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Phytochemistry 63 (2003) 265–284

PHYTOCHEMISTRY

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Fragrance chemistry, nocturnal rhythms and pollination "syndromes" in *Nicotiana*

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Received 18 December 2002; received in revised form 5 February 2003

Abstract

GC–MS analyses of nocturnal and diurnal floral volatiles from nine tobacco species (*Nicotiana*; Solanaceae) resulted in the identification of 125 volatiles, including mono- and sesquiterpenoids, benzenoid and aliphatic alcohols, aldehydes and esters. Fragrance chemistry was species-specific during nocturnal emissions, whereas odors emitted diurnally were less distinct. All species emitted greater amounts of fragrance at night, regardless of pollinator affinity. However, these species differed markedly in odor complexity and emission rates, even among close relatives. Species-specific differences in emission rates per flower and per unit fresh or dry flower mass were significantly correlated; fragrance differences between species were not greatly affected by different forms of standardization. Flowers of hawkmoth-pollinated species emitted nitrogenous aldoximes and benzenoid esters on nocturnal rhythms. Four *Nicotiana* species in section *Alatae* sensu strictu have flowers that emit large amounts of 1,8 cineole, with smaller amounts of monoterpene hydrocarbons and α-terpineol on a nocturnal rhythm. This pattern suggests the activity of a single biosynthetic enzyme (1,8 cineole synthase) with major and minor products; however, several terpene synthase enzymes could contribute to total monoterpene emissions. Our analyses, combined with other studies of tobacco volatiles, suggest that phenotypic fragrance variation in *Nicotiana* is shaped by pollinator- and herbivore-mediated selection, biosynthetic pathway dynamics and shared evolutionary history.
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Keywords: *Alatae*; Solanaceae; Hawkmoths; Floral scent; Headspace analysis; SPME; Aldoximes

1. Introduction

Plant reproductive biologists have long asserted that non-random combinations of flower color, shape and fragrance have evolved in response to directional selection by specific classes of effective animal pollinators (Kerner von Marilaum, 1895; Faegri and van der Pijl, 1979). However, recent studies have questioned the predictive value and ecological accuracy of traditional "pollination syndromes," suggesting that this concept oversimplifies plant-pollinator interactions and underestimates spatial and temporal variation in reproductive strategy across a given plant's distribution (Herrera, 1996; Ollerton, 1996; Waser et al., 1996). These criticisms are particularly valid when plant-pollinator studies lack a phylogenetic context, because the floral traits and/or mating system of a focal species may be heavily influenced or constrained by its evolutionary history (McDade, 1992; Armbruster, 1997). For example, the night-blooming, trumpet shaped, fragrant flowers of *Datura stramonium* (Solanaceae) are quintessential "sphingophilous" (hawkmoth-pollinated) flowers (Baker, 1961; Grant, 1983), and floral morphology leads to an accurate prediction of hawkmoth visitation. However, the self-compatible flowers of *D. stramonium* are relatively small when compared with those of more subtropical *Datura* species (Grant and Grant, 1983a,b; Cavazos et

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0031-9422/03/$ - see front matter © 2003 Elsevier Science Ltd. All rights reserved.
doi:10.1016/S0031-9422(03)00113-4

al., 2000). Manipulative field studies revealed that self-pollination and unpredicted visits by honey bees greatly reduce the importance of hawkmoths as pollinators of *D. stramonium* (Motten and Antonovics, 1992; Motten and Stone, 2000). In this light, the floral morphology and nocturnal anthesis of *D. stramonium* more accurately describe its evolutionary history than its current reproductive biology.

Despite such caveats, plants that rely exclusively (or nearly so) on hawkmoths for pollen transfer have evolved repeatedly worldwide (Silberbauer-Gottsberger and Gottsberger, 1975; Nilsson et al., 1987). Their bright white visual display, sucrose-rich nectars and strong, agreeable fragrances present a compelling case for convergent evolution (Haber and Frankie, 1989; Thompson, 1994). Like any sexual signal, floral fragrances and visual displays may also attract predators (see Ryan, 1990). As a result, there is a potential for balancing selection on floral signals between pollinators and herbivores (Baldwin et al., 1997). Different hawkmoth species have been shown to select for flower number (Schemske, 1980), pale coloration (Ippolito, 2000) corolla size, shape and height (Herrera, 1993; Mothershead and Marquis, 2000), and nectar spur/tube length (Nilsson, 1988; Alexandersson and Johnson, 2001). However, the degree to which hawkmoth behavior might select upon variation in fragrance chemistry has not been directly addressed. One major impediment is the inherent complexity of floral scent. It is difficult to determine a priori which fragrance components to study, given that hawkmoth-pollinated orchids, cacti and Nyctaginaceae, for example, typically emit up to 100 different volatiles (Kaiser, 1993; Kaiser and Tollsten, 1995; Levin et al., 2001). A combination of behavioral, phylogenetic and physiological analyses might eventually dissect complex fragrance blends into signal (pollinator attractants) and noise (historical and biosynthetic artifacts) (Raguso, 2001; Schiestl and Marion-Poll, 2002). This paper represents a first step towards such a goal.

We have initiated a multidisciplinary analysis of hawkmoth-pollinated plants in order to understand how their floral traits, especially scent, have evolved in three distantly related angiosperm families. Further, we are interested in determining how such shifts might be constrained by evolutionary history. Phase I of our study provided evidence for convergent evolution of potential "signal" compounds (indole and sesquiterpene alcohols) among hawkmoth-pollinated four o'clock plants (Nyctaginaceae), but also revealed significant phylogenetic patterns embedded in fragrance chemistry (Levin et al., 2001; in revision). Specifically, lactones and eugenol-related compounds were shared-derived traits in one lineage of *Acleisanthes* (Nyctaginaceae). However, the fact that nearly all species in this genus are pollinated by the same guild of hawkmoths (Spellenberg and Delson, 1977; Levin et al., 2001) suggests that lactones and eugenol-related compounds may not be essential for hawkmoth pollination, and may instead reflect a recent common ancestry. Interestingly, fragrance in plants of the genus *Mirabilis* showed much lower biosynthetic diversity than in the closely related *Acleisanthes*, presumably due to ancestral losses of physiological function. Nevertheless, the fragrances of *Mirabilis jalapa, M. longiflora* and *M. multiflora* are sufficient to attract several hawkmoth species in natural populations (Grant and Grant, 1983b; Martinez del Río and Búrquez, 1986; Hodges, 1995).

Our studies of floral scent chemistry in Nyctaginaceae further demonstrated significant variation between species; fragrance blends were species-specific. Certainly, some of this variation may be generated by neutral genetic drift (Ackerman et al., 1997). However, recent studies with *Manduca sexta* have revealed odor-based associative learning on par with honeybees and noctuid moths (Daly and Smith, 2000), suggesting that some hawkmoths can distinguish between flowers as alternative nectar sources by learning their specific fragrance blends. Thus, fragrance should play several distinct roles in hawkmoth-flower interactions: attraction to flowers from a distance (Raguso and Willis, 2003), elicitation of feeding behavior (in combination with visual cues; Raguso and Willis, 2002) and learned reinforcement of floral constancy through odor discrimination (Daly et al., 2001).

In this paper we report phase II of our study, in which we characterize fragrance chemistry in nine species of wild tobacco (*Nicotiana*; Solanaceae). The genus *Nicotiana* comprises more than 70 species and is primarily South American in distribution, with distinct lineages occurring in southern North America, Australia and some South Pacific islands (Goodspeed, 1954; Aoki and Ito, 2000). Previous studies have investigated circadian rhythms in fragrance emission from several night-blooming, putatively hawkmoth-pollinated tobaccos, including *N. sylvestris* (Loughrin et al., 1990a, 1991), *N. suaveolens* (Loughrin et al., 1992, 1993; Dudareva et al., 1999; Kolosova et al., 2001) and *N. attenuata* (Euler and Baldwin, 1996; Baldwin et al., 1997). Some of our study species (Table 1) were from the South American section *Alatae*, a putatively monophyletic group noteworthy for its diversity of floral morphology, blooming phenology and mating systems (Goodspeed, 1954; Ippolito, 2000). Studies using chromosome number, DNA sequence data from the nuclear ribosomal spacers (Buckler et al., 1997) and the chloroplast matK gene (Aoki and Ito, 2000) suggest that section *Alatae s.s.* comprises five self-incompatible, inter-fertile species. These include hawkmoth-pollinated *N. alata*, hummingbird-pollinated *N. forgetiana, N. langsdorffii* and *N. mutabilis* (not studied here), and small moth/bee pollinated *N. bonariensis* (Ippolito,

Table 1
Floral biology and taxonomic affiliation of *Nicotiana* species included in this study

Species	Section	Flower color, depth	Pollinators, evidence
N. rustica	*Rusticae*	white/green, 17.3±0.3	Moth/bee?, morphology
N. suaveolens	*Suaveolentes*	white, 42.5±0.5	HM?, morphology
N. sylvestris	nr. *Alatae* s.l.	white, 96.5±0.6	HM, self?, Cocucci, 1988
N. longiflora	*Alatae* s.l.	white, 112.8±1.3	HM, Cocucci, 1988
N. plumbaginifolia	*Alatae* s.l.	white, 35.9±0.3	HM, self, Cocucci, 1988
N. langsdorffii	*Alatae* s.s.	green, red, 22.1±0.3	HB, Ippolito, 2000
N. bonariensis	*Alatae* s.s.	white, 15.8±0.2	Moth/bee?, Ippolito, 2000
N. forgetiana	*Alatae* s.s.	red, 38.4±0.2	HB, Ippolito, 2000
N. alata	*Alatae* s.s.	white, 80.5±1.0	HM; Ippolito, 2000

Measurements of flower depth are means±S.E. in mm, N=15–20 flowers per species. HM=hawkmoths, HB=hummingbirds.

2000). The sister group to this lineage is a pair of closely related species sometimes included in *Alatae*, *N. longiflora* and *N. plumbaginifolia*. Both species are self-compatible and are visited by hawkmoths; the former is facultatively out-crossed by the moths whereas the latter, like *D. stramonium*, has much smaller flowers that often self-pollinate before opening (Cocucci, 1988; Ippolito, 2000). For outgroup comparision, we included three *Nicotiana* species with different degrees of relatedness to section *Alatae*. Goodspeed (1954) placed the South American *N. sylvestris* within section *Alatae*, but subsequent studies do not strongly support this hypothesis (Aoki and Ito, 2000). Ippolito (2000) concluded that the Australian section *Suaveolentes* (represented in our study by *N. suaveolens*) is most closely related to *Alatae*. Both *N. sylvestris* and *N. suaveolens* exhibit floral traits putatively associated with hawkmoth pollination (Grant, 1983). For contrast, we included *N. rustica*, a more distantly related South American species with racemes of small whitish-green, self-compatible flowers (Goodspeed, 1954).

