 2003; Hanifin et al. 2008; Johnson et al. 2010; Raguso 2008; Thompson 2013). Attractants are a particularly intriguing class of compounds in coevolving interactions, because they actively attract mutualists but may simultaneously attract enemies (Theis and Adler 2012).

Mutualistic interactions between plants and specialized pollinating floral parasites such as yucca moths or fig wasps have become models for studies of chemical attractants. In total, there now are over one thousand identified plant species from highly divergent families that are known to have coevolved pollination interactions with floral parasites (*e.g.*, Herre et al. 2008; Ibanez *et al.* 2009; Kawakita 2010; Pellmyr et al. 2007; Thompson et al. 2013). The majority of these plant species are pollinated exclusively by their coevolved partners (Dufaÿ and Anstett 2003), but some are visited also by

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generalist pollinators in some populations (e.g., Reynolds *et al.* 2012; Thompson and Cunningham 2002). These latter examples have become useful models for studies of the evolutionary and coevolutionary processes that may lead to obligate mutualism by shaping local specificity in the interactions (Thompson et al. 2013).

Chemical cues have the potential to augment or even be the major factor shaping interactions between some plants and pollinating floral parasites (Hossaert-McKey et al. 2010; Raguso 2008; Schaefer et al. 2004). Some pollinating floral parasites are known to respond more strongly to the floral scent signal of their specific hosts in areas where several potential host plant species occur in sympatry and attract different but closely related insect pollinators (Chen and Song 2008; Hossaert-McKey et al. 2010; Okamoto et al. 2007; Proffit et al. 2007; Svensson et al. 2010). Additionally, recent evidence suggests that floral scent signals are geographically and phylogenetically variable in some of these highly specific pollination systems (Friberg et al. 2013; Soler et al. 2011; but see Svensson et al. 2011).

Recent work on the coevolving interactions between woodland star plants (Saxifragaceae: *Lithophragma*) and their pollinating floral parasites in the *Greya* moth genus (Prodoxidae) has shown that these closely related plants have an extraordinarily diverse set of floral scents that vary markedly among species and even subspecies (Friberg et al. 2013), at levels comparable to differences between genera in other studies (e.g., Kaiser and Tollsten 1995; Levin et al. 2001). The production of such a diverse array of compounds is likely to incur energetic (Gershenzon 1994; Wright and Schiestl 2009) and ecological costs, because the same compounds that attract mutualistic insects also may attract antagonistic herbivores and seed predators (Irwin et al. 2004; Proffit et al. 2007; Schiestl et al. 2011; Theis 2006; Theis and Adler 2012; Wright and Schiestl 2009).

The floral scent varies between different *Lithophragma* species, is consistent, and could potentially facilitate pollinator specificity (Friberg et al. 2013). However, if these complex scents are to function effectively as attractants for specialized pollinators, they should (i) also be most apparent during the time of day when the specialized pollinators are actively searching for plants in order to reduce the risk of eavesdropping from herbivores and seed predators. Furthermore, and most importantly, (ii) the pollinators should be attracted specifically to the scent of their particular host plant species. We tested these predictions by comparing patterns of visual and chemical divergence for three species of *Lithophragma* plants, and their impact on the attraction and oviposition preference of each local population of the specialized pollinating floral parasite moth *Greya politella*.

Methods and Materials

Study System The coevolving interaction between *Lithophragma* plants and *Greya* moths is distributed across the western United States and south-western Canada. At least five of the nine species in the *Lithophragma* genus are involved in a mutualistic relationship with the *G. politella* moth species complex (Rich et al. 2008; Thompson 2010; Thompson et al. 2013). These four cryptic moth taxa are defined by genetically distinct mitochondrial haplotype clusters (Rich et al. 2008), and also show evidence of morphological divergence, albeit with partly overlapping morphological distributions (Thompson et al. 2013). The plants and insects appear in different combinations at different sites. Thereby, the interaction range includes sites with pairwise interactions (1 moth subspecies/1 plant species) as well as sites where up to three *Lithophragma* species are pollinated by the same subspecies of moth (Rich et al. 2008; Thompson et al. 2013; JNT unpublished data). To our knowledge, there are no sites that include multiple subspecies of *G. politella*.

Females of these diurnal moths act as efficient pollinators while ovipositing into the floral ovaries (Davis et al. 1992; Thompson et al. 2013). Pollen adhering to a female's abdomen transfers to the stigma when she inserts her abdomen into the floral corolla. The moths are intimately associated with the host plants in all life stages. Adults mate, take nectar, and usually rest only on their local host plant species. Females oviposit in the flowers, the early-instars feed on developing seeds, mid-instars overwinter in the root system, and late instars feed on and pupate rolled up in *Lithophragma* leaves (Davis et al. 1992). The cost to the plant of hosting the growing larvae is outweighed in most populations that have been studied by the benefit of the moths being specialized and effective pollinators (Thompson and Cunningham 2002; Thompson et al. 2010, 2013). In some habitats, however, the mutualism is swamped by non-*Greya* co-pollinators (e.g., bombyliid flies, andrenid bees) that pollinate without ovipositing into the flowers and hence do not cost the plant any seeds (Thompson and Cunningham 2002; Thompson and Fernandez 2006).

