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Making sense of nectar scents: the effects of nectar secondary metabolites on floral visitors of *Nicotiana attenuata*

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Summary

Flowers produce a plethora of secondary metabolites but only nectar sugars, floral pigments and headspace volatiles have been examined in the context of pollinator behavior. We identify secondary metabolites in the headspace and nectar of glasshouse- and field-grown *Nicotiana attenuata* plants, infer within-flower origins by analyzing six flower parts, and compare the attractiveness of 16 constituents in standardized choice tests with two guilds of natural pollinators (*Manduca sexta* moths and *Archilochus alexandri* and *Selasphorus rufus* hummingbirds) and one nectar thief (*Solenopsis xyloni* ants) to determine whether nectar metabolites can 'filter' flower visitors: only two could. Moths responded more strongly than did hummingbirds to headspace presentation of nicotine and benzylacetone, the most abundant repellent and attractant compounds, respectively. For both pollinators, nectar repellents decreased nectaring time and nectar volume removed, but increased visitation number, particularly for hummingbirds. Fewer ants visited if the nectar contained repellents. To determine whether nicotine reduced nectar removal rates in nature, we planted transformed, nicotine-silenced plants into native populations in Utah over 2 years. Plants completely lacking nicotine in their nectar had 68–70% more nectar removed per night by the native community of floral visitors than did wild-type plants. We hypothesize that nectar repellents optimize the number of flower visitors per volume of nectar produced, allowing plants to keep their nectar volumes small.

Keywords: pollinators, nectar constituents, putrescine *N*-methyl transferase, secondary compounds, nicotine, VOCs.

Introduction

In many species, pollination succeeds only with the help of nectar-seeking pollinators and plants offer a variety of rewards to attract them. Floral nectar is the most common reward that plants produce (Simpson and Neff, 1983). Nevertheless, how plants manage to attract fitness-enhancing pollinators while simultaneously repelling nectar thieves that provide no pollination services is not well understood. Nectar is chemically complex; it contains primary metabolites such as sugars and amino acids that are thought to enhance plant fitness by attracting pollinators but also secondary metabolites, such as alkaloids, phenolics and non-protein amino acids (Baker, 1977, 1978) that are thought to repel nectar robbers (Stephenson, 1981). Non-sugar nectar constituents are thought to inhibit nectar consumption and floral visits by nectar robbers or less effective pollinators (Feinsinger and Swarm, 1978; Stephenson, 1982), but often these compounds are also toxic to nectar feeders and potential pollinators (Bell, 1971; Crane, 1977). In comparative studies, the repellent effects of nectar toxins on pollinators were found to be greater than their deterrent value to nectar robbers (Adler and Irwin, 2005); hence it is not clear if plants realize a fitness benefit from producing toxic nectars. Numerous hypotheses about the adaptive value of toxic nectars have been proposed (reviewed by Adler, 2000; Raguso, 2004). Toxic nectars could increase pollinator fidelity, repel nectar robbers, improve pollen transfer by intoxicating pollinators and increase nectar's 'shelf life' through its antimicrobial properties. By changing the cocktail of constituents in their nectar, plants might be able to favor some pollinators and discourage others. Alternatively, toxic nectars may have no adaptive value in a plant's interactions with floral visitors and may perhaps be simply an unavoidable consequence of protecting leaves and reproductive structures from herbivore attack with defensive

chemicals. In contrast to the plethora of hypotheses, the data available to test these potential functions are limited.

To test hypotheses about the function of nectar constituents, pollination biologists have added purified secondary metabolites to nectar or diluted naturally produced nectar with sugar solutions (Adler and Irwin, 2005; Tadmor-Melamed *et al.*, 2004). The data on how floral visitors respond to these manipulated nectars, have been interpreted as evidence for the influence of individual metabolites on the interaction between the plant and the floral visitor. Manipulated nectars either contain increased concentrations of a targeted metabolite beyond the range normally found in nature or they alter the concentrations of the other unmeasured metabolites in nectar while also frequently altering the standing volume of nectar in a flower, a trait which in laboratory studies has been shown to dramatically influence the interaction between the plant and the floral visitor by increasing herbivore oviposition rates (Adler and Bronstein, 2004). Genetic manipulation of nectar constituents has the potential to dissect the cocktail of metabolites that occur in nectar and provide a means of resolving the functional roles of secondary metabolites in nectar. However, very little is known about the origin of the metabolites that are commonly found in nectar. As this information is required to make a genetic approach successful, we examined their origins.