We collected fragrance from living, intact flowers and evaluated the scent profiles of species in the context of related species. In order to identify the sources of specific volatiles, we contrasted headspace samples from flowers with those from vegetative parts. For species of section *Alatae*, we also contrasted fragrance from whole flowers with odors from dissected floral organs. Further, we collected headspace volatiles during day and night, to evaluate whether the rhythm of scent emission might also change with evolutionary shifts in pollinator class, i.e. species with diurnal pollinators should emit odors on a diurnal rhythm (or not at all). We predicted that hawkmoth-pollinated species should share nitrogenous compounds, benzenoid esters and/or terpenoid alcohols, and that fragrance should be lost or greatly attenuated in hummingbird-pollinated taxa. Lastly, we predicted that interspecific fragrance variation always would exceed intraspecific variation, and we sought to determine whether components of fragrance chemistry have phylogenetic signal and are more reflective of shared ancestry than mode of pollination.

2. Results and discussion

We identified 125 volatile compounds from floral and vegetative organs of nine *Nicotiana* species (Table 2). Nearly half of these compounds are isoprene derivatives, including 23 monoterpenoids (hydrocarbons and alcohols) and 27 sesquiterpenoids with diverse hydrocarbon skeletons. Sixteen nitrogen (N)-bearing volatiles are present in our study species, including benzenoid shikimate-pathway products (indole, phenyl acetonitrile) the valine-, leucine- and isoleucine-derived aldoximes, nitriles and nitro-compounds (Kaiser, 1993), nicotine and methyl nicotinate (Table 2). The remaining compounds comprise 27 shikimate-derived benzenoid alcohols, aldehydes and esters (Dudareva and Pichersky, 2000) and 26 derivatives of fatty-acid catabolism, with several products of the lipoxygenase cascade (Croft et al., 1993). The number and biosynthetic complexity of scent compounds varied markedly among species (mean±S.E.=31±5 compounds), ranging from *N. langsdorffii*, whose 14 volatiles are nearly exclusively monoterpenoids, to *N. alata,* whose 69 compound blend includes products from all biosynthetic pathways identified in this study (Table 2). These data may be compared with other fragrance surveys of plant genera in which hawkmoth-pollination is prevalent. *Nicotiana* odors are more complex than those of *Narcissus* (Amaryllidaceae) (19±3 compounds, Dobson et al., 1997) and *Mirabilis* (Nyctaginaceae) (24±4, Levin et al., 2001) but less complex than those of *Acleisanthes* (Nyctaginaceae) (53±6, Levin et al., 2001) and *Oenothera* Sect. *Pachylophus* (Onagraceae) (65±9, R.A. Raguso, unpubl. data).

In general, tobacco vegetation is glandular and scented (Goodspeed, 1954; Sasaki et al., 1984; Andersen et al., 1986). Monoterpene hydrocarbons and nicotine were emitted only by vegetation in some species (e.g. *N. longiflora*). However, no volatiles were unique to leaf or calyx tissues across all species studied (Table 2). In an extreme case, 25 of 27 volatiles emitted by *N. plumbaginifolia* were common to flowers, stems and leaves. Nitrogenous aldoximes, benzenoid aldehydes and benzenoid

Table 2
Volatile compounds emitted by *Nicotiana* species

Compound	RT	# spp.	*N. rustica*		*N. suaveolens*		*N. sylvestris*		*N. longiflora*		*N. plumbaginifolia*	*N. langsdorffii*		*N. bonariensis*		*N. forgetiana*		*N. alata*	
			PM *n*=6	AM *n*=5	PM *n*=9	AM *n*=4	PM *n*=10	AM *n*=3	PM *n*=8	AM *n*=3	PM *n*=8	PM *n*=6	AM *n*=6	PM *n*=3	AM *n*=3	PM *n*=10	AM *n*=10	PM *n*=8	AM *n*=3
Total no. of compounds			22	22	41	25	19	17	28	22	27	14	14	23	11	32	20	69	49
No. shared by flowers and veg. tissues			14	14	6	6	6	6	11	10	25	1	1	4	4	3	3	4	4
No. only floral compounds			8	8	31	15	13	11	10	6	2	8	8	18	6	29	17	65	45
No. only veg. compounds			0	0	4	4	0	0	7	6	0	5	5	1	1	0	0	0	0
ng Scent/flower/h			3.46± 1.38	0.65± 0.24	2.28± 0.47	0.57± 0.16	0.42± 0.07	0.40± 0.14	0.93± 0.22	0.41± 0.05	0.48± 0.18	1.38 ±0.51	0.54± 0.19	1.33± 0.23	0.34± 0.05	5.25± 0.61	1.06± 0.18	81.02± 17.73	7.36± 0.45
ng Scent/fresh mass flowers/h			25.27± 9.84	4.78± 1.68	16.16± 3.78	4.08± 1.18	1.01± 0.18	0.96± 0.34	2.36± 0.56	1.04± 0.14	7.35± 2.68	15.81± 5.77	6.28± 2.13	22.91± 4.02	5.78± 0.84	29.31± 3.39	5.93± 0.98	111.75± 24.45	11.30± 1.34
ng scent/dry mass flowers/h			129.50± 51.59	24.20± 8.86	102.49± 21.51	26.93± 7.70	6.74± 1.15	6.57± 2.05	8.58± 2.03	3.77± 0.51	37.44± 17.63	74.27± 29.00	27.00± 9.38	57.77± 10.15	14.57± 2.12	125.52± 13.22	24.12± 3.92	659.67± 143.34	73.35± 11.58
*Monoterpenes (**9**)*																			
α-Pinene*	2.26	7			0.14± 0.08	0.85± 0.18			*1.29± 0.32*	*0.92± 0.82*	**8.63± 4.10**	1.55± 0.39	0.69± 0.64	0.51± 0.28	3.43± 0.89	1.37± 0.20	1.02± 0.55	0.36± 0.09	0.33± 0.01
Camphene*	2.72	2							*0.02± 0.02*	*0.75± 0.38*				0.30± 0.23	ND	0.57± 0.23	0.04± 0.02		
β-Pinene*	3.42	7			0.19± 0.05	0.81± 0.04			*1.34± 1.34*	*ND*	**3.44± 0.86**	3.93± 0.85	3.54± 0.66	**0.76± 0.25**	**3.26± 0.97**	2.86± 0.44	2.65± 0.16	1.12± 0.30	0.84± 0.13
Sabinene*	3.71	7			0.37± 0.09	2.12± 0.27			*0.02± 0.01*	*1.03± 0.19*	**2.82± 0.67**	8.79± 2.18	7.74± 2.14	1.46± 0.99	13.69± 1.93	6.99± 1.23	4.39± 1.33	**2.75± 0.83**	**1.86± 0.28**
β-Myrcene*	4.65	8			0.18± 0.06	1.46± 0.14	**1.05± 0.17**	**2.28± 0.47**	*0.37± 0.10*	*0.91± 0.15*	**1.22± 0.50**	6.75± 1.67	10.02± 2.76	**0.80± 0.67**	**6.16± 2.53**	**6.10± 1.10**	**3.91± 1.97**	**2.72± 0.75**	**2.02± 0.90**
Limonene*	5.35	7			**0.52± 0.09**	**2.58± 0.32**			*3.57± 0.70*	*2.69± 0.18*	**3.44± 0.40**	7.29± 1.64	7.11± 1.98	**1.77± 0.98**	**7.80± 1.23**	**6.99± 1.08**	**4.98± 1.19**	**2.46± 0.79**	**1.96± 0.44**
Z-β-Ocimene*	6.1	2														0.06± 0.02	ND	0.05± 0.01	0.52± 0.25
E-β-Ocimene*	6.42	7			**0.07± 0.01**	**2.17± 0.11**	**2.96± 0.67**	**6.89± 0.98**	*0.67± 0.10*	*3.99± 0.03*		*0.49± 0.11*	*0.47± 0.13*	*0.24± 0.11*	*2.99± 0.71*	0.54± 0.09	0.15± 0.05	0.21± 0.05	0.14± 0.06
α-Terpinolene*	6.97	1																0.11± 0.02	0.19± 0.19
*Oxygenated monoterpenoids (**14**)*																			
1,8-Cineole*	5.55	6			**3.87± 1.31**	**11.46± 2.29**			1.81± 0.64	ND		68.35± 16.97	62.44± 21.25	8.98± 7.26	56.4± 17.37	52.94± 11.41	77.96± 17.23	21.11± 5.02	12.56± 3.09
E-Furanoid linalool oxide*	9.47	1																0.32± 0.07	1.01± 1.01
111 (10), 93 (13), 81 (12), 71 (27), 55 (12), 43 (100), 41 (26)	9.79	1																0.11± 0.03	ND
Z-Furanoid linalool oxide*	9.87	1																0.60± 0.16	1.70± 0.05
Camphor*	10.27	1									**4.98± 1.35**								
Linalool*	10.95	6					5.33± 1.72	8.45± 1.46	2.17± 0.30	1.69± 0.96	**2.54± 0.56**	*0.78± 0.17*	*2.14± 0.28*	0.31± 0.16	ND			27.45± 8.02	10.00± 1.36

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Table 2 (*continued*)