We chose three focal plant species from populations that strongly depend upon *G. politella* for pollination. These species are self-incompatible (Thompson et al. 2013), and previous studies have shown that the moths are the dominant pollinator of these plants at the study sites (Rich et al. 2008; Thompson and Cunningham 2002; Thompson et al. 2013). Two of the plant species were chosen because they emit very different floral scent bouquets: *Lithophragma parviflorum* from Turnbull Wildlife Refuge (47 24.0'N, 117 34.0'W) in eastern Washington, and *L. cymbalaria* from the UC Santa Barbara Sedgwick Reserve in California (34 42.871'N, 120 2.999'W) (Friberg et al. 2013). The floral scent of *L. parviflorum* from Turnbull is dominated by monoterpenes

and some benzenoid esters, whereas *L. cymbalaria* from Sedgwick emits a complex volatile bouquet, including monoterpenes, benzenoid esters, and nitrogenous aromatics. The floral scent of the third focal plant, *L. bolanderi* from Marble Falls in Sequoia National Park, California (36 31.198'N, 118 48.024'W), had not been investigated prior to this study.

Lithophragma bolanderi is a close relative to *L. cymbalaria*, whereas *L. parviflorum* belongs to a different clade in the *Lithophragma* genus (Soltis et al. 1992). The three species differ in floral morphology (Thompson et al. 2013), and occur in different parts of the genus's range. The Turnbull population of *L. parviflorum* grows in Ponderosa pine woodland with a rich understory of herbs and shrubs, whereas the two Californian sites are situated in a dense oak foothill with a rich understory (*L. bolanderi* at Marble Falls), and in oak woodland with scattered pine trees and adjacent chaparral foothills (*L. cymbalaria* at Sedgwick).

Patterns of Floral Scent Emission Plants were grown under common garden greenhouse conditions (see online resource 1 (OR1) for detailed growth conditions). At the onset of flowering, they were transferred to a growth chamber (Conviron E-15, Pembina, ND, USA) specifically programmed for each experiment. A plant was allowed to acclimate for at least 5 d before scent collection. Floral scent was collected using dynamic headspace techniques (Raguso and Pellmyr 1998) and both scent collection and the subsequent gas chromatography/mass spectrometry (GC/MS) analysis was performed in exact accordance with the protocols outlined in (Friberg et al. 2013) (also see OR1 for a detailed description).

The floral scent signaling was analyzed in a two-step experiment. First, we reproduced the natural variation of warm days and cool nights that plants experience in the field (day: 11 h light (230 μmol photons/m^2/s), 20 °C, dusk (85 μmol/m^2/s): 1 h, 15 °C, night: 11 h dark, 10 °C, and dawn (85 μmol/m^2/s): 1 h, 15 °C). We collected scent from individuals from each species ($n_{L.\ bolanderi}$=10 (5 seed families); $n_{L.\ cymbalaria}$=12 (11); $n_{L.\ parviflorum}$=10 (7)) for 2 h under both day and night conditions. Daytime collections started 1 h after dawn, and nighttime collections started 1 h after dusk. Half of the plants had daytime collections made first, and half had nighttime collections made first.

Thereafter, we attempted to disentangle the effects of daylight and temperature on the daily pattern of floral scent emission discovered in the first experiment. The light conditions and overall protocols were identical to the previous experiment, while temperature was kept constant at either 10 °C or 20 °C. We applied these treatments to individuals of *L. parviflorum* from Turnbull (*N* =25 (11 seed families)) and *L. bolanderi* (*N* =24 (5)) from Marble Falls.

The multivariate variation in daytime floral scent composition was analyzed using the software PRIMER 6.1.11 (Clarke 1993; Clarke and Gorley 2006). Data were square-root transformed to approach normality. We generated Bray-Curtis similarities and applied multidimensional scaling (MDS) to the data. We used analysis of similarity (ANOSIM) to reveal patterns of statistical significance, and determined the average similarities and dissimilarities within and between plant species using the SIMPER function (Clarke 1993).

Scent variation also was evaluated by calculating the standardized emission rate of all volatiles from each sample (SEM; ng volatiles/flower/h) (see Friberg et al. 2013; Svensson et al. 2005 for rationale). The magnitude of the scent signal during day- and nighttime was compared between species and treatments in multivariate analyses of variance (MANOVA) models using Statistica 10 (StatSoft Inc 2011). Data were log-transformed before analysis to equalize variances between groups. The MANOVA models used the standardized emission rate of each individual at daytime and nighttime as the repeatedly measured response variable, and the order (day first/night first), the plant species, the temperature (when applicable), and the interaction between effects of species and temperature as factors. The number of compounds emitted at daytime and nighttime was analyzed in similar models in order to detect also qualitative effects of temperature and light conditions.

The second experiment disentangled the effects of light condition and temperature on scent signaling, using *L. bolanderi* and *L. parviflorum.* Compounds were subdivided into two major groups, monoterpenes and aromatics (see supplemental Table 1 (S1) in OR2). The standardized emission rate of each compound group at daytime and nighttime was then used as the repeatedly measured response variable in species-specific MANOVA models with collection order and experiment temperature as factors. The number of monoterpenes and aromatic compounds emitted at daytime and nighttime were analyzed similarly in MANOVA models.