While the sugar constituents of nectar are frequently from nectaries located at the base of flowers (Fahn, 1979), the origins of the other secondary metabolites are largely unknown. The volatile organic compounds (VOCs) found in nectar are thought to be a hydrophilic subset of the compounds emitted by the surrounding floral tissues (Raguso, 2004). Given that flower scents are known to have both positive (Gabel *et al.*, 1992; Haynes *et al.*, 1991; Honda *et al.*, 1998; Raguso and Willis, 2002) and negative (Ômura *et al.*, 2000) consequences for interactions between plants and floral visitors, the function of VOCs in nectar is probably similarly complex. As with any tests of putative adaptive functions of traits that mediate a plant's ecological interactions, bioassays must be performed with relevant organisms.

Here we provide an analysis of a native plant's interactions with its floral visitors to elucidate the ecological and evolutionary consequences of nectar secondary metabolites. We first identified the constituents of the floral nectar of *Nicotiana attenuata* (Solanaceae). This diploid tobacco is native to the Great Basin Desert, and has been developed as a model system for studying the genetic basis of traits important for ecological interactions (Baldwin, 2001). By analyzing six different parts of the flower, in addition to the floral headspace, we inferred the origin of the nectar constituents. Next, we tested the preferences of representatives of three guilds of floral visitors, hawkmoths (*Manduca sexta*), hummingbirds (*Archilochus alexandri* and *Selasphorus rufus*) and ants (*Solenopsis xyloni*) for 16 individual nectar constituents at standard concentrations in solutions containing the sugars commonly found in nectars at their natural concentrations. Nicotine, an alkaloid synthesized in the roots of *N. attenuata* (Winz and Baldwin, 2001), was found to be the most abundant repellent in nectar. With choice trials in which scent compounds were presented either in nectar or headspace, we determined if the presentation influenced how attractive the compounds were to moths and hummingbirds. Finally, we characterized the nectar composition of genetically transformed plants expressing a key nicotine biosynthetic enzyme, putrescine-*N*-methyl transferase (PMT), in an inverted repeat orientation (Steppuhn *et al.*, 2004) to silence nicotine expression. These nicotine-silenced plants were planted into a native population of *N. attenuata* to determine whether the presence of this toxin in nectar influenced the removal of nectar by the plant's native floral visitor community. The results of these experiments which analyzed the interplay of different nectar secondary metabolites in attracting and repelling flower visitors inspired a new hypothesis for the presence of toxins in nectar: that they optimize the number of flower visitors per volume of nectar produced.

Results

Volatile organic compounds

Flower headspace, nectar samples and samples of the flower parts – corolla limb, corolla tube, gynecium plus nectary, anthers, stigma plus style, and sepals – were analyzed by solid-phase microextraction gas chromatography-mass spectrometry (SPME GC-MS). Additionally, we analyzed plant sap, which was expressed from stems. The accumulation of compounds in the different flower parts was standardized by adding fresh mass and comparing it with an internal standard.

We detected 16 VOCs that were common to both the floral and the nectar headspace of *N. attenuata* (Table 1, Figure 1). More than half of all compounds found in the headspace of intact flowers were also found in the SPME analysis of nectar (65.4%) or in the headspace of the six different flower parts (76.9%). One-fourth (23.1%) of all floral headspace compounds were not found again in any part of the flower and are probably secreted directly into the nectar. More than half (68.8%) of the nectar VOCs were detected in at least one of the analyzed flower parts.