Compound	RT	# spp.	*N. rustica*		*N. suaveolens*		*N. sylvestris*		*N. longiflora*		*N. plum-baginifolia*	*N. langsdorffii*		*N. bonariensis*		*N. forgetiana*		*N. alata*	
			PM *n* = 6	AM *n* = 5	PM *n* = 9	AM *n* = 4	PM *n* = 10	AM *n* = 3	PM *n* = 8	AM *n* = 3	PM *n* = 8	PM *n* = 6	AM *n* = 6	PM *n* = 3	AM *n* = 3	PM *n* = 10	AM *n* = 10	PM *n* = 8	AM *n* = 3
136 (11), 93 (25), 81 (43), 59 (100), 43 (64), 41 (43)	12.54	1																0.31 ± 0.10	0.07 ± 0.07
α-Terpineol*	12.75	5			0.10 ± 0.06	ND	3.53 ± 0.85	5.47 ± 1.85				*0.24 ± 0.05*	*0.27 ± 0.06*			0.89 ± 0.23	0.57 ± 0.18	3.23 ± 1.20	0.96 ± 0.26
E-Pyranoid linalool oxide*	13.25	1																0.04 ± 0.01	ND
Z-Pyranoid linalool oxide*	13.55	1																0.13 ± 0.04	ND
Nerol*	13.95	1																0.02 ± <0.01	0.20 ± 0.10
Geraniol*	14.43	2														0.25 ± 0.08	0.21 ± 0.18	0.05 ± 0.01	0.77 ± 0.06
109 (2), 85 (13), 82 (100), 71 (59), 67 (48), 55 (10), 43 (83), 41 (22)	15.42	1																0.05 ± 0.02	ND
109 (28), 82 (66), 71 (41), 67 (90), 55 (46), 43 (100), 41 (68)	17.24	1																0.03 ± 0.01	0.33 ± 0.21
*Irregular terpenoids (**6**)*																			
E-4,8-Dimethylnona-1,3,7-Triene**	7.46	1									**0.41 ± 0.24**								
1,3,3-Trimethyl-7-oxabicyclo[4.1.0] heptan-2,5-dione**	12.45	2	9.89 ± 7.81	5.66 ± 2.87										39.14 ± 18.29	4.51 ± 3.51				
2,6,6-Trimethy-2-cyclo hexene-1,4-dione (4-oxo-isophorone)**	12.73	2	0.96 ± 0.29	1.12 ± 0.29										6.79 ± 2.21	0.75 ± 0.75				
2,6,6-Trimethyl 1,4-cyclo-hexa-dione**	13.85	2												0.47 ± 0.15	ND				
E-Geranyl acetone*	14.61	2									**2.00 ± 1.25**							**0.08 ± 0.02**	ND
150 (28), 121 (20), 95 (22), 93 (26), 91 (44), 84 (32), 82 (30), 81 (80), 79 (48), 77(27), 69 (100), 53 (38), 46 (22), 41 (64)	15.55	1																0.01 ± <0.01	ND
*Sesquiterpenes (**17**)*																			
α-Zingiberene**	11.33	1																0.08 ± 0.05	1.45 ± 0.33
α-Cedrene*	11.53	2	**4.17 ± 2.91**	**10.20 ± 8.78**												**0.26 ± 0.08**	**0.82 ± 0.27**		
β-Caryophyllene*	11.63	6	**0.05 ± 0.05**	**2.79 ± 2.79**	**1.11 ± 0.23**	**11.80 ± 1.96**	**19.00 ± 4.71**	**48.40 ± 6.02**	**6.11 ± 0.46**	**14.89 ± 1.92**	**11.31 ± 4.10**							0.07 ± 0.01	0.73 ± 0.38
Sativene**	11.94	1	**0.86 ± 0.45**	**0.86 ± 0.55**															

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Table 2 (*continued*)

Compound	RT	# spp.	*N. rustica*		*N. suaveolens*		*N. sylvestris*		*N. longiflora*		*N. plumbaginifolia*	*N. langsdorffii*		*N. bonariensis*		*N. forgetiana*		*N. alata*	
			PM $n=6$	AM $n=5$	PM $n=9$	AM $n=4$	PM $n=10$	AM $n=3$	PM $n=8$	AM $n=3$	PM $n=8$	PM $n=6$	AM $n=6$	PM $n=3$	AM $n=3$	PM $n=10$	AM $n=10$	PM $n=8$	AM $n=3$
1,2,3,4,4a,7-Hexahydro-,6-dimethyl-4-(1-methylethyl)-naphthalene	12.05	1	**0.07± 0.05**	**0.53± 0.53**															
Z-β-Farnesene	12.13	1																0.12± 0.02	1.71± 0.66
189 (30), 162 (7), 133 (6), 121 (73), 119 (87), 107 (21), 105 (39), 93 (61), 91 (34), 81 (37), 79 (51), 77 (33), 72 (20), 68 (19), 67 (23), 59 (31), 55 (27), 53 (32), 43 (100), 41 (54)	12.46	5	**<0.01**	**0.19± 0.19**														0.07± 0.01	1.02± 0.44
E-β-Farnesene**	12.46	5			0.16± 0.03	0.09± 0.09			0.77± 0.49	ND		0.68± 0.21	2.23± 0.14			0.46± 0.18	1.41± 0.89	0.20± 0.09	1.08± 0.39
[M+ 204], 147 (6), 121 (47), 119 (100), 107 (22), 105 (43), 93 (57), 91 (44), 79 (41), 77 (25), 73 (20), 55 (20), 53 (27), 43 (64), 41 (44)	12.74	1	**0.05± 0.03**	**0.44± 0.44**															
α-Humulene*	12.91	5			**0.15± 0.09**	**1.15± 0.44**	**1.28± 0.52**	**1.81± 0.91**	**0.18± 0.05**	**0.82± 0.82**	**0.67± 0.61**							0.01± 0.01	ND
[M+ 204], 161 (16), 133 (20), 121 (19), 119 (17), 107 (28), 105 (100), 93 (30), 91 (34), 79 (23), 77 (20), 67 (18), 55 (17), 41 (35)	12.92	1	**2.33± 1.07**	**3.86± 2.47**															
Z,*E*-α-Farnesene**	13.17	1																0.05± 0.01	ND
204 (M+, 13), 189 (17), 175 (10), 161 (31), 147 (29), 133 (28), 128 (100), 119 (57), 121 (28), 107 (66), 105 (43), 95 (25), 94 (22), 93 (63), 91 (51), 81 (26), 79 (53), 67 (28), 55 (29), 53 (24), 51 (22), 41 (69)	13.34	1	**1.62± 0.29**	**2.52± 2.51**															
Valencene**	13.52	1	**0.04± 0.02**	**0.17± 0.17**															
E,*E*-αFarnesene*	13.53	2			0.14± 0.04	0.91± 0.54					**0.58± 0.46**								
β-Sesquiphellandrene**	13.57	1																0.04± 0.04	0.03± 0.03
204 (M+, 58), 189 (25), 161 (100), 147 (13), 133 (31), 119 (52), 105 (76), 93 (29), 91 (44), 81 (79), 79 (26), 77 (28), 55 (34), 41 (73)	13.62	2	**<0.01**	**<0.01**					**2.04± 0.32**	**7.50± 1.33**									

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Table 2 (*continued*)

Compound	RT	# spp.	*N. rustica*		*N. suaveolens*		*N. sylvestris*		*N. longiflora*		*N. plumbaginifolia*	*N. langsdorffii*		*N. bonariensis*		*N. forgetiana*		*N. alata*	
			PM n=6	AM n=5	PM n=9	AM n=4	PM n=10	AM n=3	PM n=8	AM n=3	PM n=8	PM n=6	AM n=6	PM n=3	AM n=3	PM n=10	AM n=10	PM n=8	AM n=3
Oxygenated sesquiterpenoids (***10***)																			
220 (M+, 12), 192 (11), 177 (5), 149 (25), 147 (24), 135 (19), 121 (100), 108 (58), 107 (37), 93 (38), 91 (22), 82 (26), 81 (79), 67 (21), 55 (29), 43 (40), 41 (90)	15.5	1	**0.09±0.07**	**1.47±1.47**															
Z-Nerolidol*	16.15	1																0.16±0.05	0.16±0.16
Caryophyllene oxide*	16.43	2					**2.57±1.19**	**1.84±0.79**	**1.15±0.12**	**<0.01**									
E-Nerolidol*	16.46	2														0.50±0.19	0.03±0.03	4.15±1.50	10.45±1.74
222 (M+, 12), 207 (12), 191 (47), 179 (16), 149 (13), 137 (18), 135 (24), 121 (50), 119 (17), 109 (31), 108 (52), 107 (34), 105 (32), 95 (30), 93 (34), 82 (47), 81 (48), 77 (23), 69 (29), 55 (41), 43 (94), 41 (100)	17.38	1	**0.42±0.14**	**0.56±0.56**															
Z,E-Farnesal	18.27	1																0.03±0.02	2.64±0.39
Farnesol isomer?	18.35	2														0.03±0.03	ND	<0.01	0.93±0.77
E,E-Farnesal	18.77	2														0.12±0.12	ND	2.67±1.14	16.13±6.50
E,E-Farnesol*	19.46	2														0.03±0.03	ND	0.13±0.05	0.43±0.27
Farnesol isomer*	19.94	1																0.02±0.01	0.02±0.01
Aromatic alcohols, aldehydes (***10***)																			
Benzaldehyde*	10.57	4	64.90±27.33	18.54±3.09	0.46±0.17	0.75±0.09	11.14±1.61	6.78±1.03										0.48±0.21	2.17±1.09
Phenylacetaldehyde*	12.12	4	1.29±0.66	0.27±0.27								<0.01	0.10±0.10			2.05±1.04	0.02±0.02	0.10±0.06	ND
Salicylaldehyde*	12.12	2			*0.19±0.10*	*2.56±0.48*												0.04±0.02	0.47±0.47
Benzyl alcohol*	14.73	5	5.56±1.63	16.52±0.97	2.65±1.23	3.45±1.85	18.82±2.19	3.62±0.71						0.43±0.27	ND			0.02±0.02	ND
2-Phenylethanol*	15.12	5	**3.03±1.41**	**4.16±1.50**	0.29±0.05	ND	**0.75±0.33**	**1.99±0.71**								0.28±0.13	0.01±0.01	0.05±0.02	0.11±0.11
Cinnamic aldehyde*	16.6	1			0.36±0.13	ND													
Eugenol*	17.72	2												0.03±0.03	ND			0.47±0.19	0.29±0.29
E-Cinnamic alcohol*	18.78	1			4.00±1.26	ND													

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Table 2 (*continued*)