Reflectance Scans Spectral reflectance was measured from the adaxial surface of petals of greenhouse grown plants of the three species using the software OOIBase (Ocean Optics, Dunedin, FL, USA) and an Ocean Optics USB2000 spectrophotometer with a PX-2 pulsed xenon lamp. The spectral measurement area was restricted to a standard 2 mm^2. We took five measurements from single petals of different flowers from each plant individual. Each individual also was scanned for distinct peaks of UV-reflectance across the adaxial petal area. No such areas of increased UV-reflectance were found. The spectrophotometer was recalibrated between each measurement on a standard black and a standard white surface (Labsphere, Inc.). We measured a total of 12 individuals from 5 different seed families of *L. bolanderi* from Marble, 13

individuals from 10 families of *L. cymbalaria* from Sedgwick, and 11 individuals of 9 families of *L. parviflorum* from Turnbull.

We calculated the average reflectance for each individual in the ultraviolet- (300–380 nm wavelengths), the violet (381–450 nm), the blue (451–475), the cyan (476–495 nm), the green (496–570 nm), the orange (571–590 nm), the yellow (591–620 nm), and the red (621–700 nm) sub-spectra. These calculations were made using the Excel-based programs BinR1.7 and ColoR 1.7 (Montgomerie 2006), and the resulting spectrum-specific reflectance data were analyzed in a MANOVA model with the reflectance in each color span as the repeatedly measured response variable, and species as grouping factor. We further tested the potential for multivariate differences in a principal component (PC) analysis (correlation matrix) in Statistica 10 (StatSoft Inc 2011) with the reflectance in each of the 8 wavelength categories as the initial variables of analysis. The variation in the first and second principal components then was analyzed in separate linear models (ANOVA) with species as grouping factor.

Olfactometer Experiment We conducted y-tube olfactometer experiments on wild-caught female moths from each of the study populations, collected during their natural flight period. Each two-choice olfactometer (Scientific Glass, Löberöd, Sweden) consisted of a transparent glass tube (15 mm diam; 110 mm long), connected to two 70 mm long terminal tubes. Moths were allowed to choose between a floral terminal including a greenhouse grown plant either of the local plant species or from one of the non-local species and a control terminal of ambient air. Visual cues were blocked during the experiment using bridal veil netting. Females were released into the tube and were considered to have made a choice when remaining in a terminal for more than 30 s. A female that had not made a choice within 10 min was removed (see OR1 for further details). The propensity to choose the floral terminal (1) or the control terminal (0) was used as a binomially distributed response variable in a logistic regression, with logit as link function, and the main and interactive effects of plant and moth origin, using the software R 2.13.2 (R Development Core Team).

Egg-laying Experiment Moth females were collected from *Lithophragma* flowers at each field site, and transported to the greenhouse. A total of 154 females (46 from Turnbull, 58 from Marble Falls, and 50 from Sedgwick) were tested in no-choice egg laying trials. Each female was tested only once by placing her in a Plexiglas tube (see supplemental Fig. 1 (S1) in OR1) together with one scape, cut so that it presented two flowers from a greenhouse-grown plant of one of the three focal species. The distance from the bottom of the scape to the first flower was held constant at 15 cm. Previous studies have shown that this experimental set-up is effective in testing for egg-laying preference in these moths (Janz and Thompson 2002). Each experimental trial was started between 18.00 and 21.00 at night and was terminated 24 h later. Flowers then were collected in 68 % ethanol and stored until dissection. Flowers were stained with 3 μl brilliant green before dissection, and this facilitated the counting of eggs in the floral ovaries.

The propensity to oviposit was analyzed in a logistic regression model with eggs or no eggs as the binomially distributed response variable, with logit as link function, and with the moth and plant origin and their interaction as categorical factors in R 2.13.2 (R Development Core Team). Among the females that chose to lay eggs, the number of eggs laid was used as a continuously distributed response variable, in a linear model with the moth and plant origin and their interaction as categorical predictors.

Results

Floral Scent Experiment 1–20 °C days, 10 °C nights The species differed strongly in the major biochemical pathways and compounds that dominated floral scent. *Lithophragma parviflorum* scent was dominated by monoterpenes and some benzenoid esters, whereas *L. cymbalaria* emitted a different blend of compounds, with dimethyl salicylate and methyl salicylate together composing half of the scent emission (see Table S1 in OR2 for a full list of compounds detected across species and treatments). Other significant compounds in *L. cymbalaria* included the nitrogenous aromatics 2-aminobenzaldehyde and indole, and the monoterpene linalool (Table S1 in OR2). Scent emission of *L. bolanderi* was similar to its close relative *L. cymbalaria*, but also included unique compounds in lower amounts, such as the phenylpropanoids cinnamyl alcohol and cinnamaldehyde, and larger concentrations of some compounds, such as the nitrogenous aromatics indole and methyl anthranilate. One *L. bolanderi* individual tested at 10 °C was dominated by the otherwise rare benzenoid ether 1,4-dimethoxybenzene both during daytime and nighttime (Table S1 in OR 2). Removing this outlier individual had no effect on the analysis of SEM or on the number of compounds emitted.