Nectar was dominated by nitrogenous compounds, sesquiterpenes and aromatic alcohols and aldehydes, which appear to originate in different tissues. To identify the source of the different headspace and nectar constituents, the different parts of the flower were dissected and analyzed separately. Each part was found to release a distinct subset of the total VOCs (Figure 2). The total floral scent comprised primarily aromatic alcohols and aldehydes emitted from the

Table 1 Volatiles found in floral headspace, nectar and flower parts

Scent compounds	RT	Headspace	Nectar	Limb	Tube	Nectary	Anthers	Stigma	Sepals
Biosynthetic class									
Samples (field + greenhouse)		6 + 6	2 + 2	2 + 2	2 + 2	2 + 2	0 + 2	0 + 2	0 + 2
Total no. of compounds		26	35	18	11	11	9	9	16
Monoterpenes		**4.33**	**0.06**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**1.41**
β-Pinene	**7.02**								0.28
Limonene	**9.80**	1.40	0.06						0.15
trans-β-ocimene	**10.79**	0.02							0.37
Unknown monoterpene	**10.93**	0.50							
Terpinolene	**11.49**	2.41							0.61
Oxygenated monoterpenoids		**1.89**	**0.29**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**
1,8-Cineole	**10.04**	0.06							
Unknown monoterpene alcohol	**12.41**	1.09							
Linalool	**15.59**		0.29						
Geraniol	**20.14**	0.74							
Sesquiterpenes		**2.94**	**21.84**	**2.87**	**34.22**	**0.00**	**38.46**	**41.98**	**0.00**
Longifolene	**15.73**		0.92						
iso-Caryophyllene	**15.89**		0.55	0.03					
cis-α-Bergamotene	**16.09**	0.76	3.42	1.47	26.21		15.76	9.85	
unknown sesquiterpene	**16.18**		13.83	0.03	6.84		17.42	21.71	
trans-Caryophyllene	**16.22**	2.18	0.56	1.35				8.35	
β-Cedrene	**16.48**		0.55						
Unknown sesquiterpene	**16.60**		1.10						
Germacrene A	**19.08**	trace	0.93		1.17		2.96	2.07	
Farnesene	**19.11**						2.32		
Oxygenated sesquiterpenoids		**0.50**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**6.33**	**0.79**
Unknown sesquiterpene alcohol	**21.54**	0.50						6.33	0.79
Diterpenes		**0.00**	**5.10**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**
Phytol	**22.27**		5.10						
Aromatic alcohols, aldehydes		**63.07**	**18.64**	**79.87**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**
Benzaldehyde	**15.11**	6.11	2.01	0.33					
Benzylacetone	**20.32**	49.06	15.77	57.42					
Benzyl alcohol	**20.86**	6.38		1.02					
Phenyl ethyl alcohol*	**21.41**	0.62							
α-Methylbenzenepropanol*	**22.91**	0.90	0.85	21.09					
Aromatic esters		**0.20**	**2.46**	**0.00**	**0.00**	**21.16**	**0.00**	**0.00**	**0.00**
Methyl benzoate	**16.77**	0.15	0.16			0.08			
Ethyl benzoate	**17.10**					4.03			
Methyl salicylate	**19.22**	0.05	1.60			17.05			
Ethyl salicylate	**19.73**		0.70						
Fatty acid derivatives		**5.58**	**10.66**	**10.28**	**24.47**	**28.82**	**1.02**	**0.00**	**16.86**
trans-2-Hexenal	**10.31**		0.06						
Hexyl acetate	**11.45**	3.56	0.08	0.23					0.27
1-Hexanol	**12.70**		3.25	1.05	4.06	4.45			0.21
cis-3-Hexenyl propionate	**13.26**								1.19
cis-3-Hexen-ol	**13.31**	1.02	6.18	5.23	12.82	6.82	1.02		5.40
cis-3-Hexenyl isobutyrate	**13.35**				2.13				0.37
cis-3-Hexenyl butyrate	**14.22**	0.78	0.33	1.22	1.64	6.26			3.50
cis-3-Hexenyl-alpha-methyl-butyrate	**14.63**								0.43
Unknown c*is*-3-hexenyl ester	**16.72**	0.22	0.36	2.55	11.39	11.29			
cis-3-Hexenyl tiglate	**17.40**				3.82				5.49
cis-3-Hexenyl benzoate	**24.74**		0.40						
Nitrogenous compounds		**0.44**	**30.85**	**2.48**	**29.79**	**41.59**	**57.89**	**48.24**	**80.94**
Nicotine	**20.65**	0.44	23.39	2.41	29.79	41.59	37.55	32.84	59.45
Unknown aliphatic amine	**21.34**		1.03	0.06			13.68	11.37	5.91
Unknown aliphatic amine	**23.54**		6.42				6.66	4.03	15.58
Unknown compounds		**21.03**	**10.11**	**5.51**	**11.51**	**8.43**	**2.63**	**3.45**	**0.00**
A 67, 59, 75, 101, 82, 71	**13.16**		0.20						
B 57, 67, 70, 81, 82, 96	**13.46**	17.30	0.98						
C 95, 105, 108, 81, 67, 77	**14.81**	2.44	2.19	0.14		0.16			