Compound	RT	# spp.	*N. rustica*		*N. suaveolens*		*N. sylvestris*		*N. longiflora*		*N. plumbaginifolia*	*N. langsdorffii*		*N. bonariensis*		*N. forgetiana*		*N. alata*	
			PM $n=6$	AM $n=5$	PM $n=9$	AM $n=4$	PM $n=10$	AM $n=3$	PM $n=8$	AM $n=3$	PM $n=8$	PM $n=6$	AM $n=6$	PM $n=3$	AM $n=3$	PM $n=10$	AM $n=10$	PM $n=8$	AM $n=3$
Hydrocinnamic alcohol	16.95	1			< 0.01	< 0.01													
E-Isoeugenol*	19.36	1																0.28 ± 0.13	< 0.01
*Aromatic Esters (**17**)*																			
Methyl benzoate*	11.9	3			57.45 ± 15.56	35.13 ± 14.70	6.81 ± 3.91	1.83 ± 0.59										< 0.01	1.99 ± 0.12
Phenylmethyl (benzyl) acetate*	13.6	2					3.63 ± 0.46	0.53 ± 0.30						27.90 ± 25.59	0.25 ± 0.25				
Methyl salicylate*	13.75	3			**0.51 ± 0.07**	**7.10 ± 1.95**						**0.24 ± 0.05**	**1.04 ± 0.25**	< **0.01**	< **0.01**			0.03 ± 0.01	0.09 ± 0.08
Isobutyl benzoate*	13.93	1																0.04 ± 0.02	ND
2-Phenylethyl acetate*	14.27	3			0.07 ± 0.01	ND										0.18 ± 0.08	ND	0.02 ± 0.01	< 0.01
Phenylmethyl (benzyl) isovalerate	15.07	2			0.07 ± 0.03	0.44 ± 0.44												0.07 ± 0.02	ND
Amyl benzoate*	15.24	4			0.10 ± 0.02	ND								0.07 ± 0.07	ND	0.05 ± 0.02	ND	0.27 ± 0.06	0.12 ± 0.04
Isobutyl salicylate	15.35	1																< 0.01	ND
Phenylmethyl (benzyl) valerate	15.38	3			< 0.01	0.65 ± 0.38	3.26 ± 2.78	0.04 ± 0.02											
Phenylmethyl (benzyl) ester	16.21	2			0.05 ± 0.02	ND								0.02 ± 0.02	ND				
Amyl salicylate*	16.74	1																0.25 ± 0.06	0.75 ± 0.50
Methyl cinnamate*	16.95	1			0.25 ± 0.09	ND													
Z-3-Hexenyl benzoate*	17.45	1			0.05 ± 0.01	0.09 ± 0.09													
Cinnamyl acetate	17.64	1			0.03 ± 0.01	ND													
Prenyl salicylate	18.11	1																< 0.01	ND
Benzyl benzoate*	21.84	4			18.79 ± 6.25	2.54 ± 1.54	3.18 ± 0.45	ND						0.94 ± 0.94	ND			0.02 ± 0.01	ND
Benzyl salicylate*	23.09	4			5.48 ± 2.55	8.11 ± 3.00	6.07 ± 0.87	ND						1.34 ± 1.34	ND			0.08 ± 0.04	< 0.01 ± < 0.01
*Fatty acid derived alcohols, ketones (**12**)*																			
98 (M +, 20), 83 (100), 55 (87), 43 (91)	4.13	1												0.17 ± 0.10	ND				
71 (38), 70 (36), 57 (38), 55 (58), 43 (100), 42 (80), 41 (69)	5.59	1														0.20 ± 0.16	ND		
97 (M + ?, 2), 84 (21), 69 (24), 61 (17), 56 (23), 55 (28), 43 (100)	6.19	1												0.67 ± 0.57	ND				

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Table 2 (*continued*)

Compound	RT	# spp.	*N. rustica*		*N. suaveolens*		*N. sylvestris*		*N. longiflora*		*N. plumbaginifolia*	*N. langsdorffii*		*N. bonariensis*		*N. forgetiana*		*N. alata*	
			PM n=6	AM n=5	PM n=9	AM n=4	PM n=10	AM n=3	PM n=8	AM n=3	PM n=8	PM n=6	AM n=6	PM n=3	AM n=3	PM n=10	AM n=10	PM n=8	AM n=3
E-3-Hexen-1-ol*	7.75	1														0.01± 0.01	ND		
Dimethylcyclohexanone isomer	8.37	2							4.84± 0.76	7.00± 0.70	**22.87± 5.47**								
Dimethylcyclohexanone isomer	9.03	1									**0.32± 0.20**								
Z-3-Hexen-1-ol*	8.53	5			*0.21± 0.04*	*0.55± 0.32*	1.71± 0.33	3.09± 1.01			**2.25± 0.84**	*0.60± 0.20*	*1.35± 0.23*			0.14± 0.06	ND		
70 (47), 55, (100), 43 (65), 42 (45), 41 (74), 40 (20)	9.29	3			0.41± 0.08	0.48± 0.48	6.39± 4.15	3.42± 0.96			**1.29± 0.44**								
70 (57), 69 (62), 57 (27), 55 (42), 42 (64), 41 (100)	9.39	2					1.18± 0.31	1.59± 0.55			**1.62± 0.52**								
84 (M+?, 52), 71 (22), 69 (78), 57 (72), 56 (24), 55 (51), 43 (100), 41 (97)	10.52	1									**0.30± 0.25**								
87 (11), 84 (34), 83 (28), 70 (46), 69, (43),68 (17), 56 (71), 55 (62), 43 (42), 42 (65), 41 (100)	11	1	0.36± 0.27	0.73± 0.65															
Z-Jasmone*	15.95	1					1.32± 0.41	1.98± 0.92											
*Fatty-acid derived esters (**14**)*																			
Methyl-2-methyl Butyrate	2.3	1							**0.17± 0.10**	**ND**									
Methyl-3-methyl butyrate	2.37	1							**0.14± 0.05**	**0.40± 0.20**									
Methyl-3-methyl pentanoate	4	1									**9.12± 1.55**								
3-Methyl pentanoate	4.15	1							**9.82± 1.88**	**0.52± 0.11**									
Methyl-4-methyl pentanoate	4.4	1							**0.54± 0.13**	**14.68± 2.76**									
Methyl-5-methyl hexanoate	6.25	2							**1.14± 0.27**	**1.67± 0.85**	**0.12± 0.06**								
Methyl-4-methyl hexanoate	6.65	2							**2.41 ±0.43**	**4.32 ±0.28**	**5.33 ±1.11**								
Isoamyl isovalerate	7.2	1																0.02 ±0.01	ND
87 (26), 74 (89), 73 (32), 59 (21), 57 (57), 55 (51), 43 (100), 42 (24), 41 (93)	7.44	2									**3.27± 1.09**							0.02± 0.01	ND
Z-3-Hexenyl acetate*	7.55	5			*0.25± 0.07*	*2.74± 0.63*					**8.25± 1.68**	*0.32± 0.04*	*0.86± 0.15*			0.17± 0.04	0.51± 0.19	0.04± 0.01	0.53± 0.17
81 (16), 70 (41), 69 (34), 61 (21), 57 (24), 56 (25), 55 (38), 43 (100), 42 (32), 41 (43)	8.01	1									**0.65± 0.28**								

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Table 2 (*continued*)

Compound	RT	# spp.	*N. rustica*		*N. suaveolens*		*N. sylvestris*		*N. longiflora*		*N. plum-baginifolia*	*N. langsdorffii*		*N. bonariensis*		*N. forgetiana*		*N. alata*	
			PM *n* = 6	AM *n* = 5	PM *n* = 9	AM *n* = 4	PM *n* = 10	AM *n* = 3	PM *n* = 8	AM *n* = 3	PM *n* = 8	PM *n* = 6	AM *n* = 6	PM *n* = 3	AM *n* = 3	PM *n* = 10	AM *n* = 10	PM *n* = 8	AM *n* = 3
Methyl-5-methyl heptanoate?	8.32	3							**1.21 ± 0.13**	**1.26 ± 0.64**	**0.24 ± 0.20**							< 0.01	0.94 ± 0.59
93 (43), 87 (38), 80 (15), 74 (100), 73 (15), 60 (18), 57 (31), 45 (17), 41 (87)	12.63	1									< 0.01								
4-Methylhexanoic acid	15.47	2									0.48 ± 0.45							< 0.01	0.01 ± 0.01
*Nitrogenous compounds (**16**)*																			
2-Methylbutylnitrile**	3.05	3							1.48 ± 0.37	0.12 ± 0.12						< 0.01	ND	< 0.01	ND
3-Methylbutylnitrile**	3.18	2														< 0.01	ND	< 0.01	ND
Nitro-2-methylbutane**	7.57	3							2.99 ± 0.33	10.20 ± 0.68						0.08 ± 0.04	ND	0.04 ± 0.02	ND
Nitro-3-methylbutane**	7.82	2														1.88 ± 0.61	0.10 ± 0.10	0.82 ± 0.22	2.47 ± 1.14
Isobutyraldoxime	8.59	2							0.89 ± 0.54	ND								0.12 ± 0.03	ND
Isobutyraldoxime	8.86	2							0.61 ± 0.06	ND								0.05 ± 0.03	ND
2-Methylbutyloxime**	10.11	5			0.31 ± 0.07	ND			38.81 ± 4.88	18.53 ± 1.42				0.85 ± 0.17	ND	1.30 ± 0.29	ND	0.88 ± 0.23	0.94 ± 0.12
3-Methylbutyloxime**	10.26	3			0.23 ± 0.06	ND										7.00 ± 2.02	4.34 ± 1.18	12.77 ± 2.96	8.34 ± 1.75
2-Methylbutyloxime**	10.32	4			0.05 ± 0.04	ND			13.50 ± 1.95	6.15 ± 0.48						0.81 ± 0.23	ND	1.07 ± 0.23	2.49 ± 0.74
3-Methylbutyloxime**	10.77	3			0.16 ± 0.08	ND										4.52 ± 0.97	4.56 ± 1.33	10.82 ± 1.94	7.77 ± 1.64
Methyl nicotinate*	13.79	1			0.17 ± 0.04	ND													
Nicotine*	14.75	2	**4.03 ± 1.85**	**21.44 ± 9.85**	*0.17 ± 0.09*	ND													
Phenylacetonitrile*	15.36	2	0.01 ± < 0.01	0.04 ± 0.02															
154 (M + ?, 38), 121 (8), 112 (18), 98 (100), 93 (19), 69 (9), 43 (97), 41 (23)	16.21	1	0.13 ± 0.07	0.24 ± 0.17															
Methyl anthranilate*	18.46	1			0.03 ± 0.03	ND													
Indole*	20.25	1			0.18 ± 0.03	ND													
125 Total compounds																			

Compounds marked with * were identified by co-chromatography with known standards, those with ** using essential oils or natural products for which published GC–MS data are available. For remaining compounds, putative names are provided when MS showed > 90% identity with NIST and Wiley library spectra. MS of unidentified compounds are given in descending order of m/z, with% abundance relative to the base peak (100) in parentheses. Italics indicate compounds emitted only by vegetative tissues, bold face indicates compounds emitted by both flowers and non-floral tissues. ND = compound not detected using our protocols.

esters were the only compounds that were emitted exclusively by floral tissues. The presence of aldoximes in flowers is unusual and appears to be correlated with nocturnal anthesis and hawkmoth pollination (Kaiser, 1993; Knudsen and Tollsten, 1993). The cytochrome P-450 catalyzed conversion of amino acids to aldoximes is the first committed step in the biosynthesis of glucosinolate defense compounds in the vegetative tissues of mustards and other plants (Halkier and Du, 1997; Kliebenstein et al. 2001). A similar mechanism has been invoked for the biosynthesis of cyanogenic glucosides in plant tissues (Møller and Poulton, 1993).