Daytime scent composition of plants exposed to natural temperature variation (day 20 °C, night 10 °C) differed significantly among the three plant species (ANOSIM: Species R=0.938; P<0.01, all contrasts significant at the P<0.01 level) (Fig. 1a). The average similarity within species (SIMPER) varied from 67 % (*L. cymbalaria*) and 72 % (*L. bolanderi*) to 78 % (*L. parviflorum*). Similarity between species varied from 4 % (*L. parviflorum-L. bolanderi*) and 5 % (*L. parviflorum-L cymbalaria*) to 54 % (*L. bolanderi-L. cymbalaria*). A separate analysis including only *L. bolanderi*

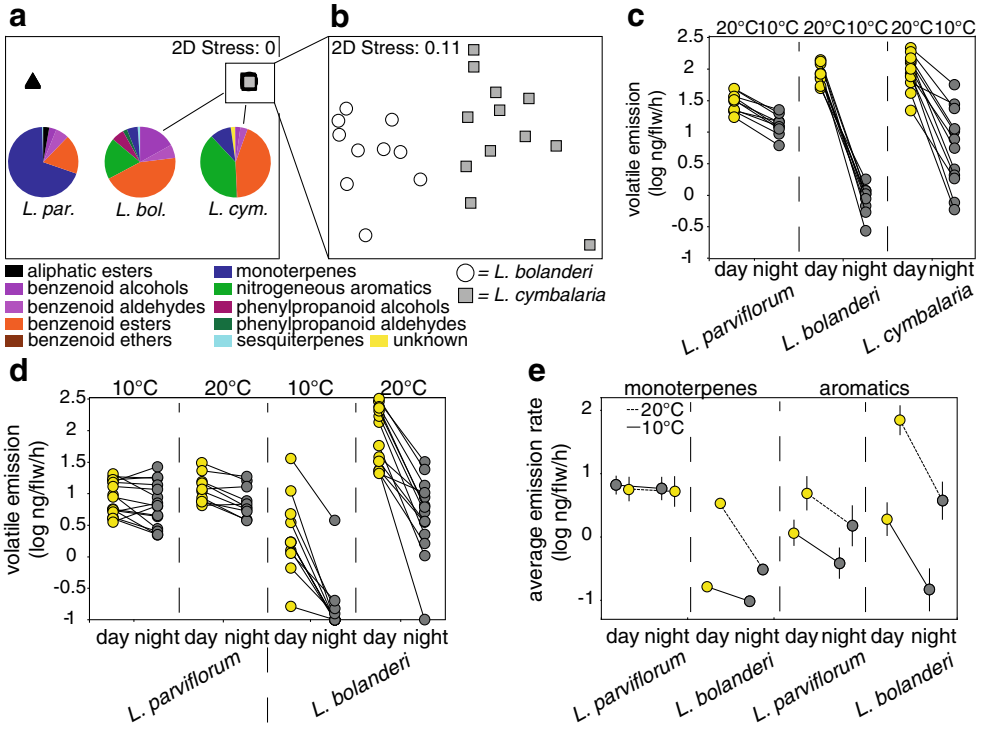

Fig. 1 Floral scent variation presented as **a** a multidimensional scaling (MDS) plot of the three study species (*Lithophragma parviflorum*, *L. bolanderi,* and *L. cymbalaria*) embedded with pie charts showing the average floral scent emission of each species at the compound group level under daytime conditions (20 °C); **b** a MDS plot comparing only the scent distributions of *L. bolanderi* (open circles) and *L. cymbalaria* (grey squares) (daytime, 20 °C); **c** the total emission rate (SEM, ng scent/flower/h) of each individual from the three study species at day (20 °C; yellow circles) and at night (10 °C; dark grey circles); **d** the SEM of *L. bolanderi* and *L. parviflorum* individuals at day and at night in constant temperatures (10 °C/20 °C); and **e**, the average total scent emission (mean SEM ±95 % confidence intervals) of monoterpenes and aromatic compounds of *L. bolanderi* and *L. parviflorum* at day and at night in constant temperatures (10 °C=*whole line*, 20 °C=*dashed line*)

and *L. cymbalaria* showed no overlap in multivariate scent distributions among these species (ANOSIM: R=0.763; P<0.01; Fig. 1b).

All three species were most fragrant during the day (Fig. 1c), but the magnitude of the daily variation in floral scent varied among species (Table 1). Whereas *L. bolanderi* emitted on average 121 times as much scent in the day than at night, *L. cymbalaria* emitted 29 times more scent at daytime, and the daytime signaling of *L. parviflorum* was only 2.3 times greater than at night (Fig. 1c). The number of compounds emitted varied in a similar fashion between day and night; a significantly higher number of compounds were detected during day than at night in all species. The number of scent compounds that were emitted during day and night, however, was species-specific (Table 1; Fig. S2 in OR1).

Table 1 Multivariate analysis of variance (MANOVA) on daily patterns (day/night) of floral scent signaling in terms of the standardized emission rate, and the number of compounds emitted by *Lithophragma bolanderi*, *L. cymbalaria*, and *L. parviflorum* grown under varying temperatures (Day 20 °C, Night 10 °C)

	Standardized emission rate (SEM)					Number of compounds				
	SS	*df*	MS	*F*	*P*	SS	*df*	MS	*F*	*P*
Order (O)	0.11	1	0.11	0.703	0.41	5.06	1	5.06	0.880	0.36
Species (S)	2.15	2	1.08	7.0	0.004	10.9	2	5.45	0.947	0.40
Error	4.33	28	0.155			161.0	28	5.75		
Time of day (TD)	22.4	1	22.38	409.2	<0.001	1095.6	1	1095.6	353.2	<0.001
TD * O	0.35	1	0.35	6.3	0.018	2.25	1	2.25	0.725	0.40
TD * S	6.71	2	3.36	61.4	<0.001	48.3	2	24.2	7.79	0.002
error	1.53	28	0.055			86.9	28	3.10		

The total SEM varied slightly with the order in which the plants were measured. This effect was detected only at night, so that individuals that were first measured at daytime tended to emit less scent at night than the individuals that were first measured at night (Table 1). There were no detectable order effects in the number of compounds emitted.