Table 1 Continued

Scent compounds	RT	Headspace	Nectar	Limb	Tube	Nectary	Anthers	Stigma	Sepals
D 69, 83, 70, 84, 57, 82	**15.63**	1.29	0.69	1.83	11.51	6.02			
E 69, 97, 83, 84, 67, 57	**17.41**		0.31						
F 68, 96, 152, 67, 109, 137	**18.01**		0.45	2.54					
G 69, 83, 67, 82, 111, 97	**19.50**		3.22			2.26			
H 69, 83, 97, 67, 57, 111	**22.51**		2.06				2.63	3.45	

Compounds identified from floral and nectar headspace and from aqueous extracts of the flower parts, corolla limb, corolla tube, gynecium with nectary, anthers and filaments, stigma and style, and the sepals by SPME-GC-MS analysis. Compounds within biosynthetic classes are listed by retention time (minutes) with a DB-5 column. Mean percentages of total emission per sample were calculated from integrated peak areas. 'Trace' refers to compounds which represent <0.01% of total volatiles. The sum of all compounds obtained from one biosynthetic class are given in bold. Compounds were identified by comparing retention times and mass spectra with those of authentic standards, except for compounds labeled *, which were identified by comparison with spectra of a Saturn GCMS Workstation 5.2 database.

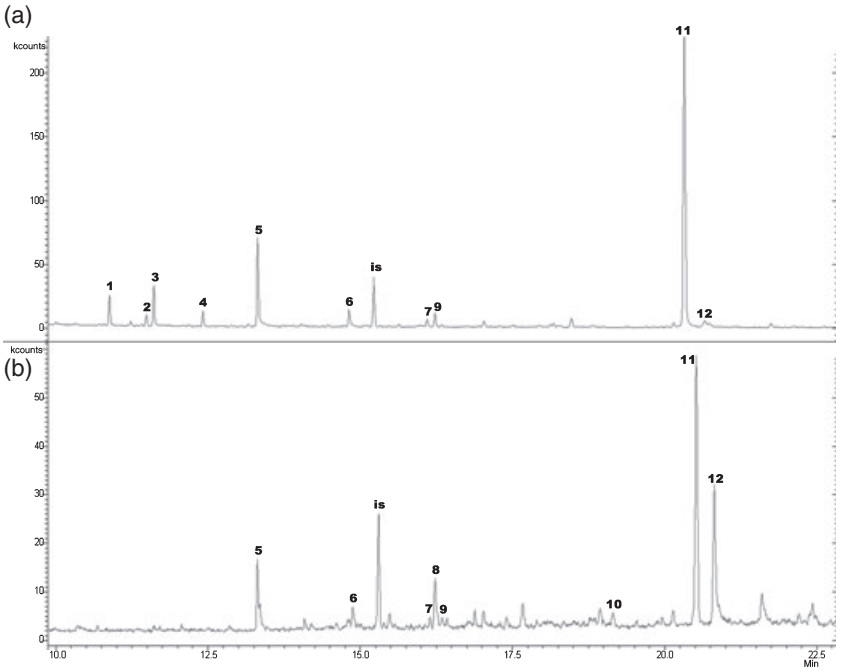

Figure 1. Gas chromatograms of *N. attenuata* headspace and nectar.
Floral headspace collected from one inflorescence (a) and a SPME analysis of a 150 μl bulk nectar collection (b).
Labeled compounds are: (1) *trans*-β-ocimen, (2) terpinolene, (3) hexyl acetate, (4) unknown monoterpene alcohol, (5) *cis*-3-hexenol, (6) *cis*-3-hexenyl butyrate, (7) *cis*-α-bergamotene, (8) unknown sesquiterpene, (9) *trans*-caryophyllene, (10) methyl salicylate, (11) benzylacetone, (12) nicotine, and (is) internal standard, tetralin.