2.1. Intraspecific vs. interspecific variation

For all species, mean intraspecific differences in nocturnal floral and vegetative scent composition were significantly lower than interspecific differences ($Z = -12.28$, $P < 0.001$; Table 3). (Bonferroni adjustment for multiple comparisons resulted in a critical value (α) of 0.005.) Thus, odors were species-specific at night. When evaluated by species, only *N. bonariensis* did not show significantly lower levels of intraspecific than interspecific variation ($P = 0.019$). Thus, the fragrance profiles of some *N. bonariensis* individuals were more similar to those of other *Nicotiana* species than they were to other *N. bonariensis* individuals. Certain distinctive compounds contributed to species-specific patterns. These include the array of unique ledene-related sesquiterpenes in *N. rustica*, cinnamic acid metabolites in *N. suaveolens* flowers and a series of methyl esters of butyric, valeric and caproic acids shared by *N. longiflora* and *N. plumbaginifolia* (Table 2). For scent emitted diurnally, mean intraspecific differences were again significantly lower than interspecific differences ($Z = -6.33$, $P < 0.001$), but the magnitude of the difference was only half that observed for nocturnal fragrance. At the level of individual species, only the diurnal odors of *N. rustica* and *N. forgetiana* had significantly greater interspecific vs. intraspecific variation in fragrance composition (Table 4). This result cannot be attributed to small sample sizes because there was no relationship between sample size and dissimilarity (data not shown). However, our study species emitted a mean of 9 ± 3 (S.E.M.) more scent compounds at night than during the day (Table 2), and we have shown previously that odor variability is positively correlated with chemical complexity (Levin et al., 2001).

It is intriguing that *Nicotiana* odors are less species-specific during the day, including hummingbird-pollinated species (*N. langsdorffii*) for which fragrance presumably is superfluous. Perhaps there is one common theme (e.g. anti-herbivore defense) that dominates the selective and/or phylogenetic controls on diurnal scent production, whereas these species may experience different selective regimes for nocturnal fragrance production. Circadian rhythms in odor emission are prominent in long-lived flowers with nocturnal pollinators, such as *Cestrum nocturnum* (Overland, 1960; Heath et al., 1992), *Angraecum sesquipedale* (Kaiser, 1993; Wasserthal,

Table 3
Differences in diurnal vs. nocturnal volatile emissions[a] (ng per g dry mass flower per h)

Species (*N*)		Total scent	Monoterpenoids	Benzenoids	N-compounds
N. rustica (4)	D[d]	15.53±2.24[b]	1.45±1.20[c]	7.94±1.47	2.57±1.01
	N	55.47±19.45	9.43±4.87	39.90±16.81	2.41±1.72
N. suaveolens (4)	D	26.92±7.70	5.85±0.91	16.76±6.58	NA
	N	55.40±5.91	5.42±0.34	44.47±5.52	NA
N. sylvestris (3)	D	6.57±2.05	1.54±0.57	0.91±0.15	NA
	N	10.03±2.71	1.26±0.35	4.63±1.35	NA
N. longiflora (3)	D	3.77±0.51	0.45±0.07	NA	1.31±0.11
	N	6.58±2.03	0.39±0.19	NA	6.28±0.98
N. langsdorfii (4)	D	27.81±11.35	26.32±11.35	NA	NA
	N	83.20±35.03	81.79±34.66	NA	NA
N. bonariensis (3)	D	14.57±2.12	6.04±1.39[c]	0.02±0.006	0.00
	N	57.77±10.14	41.97±4.81	0.89±0.87	1.22±0.51
N. forgetiana (4)	D	16.38±5.02	12.12±4.13	0.00	2.58±1.32
	N	146.15±22.26	109.00±19.08	3.00±2.68	29.78±9.76
N. alata (3)	D	73.34±11.57	24.65±3.30	1.13±0.10	15.95±2.03
	N	1018.23±238.64	701.52±151.64	11.33±4.57	221.29±40.56

[a] These data differ from Table 2 because they were collected as repeated measures from the same 3–4 individual plants.
[b] Total scent does not equal the sum of monoterpenes, aromatics and N-compounds because sesquiterpenes and other compound classes not universally present were omitted from day–night comparisons.
[c] Includes 4-oxoisophorone and related compounds.
[d] D = diurnal scent, N = nocturnal scent, NA = comparison not applicable because compound class is absent.

Table 4
Inter vs. intraspecific differences in nocturnal scent: Wilcoxon signed ranks

Species	Comparison	Mean rank	Sum ranks	Z	P[a]
N. rustica	Within-[b]	0.0	0	−3.41	<0.001
	Between-[c]	8.0	120		
N. suaveolens	Within-	0.0	0	−2.80	0.003
	Between-	5.5	55		
N. sylvestris	Within-	0.0	0	−5.84	<0.001
	Between-	23.0	1035		
N. longiflora	Within-	0.0	0	−2.80	0.003
	Between-	5.5	55		
N. plumbaginifolia	Within-	0.0	0	−4.62	<0.001
	Between-	14.5	406		
N. langsdorffii	Within-	0.0	0	−3.41	<0.001
	Between-	8.0	120		
N. bonariensis	Within-	3.5	7	−2.09	0.018
	Between-	6.0	48		
N. forgetiana	Within-	13.1	157	−4.07	<0.001
	Between-	26.6	878		
N. alata	Within-	3.7	11	−4.37	<0.001
	Between-	15.8	395		
All data	Within-	11.1	111	−12.28	<0.001
	Between-	107.7	21004		

[a] One-tailed test, $\alpha = 0.005$ due to multiple comparisons.
[b] Cases in which within-species distance ranks exceeded those between species.
[c] Cases in which between-species distance ranks exceeded those

Table 5
Inter vs. intraspecific differences in diurnal scent AM: Wilcoxon rank sum tests

Species	Comparison	Mean rank	Sum ranks	Z	P[a]
N. rustica	Within-[b]	0	0	−2.80	0.003
	Between-[c]	5.5	55		
N. suaveolens	Within-	0	0	−2.20	0.014
	Between-	3.5	21		
N. sylvestris	Within-	0	0	−1.60	0.06
	Between-	2	6		
N. longiflora	Within-	0	0	−1.60	0.06
	Between-	2	6		
N. langsdorffii	Within-	10	40	−1.14	0.13
	Between-	7.3	80		
N. bonariensis	Within-	1	1	−1.07	0.14
	Between-	2.5	5		
N. forgetiana	Within-	2	2	−2.60	0.005
	Between-	5.9	53		
N. alata	Within-	0	0	−1.60	0.06
	Between-	2	6		
All data	Within-	0	0	−6.33	<0.001
	Between-	27	1431		

[a] One-tailed test, $\alpha = 0.005$ due to multiple comparisons.
[b] Cases in which within-species distance ranks exceeded those between species.
[c] Cases in which between-species distance ranks exceeded those within species.

1997) and *Stephanotis floribunda* (Matile and Altenburger, 1988; Pott et al., 2002). Biologists have long assumed this pattern to be adaptive, increasing fitness through synchronization with pollinator behavior or metabolic efficiency (Matile and Altenburger, 1988). However, when Baldwin et al. (1997) artificially increased benzyl acetone emission rates during day and night in *N. attenuata*, the plants suffered fitness losses due to herbivory and seed predation, without concomitant increases in pollination. These findings suggest that diurnal predator avoidance and physiological constraints on biosynthetic pathway flux should be considered along with pollinator attraction as factors that potentially shape the evolution of fragrance emission rates and rhythms in tobacco flowers.

2.2. Temporal variation in emission rates

Emission rates varied extensively between species, both on a per flower basis and when standardized for fresh or dry floral mass (Fig. 1). All pair-wise comparisons were significantly correlated ($P < 0.001$), such that standardizing for differences in fresh or dry floral mass did not significantly change the rank orders of emission rates by species. Flowers of *N. alata* emitted more than twice as much fragrance as any other species, particularly at night, and related species in section *Alatae* also were strongly scented. Table 5 summarizes odor emissions for each species during day vs. night, expressed as production of total fragrance, monoterpenoids, benzenoids and N-bearing compounds (when relevant). We observed greater nocturnal emissions in all species, by factors of two (*N. sylvestris*) to greater than 10 (*N. alata*; Table 5), regardless of flower morphology or pollinator class. Unexpectedly, scent emissions per dry floral mass were higher in hummingbird-pollinated *N. langsdorffii* and *N. forgetiana* than in the hawkmoth-pollinated *N. longiflora* and *N. sylvestris*. Indeed, the latter two species were among the least fragrant in our study by any measure (Fig. 1, Table 2), a surprising result given their pollination syndrome (Grant, 1983; Knudsen and Tollsten, 1993). In contrast, flowers of *N. alata* were the most strongly fragrant even when data were standardized for floral mass.