Floral Scent Experiment 2—L. bolanderi and L. parviflorum at either 10 °C or 20° C The second experiment confirmed that both temperature and time of day affected the production of scent (SEM; Table 2; Fig. 1d) and the number of compounds emitted (Table 2). Collection order had a weak but significant effect and varied among species (Table 2). The number of compounds was determined in part by a significant three-way interaction between species, temperature and time of day (Table 2; Fig. S2 in OR1). Thus, the two species in this experiment, *L. bolanderi* and *L. parviflorum*, differed in their responses to day and night conditions at different temperatures.

The variation between species was further partitioned into variation in the response to temperature and light condition (day/night) between individual compounds and the pathways that generate these compounds. *Lithophragma bolanderi* was dominated by aromatic compounds (Table S1), and consistently emitted only one monoterpene (linalool). In *L. bolanderi*, linalool was synergistically affected by temperature and light condition, and was emitted to the greatest extent during the day in the warm treatment (Table 3, Fig. 1e). By contrast, the emission rates of the monoterpenes (e.g., α-pinene, β-pinene, sabinene, and limonene) that constituted the majority of the floral scent bouquet of *L. parviflorum* were unaffected by both light and temperature, although slightly larger numbers of monoterpenes compounds were detected during day than at night, and at 20 °C than at 10 °C (Table 4, Fig. 1e, Fig. S2 in OR1). Aromatic compounds generally were emitted in higher numbers and in larger amounts under warm daytime conditions than under cold nights in both species (Table 3, 4, Fig. 1e, Fig. S2 in OR1).

Reflectance Scans Unlike floral scent, the floral reflectance pattern was largely similar across the three species. The largest variation was present in the violet spectrum (λ=381–450 nm), where *L. bolanderi* reflected the most, *L. cymbalaria* the least, and *L. parviflorum* showed intermediate reflectance (Fig. 2a). This difference was manifested in a significant interaction term (MANOVA: *species* $F_{2,33}=693.2$, $P=0.026$; *subspectrum* $F_{7,231}=3284.2$, $P<0.001$; *species* * *subspectrum* $F_{14,231}=19.2$, $P<0.001$). The three species also formed three slightly overlapping clusters in the PC-analysis (Fig. 2b) divided primarily along the second principal component axis (ANOVA: PC1: $F_{2,33}=3.16$, $P=0.056$; PC2: $F_{2,33}=90.23$, $P<0.001$), which was affected by variation in the violet and ultraviolet wavelengths.

Olfactometer Experiment Moths from all populations were most likely to enter the floral terminal when exposed to the scent of their local host plant. This was indicated by a significant interaction effect between the moth and the plant origin (logistic regression: *moth origin* $\chi^2_2=2.29$, $P=0.32$; *plant origin* $\chi^2_2=1.74$, $P=0.42$; *moth origin*plant origin* $\chi^2_4=15.9$, $P=0.003$) (Fig. 3).

Egg-laying Experiment Female moths that were presented to flowers of the local host species were significantly more likely to oviposit (and pollinate the flower) than moths that were enclosed with plants from other species (Logistic regression:

Table 2 Multivariate analysis of variance (MANOVA) on daily patterns (day/night) of floral scent signaling in terms of the standardized emission rate, and the number of compounds emitted by *Lithophragma bolanderi* and *L. parviflorum* grown under constant temperatures (Day/Night 20 °C, Day/Night 10 °C)

	Standardized emission rate (SEM)					Number of compounds				
	SS	*df*	MS	*F*	*P*	SS	*df*	MS	*F*	*P*
Order (O)	2.060	1	2.060	7.49	0.009	40.7	1	40.7	7.17	0.010
Species (S)	3.14	1	3.14	11.42	0.002	193.3	1	193.3	34.0	<0.001
Temperature (T)	14.1	1	14.1	51.3	<0.001	634.1	1	634.1	111.5	<0.001
S*T	13.2	1	13.2	48.0	<0.001	262.4	1	262.4	46.1	<0.001
Error	12.4	45	0.275			255.9	45	5.7		
Time of day (TD)	10.5	1	10.5	138.8	<0.001	311.9	1	311.9	69.1	<0.001
TD * O	0.061	1	0.061	0.800	0.38	7.10	1	7.10	1.574	0.22
TD * S	6.67	1	6.67	88.1	<0.001	92.0	1	92.0	20.4	<0.001
TD * T	0.061	1	0.061	0.802	0.38	87.3	1	87.3	19.3	<0.001
TD x S x T	0.003	1	0.003	0.041	0.84	29.8	1	29.8	6.6	0.014
Error	3.41	45	0.076			203.0	45	4.51		