corolla limb. The monoterpenes appeared to originate in the sepals and were not detected in any other flower parts. How monoterpenes reach the nectar is unknown, as there is no direct anatomical contact between the nectaries and the sepals. Sesquiterpenes were produced predominantly by the stigma, the anthers and the corolla tube. *cis*-α-Bergamotene was seven times more abundant in extracts of anthers and four times more abundant in extracts of the stigma than in extracts of the corolla tube, from which the majority of the floral headspace arises. Aromatic esters were released from the nectaries and apparently secreted directly into the nectar. Nicotine was detected in all flower parts as well as in the floral and the nectar headspace. The amount of nicotine in the nectar headspace was 80 times that of the floral headspace as determined by the percentage of the total peak area. The amount of nicotine in the different flower parts quantified as fresh mass specific accumulation was compared with that of the corolla limb (set at one): sepals (×101.54), nectary (×12.15), stigma (×4.84), anthers (×4.83) and corolla tube (×3.49). Fatty acid derivatives, also widespread in the flower parts, were associated mainly with the sepals. Twenty times more *cis*-3-hexenol, for example, was found in the sepals than in all other parts.

The analysis of plant sap identified compounds that might have originated in expressed xylem fluid. Phytol was the dominant compound, comprising 60% of the total peak area, with *trans*-caryophyllene, *cis*-3-hexenol and methyl salicylate all in a range of 1% of the total peak area. Two unknown aliphatic compounds at 6% and 2% of the total peak area and one unknown fatty acid derivative at 8% of the total peak area were also found in the nectar. Together more than 100 compounds were found, of which only the mentioned compounds had peak areas of >0.5% of the total.