Patterns of diurnal versus nocturnal emission of fragrance varied among classes of compounds. Monoterpenoid emissions were greater at night in *N. rustica*, *N. alata, N. forgetiana, N. bonariensis* and *N. langsdorf-*

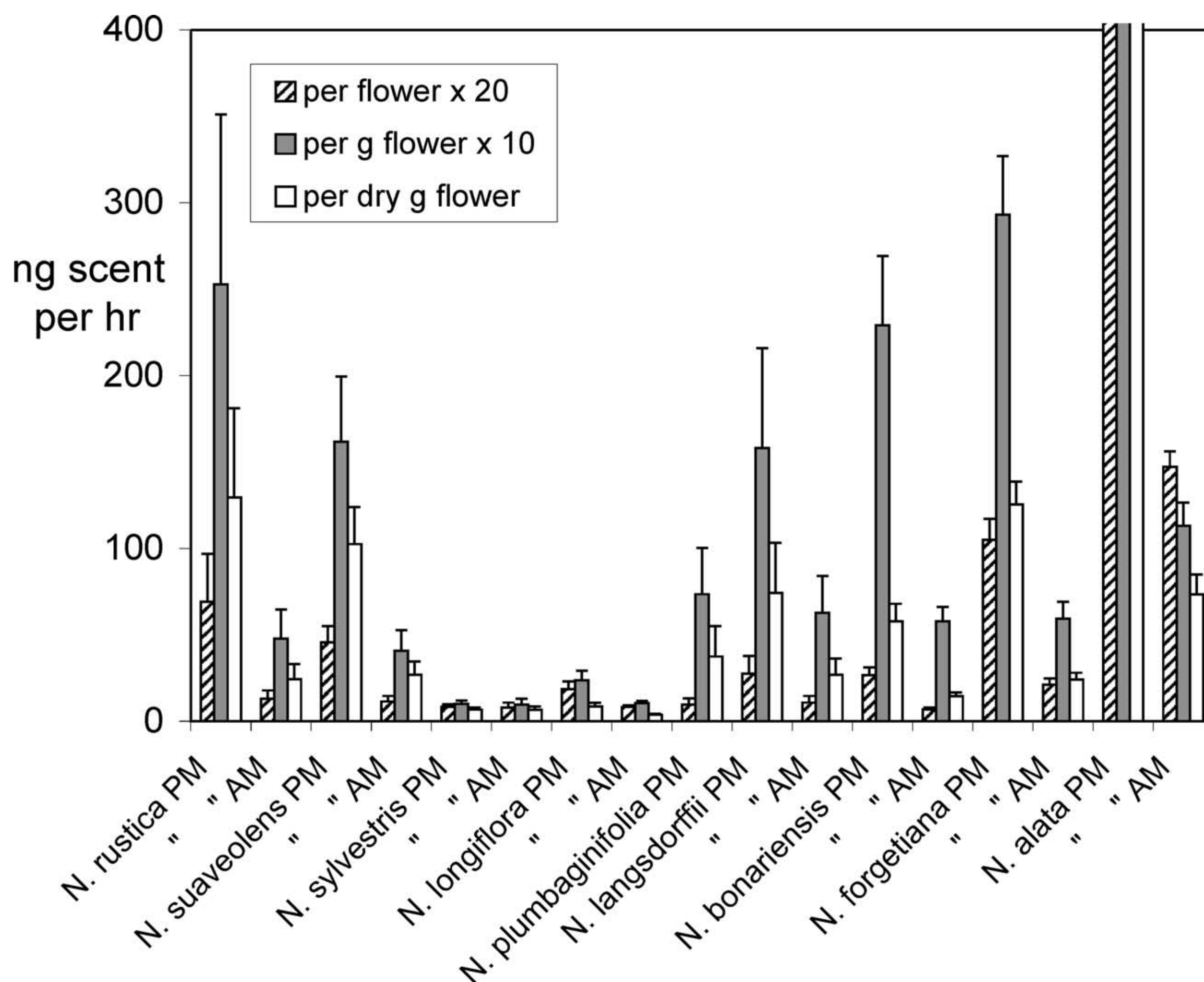

Fig. 1. Mean±S.E.M. emission rates (ng) of total fragrance for all *Nicotiana* species, expressed as ng scent per hour, on a per flower (hatched bars), per fresh floral mass (g, solid bars) and per dry floral mass (open bars) basis. Species are ordered (l to r) as in Table 1, to reflect phylogenetic relationships. Nocturnal emissions are denoted with PM, diurnal emissions with AM. Note that gross differences in scale have been adjusted for the purposes of comparison (see legend), and that emission rates for *N. alata* (PM) extend beyond upper bound of figure. Actual emission rates for *N. alata* are 81.02±17.73 ng/flower/h, 111.75±24.45 ng/g flower/h and 659.67±143.34 ng/dry g flower/h.

fii, but not in the remaining species (Table 5). Nocturnal emissions of N-bearing compounds were in excess when the compounds were aldoximes and other aliphatic amino acid derivatives (*N. alata, N. bonariensis* and *N. longiflora*), but not when the compounds (indole and phenylacetonitrile) were derived from benzenoid amino acids. Interestingly, flowers of *N. rustica* emitted 5-fold more nicotine during the day than at night, consistent with Euler and Baldwin's (1996) findings for distantly related *N. attenuata*, a North American species. Floral nicotine was not detected consistently in other species in this study.

All species emitting benzenoid compounds from flowers showed four-fold or greater excess in nocturnal emissions (Table 5). Previous studies demonstrated a pronounced nocturnal rhythm in benzenoid emissions, in the distantly related *N. sylvestris* (Loughrin et al., 1990a,b, 1991), *N. suaveolens* (Loughrin et al., 1991, 1993; Kolosova et al., 2001) and *N. attenuata* (Euler and Baldwin, 1996). A parsimonious explanation for these findings is that circadian control of benzenoid volatiles produced by the shikimate pathway is a shared ancestral trait in *Nicotiana*. It would be interesting to test whether nocturnal rhythms of benzenoid emission in all tobaccos result from diurnal reductions in substrate concentration, as was shown by Kolosova et al. (2001) for methyl benzoate in *N. suaveolens*, rather than a modulation of biosynthetic enzyme activity or gene expression.

2.3. Biosynthetic and phylogenetic components of fragrances

One of the most striking patterns to emerge from our analyses was the shared emission of 1,8 cineole, with smaller amounts of α- and β-pinene, sabinene, β-myr-

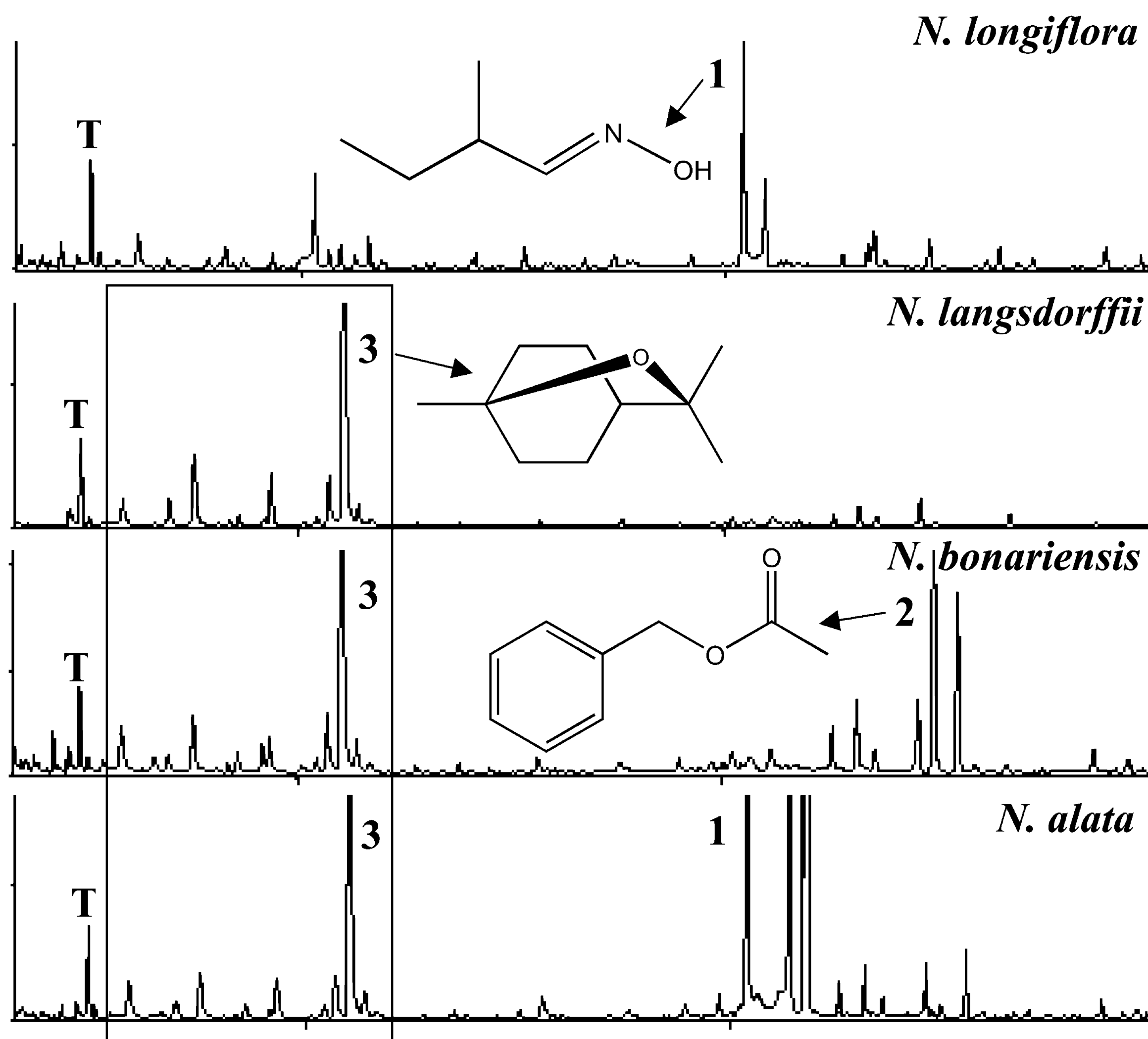

Fig. 2. GC–MS total ion chromatograms of floral headspace from related *Nicotiana* species. Hawkmoth pollinated *N. longiflora* and *N. alata* emit nitrogenous compounds such as methyl butyraldoxime (1) isomers and their derivative nitriles. These putative hawkmoth attractants are absent from hummingbird-pollinated *N. langsdorffii*, and are replaced by benzyl acetate (2) and derivatives of 4-oxoisophorone in bee/moth pollinated *N. bonariensis*. Flowers of all species in sect. *Alatae s.s.* emit 1,8 cineole (3) and related monoterpenes in proportions comparable to the known major and minor products of a single enzyme, 1,8 cineole synthase. Chromatograms are scaled to the internal standard (T), 14 ng of toluene.

cene, limonene and α-terpineol by all species of *Nicotiana* sect. *Alatae s.s.* (Table 2, Fig. 2). Varying combinations of these compounds also were emitted by *N. suaveolens* and more distantly related species, but did not show the 4- to 30-fold excess in nocturnal vs. diurnal emission shared by *N. alata, N. forgetiana, N. bonariensis* and *N. langsdorffii*. SPME analyses of dissected flowers localized >80% of all monoterpenoid emissions to the distal corolla lobes (see Euler and Baldwin, 1996) in these four species (data not shown). In contrast, flowers of *N. longiflora* emitted only 1,8 cineole, whereas vegetative tissues emitted the remaining monoterpenes (Table 2).