Table 3 Multivariate analysis of variance (MANOVA) on daily patterns (day/night) of floral scent signaling of (a) monoterpene compounds and (b) aromatic compounds in terms of the standardized emission rate, and the number of compounds emitted by *Lithophragma bolanderi* grown under constant temperatures (Day/Night 20 °C, Day/Night 10 °C)

	Standardized emission rate (sem)					Number of compounds				
	Ss	*Df*	Ms	*F*	*P*	Ss	*Df*	Ms	*F*	*P*
a) Monoterpenes										
Order (O)	2.28	1	2.28	8.29	0.009	1.47	1	1.47	7.45	0.012
Temperature (T)	10.1	1	10.1	36.8	<0.001	5.12	1	5.12	26.0	<0.001
Error	6.04	22	0.275			4.33	22	0.197		
Time of day (TD)	4.97	1	4.97	141.4	<0.001	3.22	1	3.22	17.4	<0.001
TD * O	0.059	1	0.059	1.67	0.21	0.006	1	0.006	0.032	0.86
TD * T	1.98	1	1.98	56.4	<0.001	0.043	1	0.043	0.235	0.63
Error	0.773	22	0.035			4.07	22	0.185		
b) aromatics										
Order (O)	4.32	1	4.32	13.1	0.002	60.5	1	60.5	12.6	0.002
Temperature (T)	27.2	1	27.2	82.3	<0.001	675.4	1	675.4	141.0	<0.001
Error	7.26	22	0.330			105.4	22	4.79		
Time of day (TD)	17.5	1	17.5	147.1	<0.001	287.2	1	287.2	126.0	<0.001
TD * O	0.218	1	0.218	1.83	0.19	11.4	1	11.4	5.00	0.036
TD * T	0.073	1	0.073	0.614	0.44	92.2	1	92.2	40.4	<0.001
Error	2.62	22	0.119			50.1	22	2.28		

Moth origin χ^2_2=2.73, *P*=0.26; *Plant origin* χ^2_2=9.52, *P*=0.009; *Moth origin*Plant origin* χ^2_4=47.8, *P*<0.001) (Fig. 4b). The females that did oviposit tended to differ in the degree of local specialization in terms of number of eggs laid per bout. However, this pattern could only be reciprocally tested with plants and moths from the Sedgwick and Marble Falls sites (Linear model: *Moth origin* $F_{1,41}$=1.30, *P*=0.26; *Plant origin* $F_{1,41}$=2.38, *P*=0.13; *Moth origin*Plant origin* $F_{1,41}$=6.85, *P*=0.012) due to the low overall propensity of female moths to oviposit in non-local plants (Fig. 4). Whereas the number of eggs laid by the females from Sedgwick did not differ between plants of local and non-local origin, moths

Table 4 Multivariate analysis of variance (MANOVA) on daily patterns (day/night) of floral scent signaling of (a) monoterpene compounds and (b) aromatic compounds in terms of the standardized emission rate, and the number of compounds emitted by *Lithophragma parviflorum* grown under constant temperatures (Day/Night 20 °C, Day/Night 10 °C)

	Standardized emission rate (SEM)					Number Of Compounds				
	SS	*df*	MS	*F*	*P*	SS	*df*	MS	*F*	*P*
a) Monoterpenes										
Order (O)	0.006	1	0.006	0.039	0.84	1.50	1	1.50	1.28	0.27
Temperature (T)	0.033	1	0.033	0.210	0.65	8.03	1	8.03	6.86	0.016
Error	3.45	22	0.157			25.8	22	1.17		
Time of day (TD)	0.023	1	0.023	1.08	0.31	8.17	1	8.17	13.1	0.002
TD * O	0.000	1	0.000	0.015	0.90	0.167	1	0.167	0.267	0.61
TD * T	0.001	1	0.001	0.034	0.86	0.250	1	0.250	0.400	0.53
Error	0.457	22	0.021			13.8	22	0.625		
b) Aromatics										
Order (O)	0.023	1	0.023	0.099	0.76	1.60	1	1.60	2.43	0.13
Temperature (T)	4.12	1	4.12	17.6	<0.001	15.5	1	15.5	23.4	<0.001
Error	5.16	22	0.235			14.5	22	0.660		
Time of day (TD)	2.65	1	2.65	25.9	<0.001	16.3	1	16.3	27.4	<0.001
TD * O	0.119	1	0.119	1.16	0.29	1.40	1	1.40	2.35	0.14
TD * T	0.003	1	0.003	0.032	0.86	0.871	1	0.871	1.46	0.24
Error	2.25	22	0.102			13.1	22	0.596		