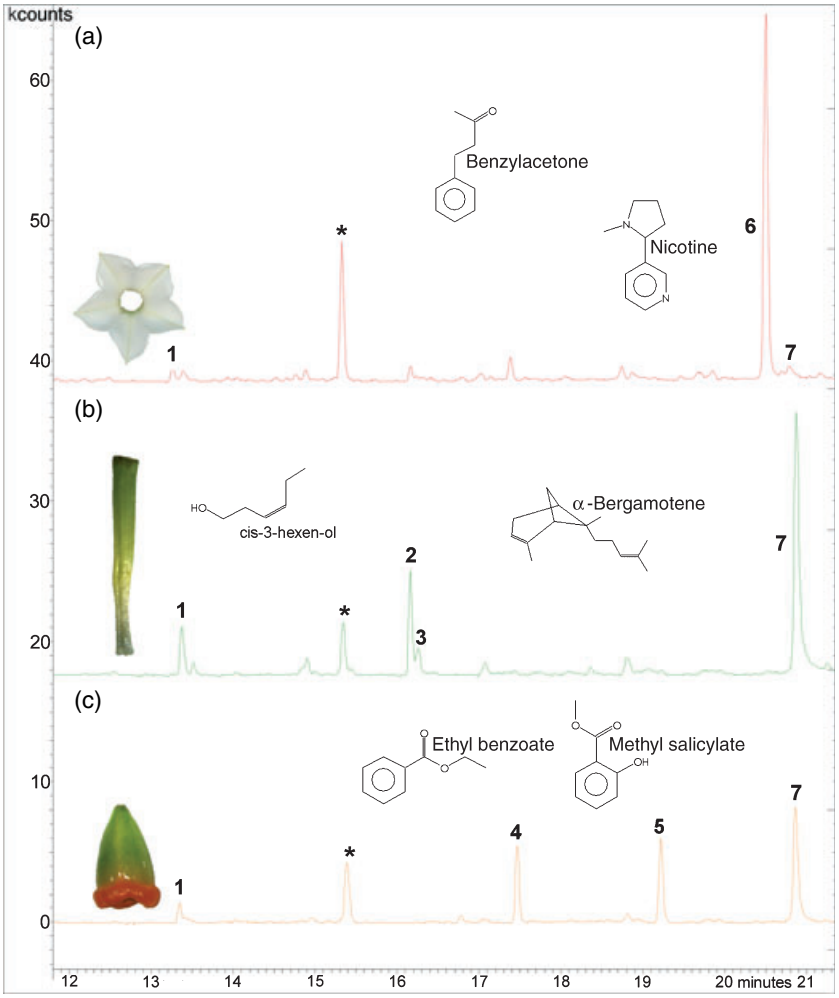

Figure 2. Gas chromatograms of SPME analysis of aqueous extracts.
SPME analysis of aqueous extracts of (a) corolla limb, (b) corolla tube, (c) gynecium with nectaries from *N. attenuata* flowers.
Labeled compounds are: (1) *cis*-3-hexenol, (2) *cis*-α-bergamotene, (3) unknown sesquiterpene, (4) ethyl benzoate, (5) methyl salicylate, (6) benzylacetone, (7) nicotine, (*) tetralin, internal standard.

Most oxygenated monoterpenoids and phenyl ethyl alcohols were only found in the floral headspace. Nine compounds (linalool, longifolene, β-cedrene, an unknown sesquiterpene, ethyl salicylate, *trans*-2-hexenal, *cis*-3-hexenyl benzoate and two unknown compounds) from a broad range of biosynthetic classes were found only in the nectar.

Floral headspace and nectar headspace share a majority of their constituent VOCs (Table 1). Approximately half of all compounds released into either the nectar or the headspace originated in a particular floral tissue: monoterpenes in sepal tissue; aromatic alcohols and aldehydes in corolla limb tissue; aromatic esters in nectary tissue; and some of the sesquiterpenes in corolla tube tissue. The other half, dominated by fatty acid derivatives and nitrogenous compounds, were found in all flower parts. Some compounds (25.7% of the total) were unique to the nectar or to the floral headspace.

Influence of nectar VOCs on pollinators

The preferences of the three natural flower visitors to *N. attenuata*, *M. sexta* (moths), *A. alexandri* plus *S. rufus* (hummingbirds) and *S. xyloni* (ants) (Figure 3), were tested for 16 VOCs of different biosynthetic classes found in the nectar or floral headspace. Each compound was tested individually at 0.1 mM in a 12.5% sucrose solution against a control sucrose solution in a circular arena with six (for moths, hummingbirds) or eight (for ants) equally spaced feeding stations. More than 60 h of observation time were required for the hummingbird trials and 110 h for the moth trials over a 2-month period. All bioassay organisms were both attracted and repelled by various nectar constituents (Figure 3). To compare the overall responses of the different visitors to all tested nectar constituents, we compared the ratio of the summed attractive compounds to the summed repellent compounds from one bioassay. Ratios of 1.0 reflect bioassay responses to the 16 nectar constituents in which the repellent and attractive compounds balance each other. With preference values of 203.7% for the constituents eliciting positive responses, the ants' response was largely neutral, compared to repellent values of 228.8% for the constituents eliciting avoidance responses, resulting in an overall ratio of 0.89. A similarly neutral ratio (0.99) was found for the hummingbirds [337.30(+)/339.54(−)], while moths