Thus, the nocturnal emission of 1,8 cineole and related monoterpenes from corolla limbs represents a shared-derived trait among these *Nicotiana* species. The relative ratios of these compounds closely resemble the pattern of major- and minor-products of the 1,8 cineole synthase enzyme (Figs. 2 and 3), as reported by Wise et al. (1998) for *Salvia officinalis* (Lamiaceae). In leaves of *S. officinalis*, 1,8 cineole synthase converts geranyl diphosphate to a blend of 1,8 cineole (79%), β-myrcene (3%), (+) sabinene (2.5%), α-terpineol (1%), and racemic mixtures of limonene (1.5%), α- and β-pinene (13%) (Wise et al., 1998). In Fig. 3 we compare these expected ratios to the mean proportions of monoterpenes emitted by *Nicotiana* sect. *Alatae* species. There is a consistent excess of sabinine, myrcene and limonene in the tobacco headspace samples. These patterns reflect a putative secondary source of emission for such compounds from vegetative tissues as well as flowers (Table 2). Also, it is possible that additional biosynthetic enzymes, such as limonene

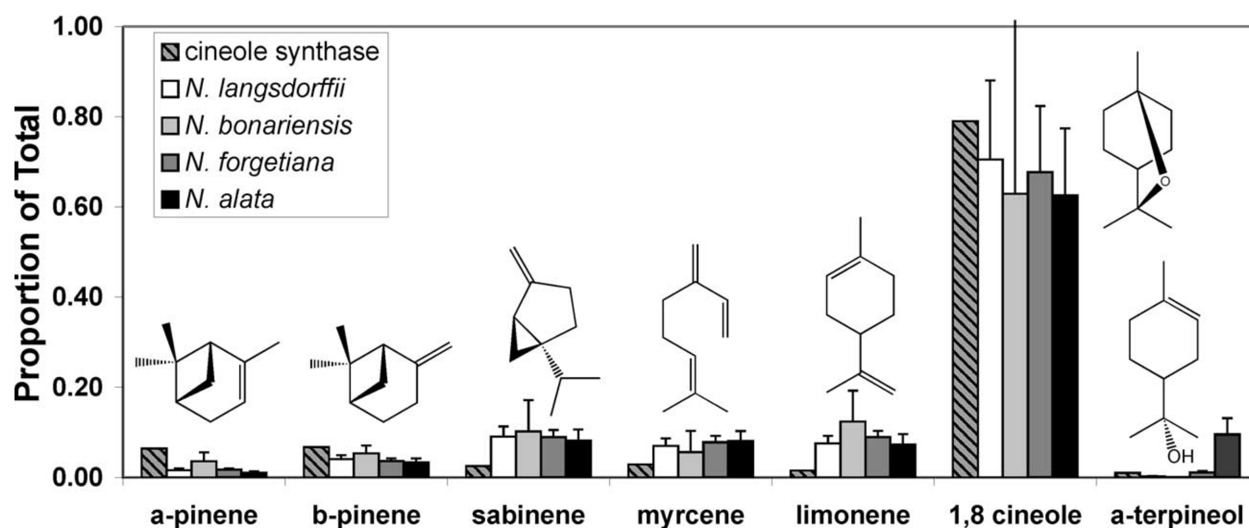

Fig. 3. Relative amounts (mean ± S.E.M.) of monoterpenoid emissions by flowers of *Nicotiana* sect. Alatae sensu strictu (solid bars), in comparison to the major and minor compounds produced by *Salvia officinalis* 1,8 cineole synthase enzyme expressed in *Escherichia coli* (hatched bars; Wise et al., 1998). Subsets of these compounds also were emitted by other *Nicotiana* species, usually by calyx, leaf or stem tissues.

synthase (Colby et al., 1993) and myrcene/ocimene synthase (Bohlmann et al., 2000), are producing these compounds as major products in *Nicotiana* species.

In the absence of behavioral assays, the importance of 1,8 cineole and related monoterpenes to reproductive fitness in these diversely pollinated *Nicotiana* species remains unclear. Volatile 1,8 cineole is released abundantly in two distinct ecological contexts; as a vegetative defense compound in plants of Mediterranean climates (e.g. mints and eucalypts; Edwards et al., 1993; Sáez, 1995) and as a floral attractant in orchids pollinated by male euglossine bees (Williams and Whitten, 1999). However, 1,8 cineole and related monoterpenoids are biosynthetically correlated traits, whose presence and relative amounts appear to co-vary, and some of these compounds may bear no ecological function. Barkman (2001) suggested that this phenomenon is widespread in plant volatile biosynthesis, such that compounds produced by the same biosynthetic pathway branch should not be treated as independent constituents of floral fragrance. Nevertheless, fragrance biosynthetic enzymes are so variable in their substrate specificity and number of catalytic products (Ross et al., 1999; D'Auria et al., 2002) that it is difficult to generalize between different enzymes or plant species.

2.4. Fragrance chemistry and pollinator affinities

Recent analyses of hawkmoth-pollinated plants led us to predict that *N. alata, N. longiflora, N. suaveolens* and *N. sylvestris* fragrances would be dominated by benzenoid esters, linalool and/or oxygenated sesquiterpenes and nitrogenous compounds (Kaiser, 1993; Knudsen and Tollsten, 1993; Miyake et al., 1998; Levin et al., 2001). Within *Nicotiana* section *Alatae s.l.*, these predictions were upheld, with nitrogenous aldoximes, linalool and caryophyllene present in *N. longiflora* and *N. alata*; flowers of the latter also produced large amounts of benzenoid esters, nerolidol and farnesol-related compounds (Table 2). These two species inhabit subtropical Brazil and Argentina, respectively, and are pollinated by a guild of long-tongued (8–12 cm) hawkmoths including *Agrius cingulatus* and *Manduca sexta* (Cocucci, 1988; Ippolito, 2000). The fragrances of *N. sylvestris* and *N. suaveolens* lack aldoximes and terpenoid alcohols but are rich in caryophyllene, benzenoid esters and benzenoid alcohols, all of which are emitted by many other hawkmoth-pollinated plants (Knudsen and Tollsten, 1993).

Do such variable fragrances contain functionally redundant hawkmoth attractants? Behavioral assays show that naïve *M. sexta* hawkmoths respond generally to plant odors as feeding cues; floral and vegetative fragrances from most *Nicotiana* species studied here are sufficient to induce feeding (Cutler et al., 1995; R.A. Raguso, unpublished data). Therefore, further studies of the adaptive role of fragrance chemistry in hawkmoth-pollinated tobaccos should test for innate, odor-specific preferences and the salience of different compounds as conditioning stimuli (Daly et al., 2001). Pollinator data are lacking for *N. sylvestris* in its native habitat. However, the combination of pendant flower posture (see Fulton and Hodges, 1999) and high levels of self-pollination in the greenhouse suggests that hawkmoth visitation may not limit reproductive fitness in *N. sylvestris*. Even less can be said about the Australian *N. suaveolens*, whose relatively short-tubed flowers suggest a spectrum of short-tongued (3–5 cm) hawkmoths, such as *Hippotion celerio, Theretra spp*. and *Hyles lineata livornicoides* (Pittaway, 1993) and other insects as pollinators. Field

observations in natural populations of both of these tobacco species would provide valuable context for ongoing studies of their floral biology.

N. bonariensis appears to have made the transition to pollination by small crepuscular moths (and potentially, bees) from an ancestor resembling *N. alata* or *N. longiflora* (Goodspeed, 1954; Ippolito, 2000). We expected to find benzenoid alcohols or aldehydes (e.g. phenylacetaldehyde) or the "lilac" alcohols and aldehydes known to attract diverse noctuid moths (Cantelo and Jacobsen, 1978; Heath et al., 1992; Plepys et al., 2002). Instead, we detected nocturnal emissions of benzyl acetate (Fig. 2), which attracts *Trichoplusia ni* (Noctuidae) moths in wind tunnels (Haynes et al., 1991), along with three cyclic diketones representing different oxidation states of 4-oxoisophorone (Fig. 4). These unusual compounds are derived from β-carotene (Kanasawud and Crouzet, 1990) and are characteristic of the odor of saffron (*Crocus sativus*) (Tarantilis and Polissiou, 1997). We also detected these compounds in the headspace of *N. rustica* (Table 2) and small moth / bee pollinated *Camissonia* species (Onagraceae; R.A. Raguso, unpublished data). Andersson (2001) identified oxophorones from floral headspace of the butterfly bush, *Buddleja davidii* (Loganiaceae) and found them to be among the most potent EAG stimulants for three European butterfly species. However, their importance (if any) as floral attractants or repellents in *Nicotiana* species awaits further investigation.

We predicted that fragrance should be absent or reduced in *N. langsdorffii* and *N. forgetiana*, which are hummingbird-pollinated. This prediction stems from the observation that hummingbirds generally ignore floral scent when foraging (Bené, 1945; van Riper, 1960) and few hummingbird-pollinated flowers emit large amounts of fragrance (Knudsen, 1993; Levin et al., 2001). We made a similar prediction for *N. plumbaginifolia*, in which greatly reduced flowers that are often cleistogamous appear to have been derived from a *N. longiflora*-like ancestor (Goodspeed, 1954). Our predictions were not upheld in terms of total scent production (Table 2). Flowers of *N. langsdorffii, N. forgetiana* and *N. plumbaginifolia* were at least as scented, per unit mass, as flowers of *N. longiflora* and *N. sylvestris* (Fig. 1). However, the putative hawkmoth attractants

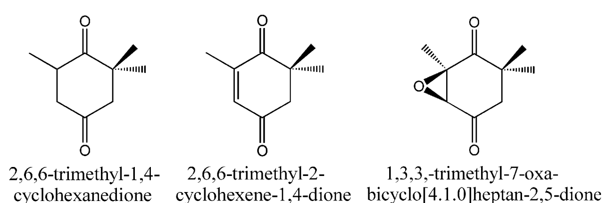

Fig. 4. 4-Oxoisophorone (center) and related compounds with fifth and sixth carbons oxidized (right) or reduced (left). These compounds were detected in *N. bonariensis* and *N. rustica*, and are derived from β-carotene (Kanasawud and Crouzet, 1990).

(linalool, nerolidol/farnesol isomers, aldoximes, benzenoid esters) emitted in large amounts by *N. alata* were either absent or much reduced in *N. langsdorffii* and *N. forgetiana*. Indeed, the aldoximes and caryophyllene oxide which dominate the fragrance of *N. longiflora* were absent in *N. plumbaginifolia*.