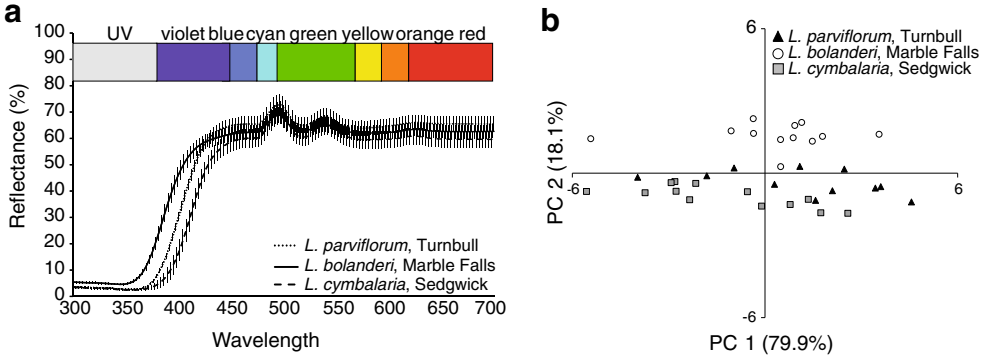

Fig. 2 Results from the spectral analysis presented as **a** the reflectance of petals from *Lithophragma bolanderi*, *L. cymbalaria,* and *L. parviflorum* across the wavelengths 300–700 nm (±95 % confidence intervals). Peaks near 490 and 540 nm are due to higher sensitivity of the sensor at these wavelengths and are not biologically relevant. The variation in visual signaling variation is also presented as **b** the principal component variation in spectral reflectance measured on the adaxial surface of petals (principal component 2 plotted against principal component 1). Percentages in parentheses show the relative contribution of each principal component for the total variation in the data

from Marble Falls laid more eggs in their local *L. bolanderi* host plants than in *L. cymbalaria* plants from Sedgwick (Fig. S3 in OR1).

Discussion

Taken together, the consistent and highly divergent floral scent differences among the *Lithophragma* species, the tailoring of the scent signal towards warm daytime conditions when the moths are active, the propensity of female *Greya* moths to orient toward the scent of local host plants, and their reluctance to oviposit in flowers of non-local plants, support the hypothesis that floral scent is a key trait shaping patterns of interaction specificity among *Lithophragma* plants and their specialized *Greya* pollinators. By comparison, the species differences in floral color were trivial. Despite their relatively close phylogenetic relationship, *L. bolanderi* and *L. cymbalaria* were most dissimilar in terms of floral color (Fig. 2). However, this dissimilarity did not seem to affect moth behavior in our experiments. Moths from each of the three populations preferred to oviposit in their local host species first, the most chemically similar non-local host second, and the least chemically similar non-local host last, regardless of the contrasting patterns of chemical and spectral similarity. This implies that similarity in scent outweighs the small spectral dissimilarity as cues for local female host preference.

Greya moths pollinate a host flower during egg-laying, and both organisms depend on the moth's ability to detect and evaluate appropriate hosts. At the same time, the floral scent evolution must fit into plant life histories in ways that minimize potential metabolic and ecological costs involved in the scent production (e.g., Gershenzon 1994; Raguso 2008; Wright and Schiestl 2009). Therefore, floral scent emission is expected to peak in association with the peak activity periods of their pollinators (Hoballah et al. 2005; Matile and Altenburger 1988; Raguso et al. 2003). In *Lithophragma,* which often depend nearly exclusively on the day-flying

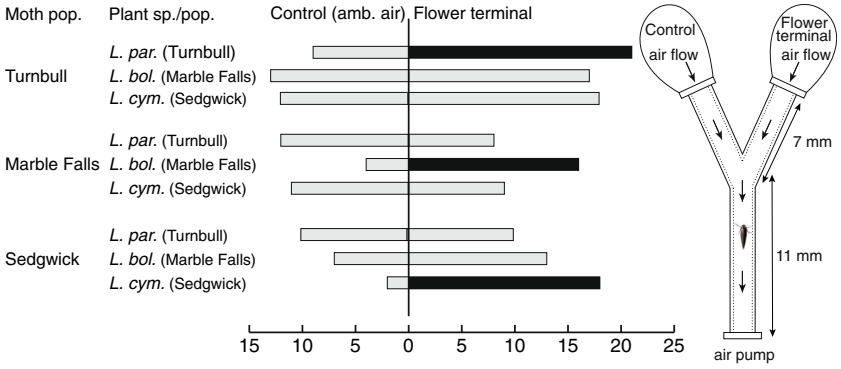

Fig. 3 The number of *Greya politella* moths from each study population that entered the Y-tube terminal with flowers rather than the terminal with ambient air. The experiments tested *Lithophragma parviflorum* from Turnbull (*L. par.*), *L. bolanderi* from Marble Falls (*L. bol.*), or *L. cymbalaria* (*L. cym.*) from Sedgwick and their associated moth populations. Moths that directed their movement towards the local plant species are indicated by black bars. Also shown is the experimental setup