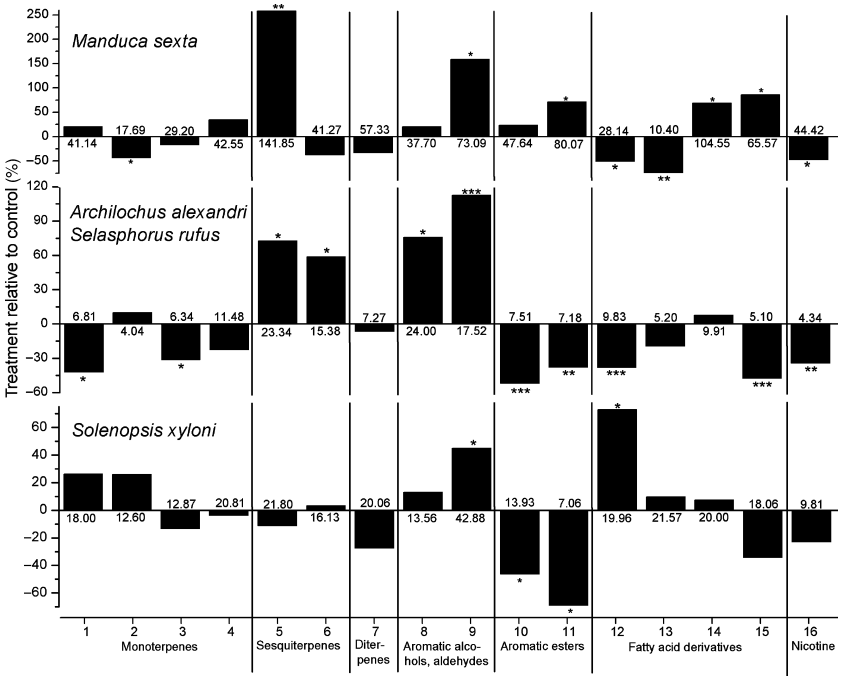

Figure 3. Relative attractiveness and repellency of volatile compounds found in *N. attenuata* flowers to three groups of flower visitors in standardized choice assays.

The relative attractiveness (+ values) and repellency (− values) of volatile compounds (organized by biosynthetic class) found in *N. attenuata* flowers to three groups of *N. attenuata* flower visitors (moths, *M. sexta*; hummingbirds, *A. alexandri* and *S. rufus*; and ants, *S. xyloni*) in standardized choice assays. Pure 12.5% sucrose solutions were used as a control nectar solution against which the same solution containing 0.1 mM volatile compound was compared.

Sixteen compounds were tested: (1) limonene, (2) ocimene, (3) linalool, (4) geraniol, (5) *cis*-α-bergamotene, (6) *trans*-caryophyllene, (7) phytol, (8) benzaldehyde, (9) benzylacetone, (10) methyl benzoate, (11) methyl salicylate, (12) 1-hexanol, (13) *cis*-3-hexenol, (14) *cis*-3-hexenyl benzoate, (15) *cis*-3-hexenyl butyrate, (16) nicotine. Mean (SE) values of the controls defined as 100% and treatment values are shown relative to the control values. The mean value of treatments (in seconds nectaring for moth and hummingbird bioassays and the number of ants feeding for ant bioassays – see Experimental procedures for details – are given by the bar). Significant differences between treatment versus control nectar as determined with Student's *t*-test for *M. sexta*, Mann–Whitney test for *A. alexandri* and *S. rufus*, and paired Student's *t*-test for *S. xyloni* are designated by the number of stars by each bar (*$P \leqslant 0.05$, **$P \leqslant 0.001$, ***$P \leqslant 0.0001$).

were largely attracted – a ratio of 2.50 [741.76(+)/297.28 (−)] – to the constituents. In summary, *M. sexta* was largely attracted by the VOC constituents of *N. attenuata* nectar, while the overall responses of ants and hummingbirds were largely neutral.

Although some compounds were more likely to influence pollinator behavior than others, compounds from the same biosynthetic class tended to evoke similar responses. The monoterpenes did not seem to tempt any floral visitors, evoking responses that were not statistically different from the control sugar solutions. To hummingbirds, limonene and linalool were repellent. Sesquiterpenes were attractive to the two pollinators and did not influence the ants. *cis*-α-Bergamotene was the most attractive compound tested for moths (Student's *t*-test, $t_{21} = 5.53$, $P < 0.0001$). Aromatic alcohols and aldehydes were attractants in all tested bioassays. All species were significantly attracted by benzylacetone. An interesting pattern was seen in the group of aromatic esters. Hummingbirds (Mann–Whitney *U*-test, $U = 919.50$, $P = 0.0002$, $n = 112$) and ants (paired Student's *t*-test, $t_{15} = 3.37$, $P = 0.004$) were significantly repelled by methyl salicylate, while moths were attracted to it ($t_{39} = 2.38$, $P = 0.022$). *cis*-3-Hexenyl benzoate was also significantly attractive to *M. sexta* ($t_{23} = 2.28$, $P = 0.032$). No clear picture emerged with regard to fatty acid derivatives. Hummingbirds were largely repelled by these compounds, while moths and ants responded very differently to compounds in this biosynthetic class. Ants were attracted to 1-hexanol ($t_{23} = -2.75$, $P = 0.012$), whereas moths were repelled by it ($t_{23} = 2.31$, $P = 0.030$). The opposite was the case for *cis*-3-hexenyl butyrate. *cis*-3-Hexenol also had a repellent effect on *M. sexta* ($t_{25} = 3.86$, $P < 0.001$). Nicotine repelled all of the floral visitors.