2.5. Comparisons to previous studies

Our fragrance analyses for *N. suaveolens* and *N. sylvestris* are comparable to previously published headspace data for these species (Loughrin et al., 1990a, 1991, 1993), despite differences in methods and amount of flowers used. In contrast, our data for fragrance chemistry and emission rates of *N. rustica* and *N. alata* differ substantially from those measured by Loughrin et al. (1990b), probably because these authors used excised flowers (see Mookherjee et al., 1990), whereas ours were on living plants. Data for *N. alata* are even less comparable, perhaps because the pink-flowered plants studied by Loughrin et al. (1990b) were of horticultural origin (presumably a *N. alata*×*N. forgetiana* hybrid), whereas our accessions were obtained near the type locality of *N. alata* in Brazil. Our data for *N. longiflora* lacked many of the larger molecular weight compounds (e.g. benzyl benzoate, pentacosane) obtained by Schlotzhauer et al. (1994) via vacuum steam distillation, a technique known to produce volatile artifacts and rearrangement products (Surburg et al., 1993). If these compounds were emitted by *N. longiflora* flowers we should have identified them, because they are detectable at picogram concentrations using our analytical methods. Schlotzhauer et al.'s (1994) data did not include the series of aldoximes and aliphatic methyl esters (Table 2) that distinguished *N. longiflora* from its relatives in our study, but did identify linalool, β-caryophyllene and several aliphatic alcohols, as did we. The nitrogenous compounds and aliphatic esters absent from Schlotzhauer et al.'s (1994) data may reside among the 11 unidentified GC peaks reported in that study, may have been lost to chemical rearrangement during distillation, or may represent true intraspecific variation.

2.6. Conclusions

Among our study species, nocturnally emitted fragrances were distinctly species-specific in chemical composition, whereas those emitted during the day were much less distinct. Many scent compounds had both floral and vegetative sources, but benzenoid esters and nitrogenous aldoximes were uniquely floral. Regardless of pollinator affinity, all species emitted more fragrance at night, especially benzenoid compounds. The fragrances of hawkmoth-pollinated species contained nitrogenous compounds, benzenoid esters and/or terpenoid alcohols, and these compounds were absent in

hummingbird-pollinated species. However, the latter species were not scentless, and in fact were more fragrant than two of the four hawkmoth-pollinated taxa in our study. Finally, the presence of aliphatic methyl esters in *N. longiflora* and *N. plumbaginifolia*, and the concerted emission of a large peak of 1,8 cineole and several smaller monoterpene peaks by flowers of all members of *Nicotiana* sect. *Alatae* appear to reflect shared evolutionary history rather than adaptation to common pollinators. These findings reinforce the notion that floral scent is a complex phenotype shaped by diverse forces including, but not restricted to, pollinator adaptation.

3. Experimental

3.1. Study taxa

Fragrance was collected from multiple individuals (see Table 2) of each species. Seed accessions obtained from T.P. Holtsford (University of Missouri, USA) were: *N. alata* TW7, *N. bonariensis* TW28, *N. longiflora* TW78, *N. forgetiana* TW50, *N. plumbaginifolia* TW106, *N. langsdorffii* TW74 and *N. sylvestris* (no accession number). *N. rustica* seeds were provided by J. Walker (Oxford University, UK), and *N. suaveolens* seeds by M. Pott and B. Piechulla (University of Rostock, Germany). Seeds were germinated under short day (12 L: 12 D) conditions in vermiculite and transplanted to 1:1 sand: potting soil mix at the four- leaf rosette stage. Later, plants were transferred to 16 cm D×12 cm tall pots and fertilized with MiracleGro® (15% N: 30% P: 15% K) every 4 weeks. Plants were grown in greenhouses under ambient temperature and photoperiod conditions, and took from 6 to 30 weeks to bloom. Mortality due to crown rot and aphid infestation greatly reduced the number of *N. bonariensis* individuals from which samples could be collected. Vouchers of all study species are housed in herbaria at the University of Arizona (ARIZ) and the University of South Carolina (USCH).

3.2. Volatile collection

Floral and vegetative odors were collected using two complementary methods. First, we used dynamic headspace collection methods (see Raguso and Pellmyr (1998) and references therein) to quantify volatile compound emission rates during diurnal and nocturnal periods. Floral volatiles were concentrated within Reynolds, Inc. (nylon resin) oven bags and were trapped on adsorbent cartridges using battery-operated diaphragm pumps (KNF Neuberger, Inc.). Glass cartridges were packed with 100 mg of the adsorbent Porapak®Q (80–100 mesh) between plugs of quartz wool and clean air was pulled over the flowers and into the adsorbent trap at a flow rate of ca. 250 ml/min, with headspace bags cinched at roughly 1 l volumes. Fragrance was collected for 10–12 h under ambient conditions in the greenhouse, with separate day and night collections performed for each species. The number of flowers included for each sample was noted, and fresh masses were recorded for flowers and vegetation enclosed within headspace bags using a Mettler, Inc. analytical balance (to 0.001 g). Subsequently, these plant tissues were dried for 24 h in an oven at 50 °C to obtain dry masses. Whenever possible, we collected fragrance from flowers on the first day of anthesis.

We also used solid phase micro extraction (SPME; Zhang and Pawliszyn, 1993) to verify the identity of compounds detected in floral and vegetative samples and improve the quality of mass spectral signal for low abundance compounds (e.g. sesquiterpenes). Headspace bags were prepared by cutting and re-sealing oven bags to 12×9 cm dimensions, using an American International Electric, Inc. impulse heat sealer. Bags were placed over living, uncut flowers and cinched with plastic ties. Simultaneous collections from empty bags and those enclosing vegetative parts were used to distinguish between floral volatiles, vegetative compounds and ambient contaminants. In other experiments, corolla limbs, floral tubes and calyces were dissected from flowers of some species, standardized for fresh mass and sealed within 9×9 cm bags to identify the tissue sources of volatile emissions. All samples were equilibrated for 15 min, then a SPME fiber coated with polydimethylsiloxane (PDMS, 100 μm film thickness) was exposed within headspace bags for an additional 15 min followed by immediate GC–MS analysis. Wound artifacts such as Z-3-hexenyl acetate and other products of the lipoxygenase cascade (Croft et al., 1993) were ignored.

3.3. Chemical and data analysis

Porapak traps were eluted with 3 ml of hexane, and the eluate was stored at −20 °C in Teflon-capped borosilicate glass vials. Before GC–MS analysis, we used a flow of gaseous N_2 to concentrate samples to 75 μl, then added 5 μl of 0.03% toluene as an internal standard. Aliquots (1 μl) of each sample were injected into a Shimadzu GC-17A equipped with a Shimadzu QP5000 quadrupole electron impact MS as a detector. All analyses were done using splitless injections on a polar GC column [diameter 0.25 mm, length 30 m, film thickness 0.25 μm (EC WAX); Alltech Associates, Inc.], with selected samples of each species also analyzed on a non-polar column [diameter 0.35 mm, length 30 m, film thickness 1.0 μm (EC-5)]. The carrier gas was helium with a flow rate of 1 ml/min and a split ratio of 12, with injection port temp. of 240 °C and detector temp. of 260 °C. The oven program began with injection at 60 °C

and a constant temp. for 3 min. Oven temperature increased by 10 °C per min until 260 °C, where it was held for 7 min, as described by Levin et al. (2001). SPME samples were introduced to the GC via direct thermal desorption in the injection port at 240 °C and were analyzed using the GC–MS parameters described above.

Compounds were tentatively identified using computerized mass spectral libraries [Wiley and NIST libraries (>120,000 mass spectra)]. The identity of many compounds was verified using retention times of known standards (Table 2). Peak areas of total ion chromatograms (TIC) were integrated using Shimadzu's Class-5000 software, and were quantified by comparison with the internal standard.

3.4. Temporal variation in volatile emissions

We compared diurnal and nocturnal fragrance production by collecting odor from the same inflorescences of 3–4 individuals per species during 8–12 h of daylight vs. night on consecutive days. We compared rank orders of emission rates per species (day and night) between all pair-wise comparisons (ng scent/flower/h, per g fresh floral mass/h and per g dry floral mass/h) using Spearman's rank correlation coefficient. This constitutes a two-tailed test of the null hypothesis that standardization of odor per unit floral mass changes the rank order of emissions between species. For each species we compared the production of volatiles (dry floral mass) in terms of total fragrance, monoterpenoids, benzenoids and nitrogenous compounds. *N. plumbaginifolia* was omitted from these analyses because the great majority of volatile emission was found to be vegetative.

3.5. Intraspecific vs. interspecific variation

We compared intraspecific variation in fragrance profiles to interspecific variation using the relative amounts of both floral and vegetative volatiles for each individual to calculate a dissimilarity matrix based on Euclidean distance (SPSS Inc., 1999). Variables were standardized to *Z*-scores, which have a mean of zero and a standard deviation of one. We then used the Wilcoxon Rank Sum Test (SPSS Inc., 1999) to compare the percent dissimilarities between conspecific individuals to those between individuals of different species. This constituted a one-tailed test of the null hypothesis that interspecific variation was not greater than intraspecific variation, with a Bonferroni adjustment of $\alpha = 0.005$ for multiple comparisons. When dissimilarity between conspecific individuals was significantly lower than that between individuals of different species, fragrance profiles were considered "species-specific" (Levin et al., 2001). Diurnal and nocturnal scent data for each species were analyzed separately to address whether odors were more or less species-specific during day and night.

Acknowledgements

We are grateful to T. Holtsford and R. Kaczorowski (University Missouri, USA), J. Walker (Oxford University, UK), M. Pott and B. Piechulla (University of Rostock, GDR) for providing seeds and to R. Olmstead for discussions of *Nicotiana* phylogeny. Special thanks to A. Chetochine, A. Fox and N. Muhammad for plant care, to D.M. Light and R. Flath for gifts of standard compounds and to P. Kennedy and H. Surburg for insights on isophorone origins. Funding was provided by the US National Science Foundation, through grant DEB9806840 to McDade and Raguso and REU supplements supporting Holmberg and Foose. Further support was provided by small grants to Levin from the American Society of Plant Taxonomists, Sigma Xi, the Department of Ecology and Evolutionary Biology at the University of Arizona, and the University of Arizona Research Training Group in the Analysis of Biological Diversification (NSF DIR-9113362, BIR-9602246).

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