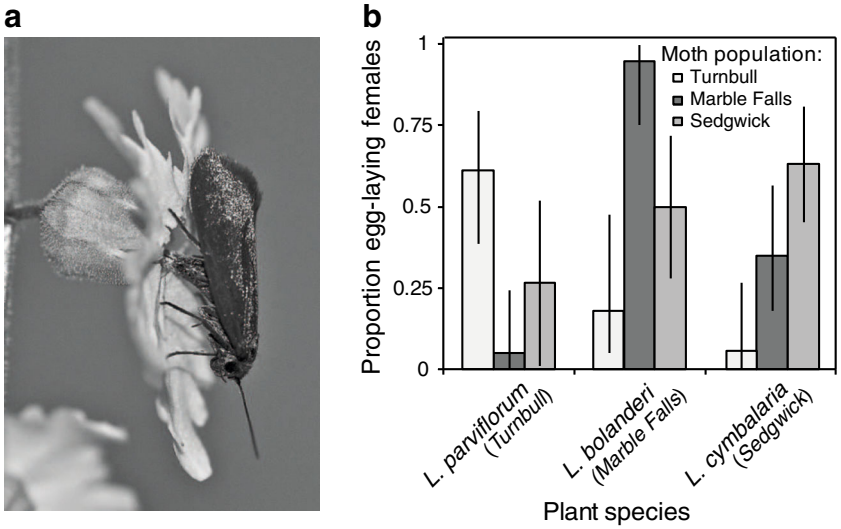

Fig 4 **a** a female *Greya politella* from the Marble Falls population in Sequoia National Park, California ovipositing into a flower of a *Lithophragma bolanderi* from the local host plant population. Note the pollen adhering to the abdomen tip. Photo: John N. Thompson. **b** The proportion of surviving *Greya politella* females that oviposited into the floral ovaries of local and non-local plants in no-choice trials. Also shown are the 95 % confidence intervals, generated with the command 'binom.test' in R 2.13.1 (R Development Core Team)

Greya moths, scent production was markedly higher in the daytime samples than in the nighttime collections.

The daytime floral scent bouquets among *L. parviflorum* and *L. cymbalaria* were similar to those reported by Friberg et al. (2013). This reinforces the observation that floral scent varies little within populations, but has diverged among species. Whereas the floral scent variation between species and subspecies of *Lithophragma* is largely genetically determined (Friberg et al. 2013), the population-variation in moth preferences could be due to site-specific adaptations or olfactory imprinting (see e.g., Arenas et al. 2009; Wright and Schiestl 2009). Any imprinting, however, would occur soon after eclosion, because the intimate association with the *Lithophragma* hosts keeps the moths continually bathed in host volatiles from the moment of egg deposition through the stages of larval and pupal development and eclosion. Adults rest on the flowers, and mating occurs on the hosts. The local preferences in the no-choice trials further indicate that even if scent preference is imprinted, it is a trait that is not easily reversed later on in the female moth's life.

Lithophragma bolanderi emitted more than 120 times as much scent in daytime conditions than at night, whereas *L. parviflorum* emitted only twice as much scent during the day than at night. A proximate explanation to these species-specific reaction norms is suggested by the finding that different compounds and compound groups had different sensitivity to the environmental cues. Whereas the monoterpenes that dominated the *L. parviflorum* scent bouquet were similar across treatments, the aromatic compounds of both *L. parviflorum* and *L. bolanderi* were all sensitive to both light and temperature cues. The larger proportional contribution of aromatic compounds to the scent bouquet of *L. bolanderi* can thus explain the larger variation between day and night in the scent signal of this species. However, not all monoterpenes were unaffected by environmental cues. Linalool, the only monoterpenoid consistently emitted by *L. bolanderi*, was synergistically affected by light and temperature, and was disproportionally emitted under daytime conditions.

The impact of temperature on floral scent emissions is by no means universal. In the perennial herb *Hesperis matronalis* (Brassicaceae), the effects of temperature on floral scent production is nearly opposite to the patterns observed for the *Lithophragma* species studied here, with aromatics being less affected by low temperature than monoterpenes (Nielsen et al. 1995). Thus, the variation in reaction norms between different compounds also could be dynamic and species-specific, and thereby reflect different ecological functions or evolutionary histories for different classes of volatiles (e.g., attractants *vs.* repellents) in each local environment (Junker and Blüthgen 2010; Theis et al. 2007). Under this hypothesis, volatile compounds with emission rates most strongly tailored towards the daytime may be especially important for interaction with *Greya* moths. Recent studies highlight the repellent functions of some floral volatiles (Junker and Blüthgen 2013; Kessler et al. 2013), suggesting that volatiles with more constant emission rates across days and nights might have functions other than pollinator attraction. Hence, it is possible that the variation in scent composition and species-specific day *vs.* night emission patterns in the three *Lithophragma* species studied here could reflect selective forces other than the specialist *Greya* moths and the sometimes significant impact of generalist pollinators (see Thompson and Cunningham 2002). However, this question will require additional field studies, whereas our present study was focused entirely on the primary question of whether *Lithophragma* floral scent attracts *Greya* moth pollinators. It is unlikely, though, that herbivores other than floral visitors contribute to the observed patterns. No specialist herbivores other than *Greya* larvae have ever been collected from *Lithophragma*, and any herbivory beyond that

caused by *Greya* larvae is rare in every population that has been studied throughout the distribution of the genus (JNT unpublished data).

At least two hypotheses could explain the underlying processes driving scent divergence in *Lithophragma* volatile attractants. One is that rare co-pollinators, which differ in composition and importance among *Lithophragma* populations (Thompson and Cunningham 2002), may contribute to divergence in floral scents (Friberg et al. 2013). Scent composition then would be a compromise between the compounds that attract the main *Greya* moth pollinators and these other pollinators. Another possibility is that the background composition of volatiles differs among habitats, favoring plants with scents that allow the moths to distinguish their host plants from the background volatiles. Such interactive effects of generalized and specific selection agents could generate a dynamic evolutionary landscape, simultaneously resulting in local specificity and population- and species divergence in this interaction as it has coevolved across environments. Trait divergence then would be reinforced by selection acting against plant immigrants that emit floral scents that do not attract the local pollinators, and against moth immigrants that are not attracted to the host plants of the novel environment. Future studies are warranted to determine whether local specialization in host signaling traits could have such a potential to restrict gene flow among different populations on both sides of a coevolutionary interaction.

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