Floral visitors reacted differently to a few particular nectar constituents but the responses in all three bioassays were similar for a quarter of all tested compounds. The sesquiterpene, *cis*-α-bergamotene, and the aromatic ketone, benzylacetone, were strongly attractive to the pollinators *M. sexta*, *A. alexandri* and *S. rufus*. The aromatic ester, methyl salicylate, and the fatty acid, *cis*-3-hexenyl benzoate, which were uniquely preferred by *M. sexta*, and the fatty acid 1-hexanol, which only attracted *S. xyloni*, are the compounds which plants could use to filter or select particular floral visitors. Collectively, these results suggest

that plants produce only a few metabolites with which they could both favor particular pollinators and discourage nectar thieves and robbers.

Taste versus odor

Benzylacetone and nicotine were shown to attract and repel, respectively, both pollinator guilds: hawkmoths and hummingbirds. Moreover, both compounds are found in both the floral headspace and nectar, albeit in different concentrations (Table 1). In the floral headspace nicotine represented only 0.4% of the total peak area, but in the nectar it represented 23.4%. Benzylacetone was the main compound in the floral headspace with 49.1% of the total peak area, but was only a minor (15.8%) constituent of the nectar. To compare the relative importance of attractant and repellent compounds in the headspace with those in nectar in different combinations, artificial flowers were fitted with cotton wool collars around the lip of their corollas. These cotton collars were impregnated with scented aqueous solutions to enhance the headspace presentation of the metabolites (Figure 4). Both hawkmoths and hummingbirds preferred benzylacetone to nicotine when these were presented in the nectar, as evidenced by the longer nectaring times at benzylacetone-containing solutions [Student's *t*-test, $t_{19} = 3.21$, $P = 0.0046$ (hawkmoths); Mann–Whitney *U*-test, $U = 1184.0$, $P < 0.0001$, $n = 176$ (hummingbirds); Figure 5]. Interestingly, in hummingbirds taste was more important than olfaction for attractants (see below).

Both pollinators preferred benzylacetone when benzylacetone and nicotine, rather than pure sugar solutions, were presented in the headspace (1 M) [$t_{20} = 2.40$, $P = 0.026$ (hawkmoths); $U = 1616.0$, $P = 0.040$, $n = 128$ (hummingbirds)], and *M. sexta* responded similarly when less concentrated solutions were used to create the headspace (1 mM) ($t_{35} = 2.39$, $P = 0.022$). To determine whether the discrimination was primarily driven by attraction to benzylacetone or aversion to nicotine, both compounds were tested against non-scented control flowers. Hummingbirds did not discriminate between benzylacetone and the flowers lacking scent ($U = 1855.0$, $P = 0.480$, $n = 129$) when these were presented only in the headspace, but decreased their nectaring time by 80% when nicotine was offered in the headspace ($U = 298.5$, $P < 0.0001$, $n = 127$). Moths' responses to headspace presentations were different. Flowers with benzylacetone-scented headspace were visited 1.5 times longer than were the untreated controls ($t_{23} = 1.24$, $P = 0.229$) and feeding times did not differ between nicotine- and non-scented headspace flowers ($t_{22} = 0.73$, $P = 0.4754$). Two experiments were conducted to compare headspace and nectar presentation of the two compounds. Benzylacetone-scented nectar coupled with a nicotine-scented headspace was compared with a nicotine-scented nectar coupled with a benzylacetone-scented headspace. *Archilochus alexandri* and *S. rufus* responded more strongly to the constituents of the nectar than to the headspace. Visits to flowers with benzylacetone-scented nectar were 1.5 longer than visits to flowers with a benzylacetone-scented headspace, despite the nicotine headspace of the former and the

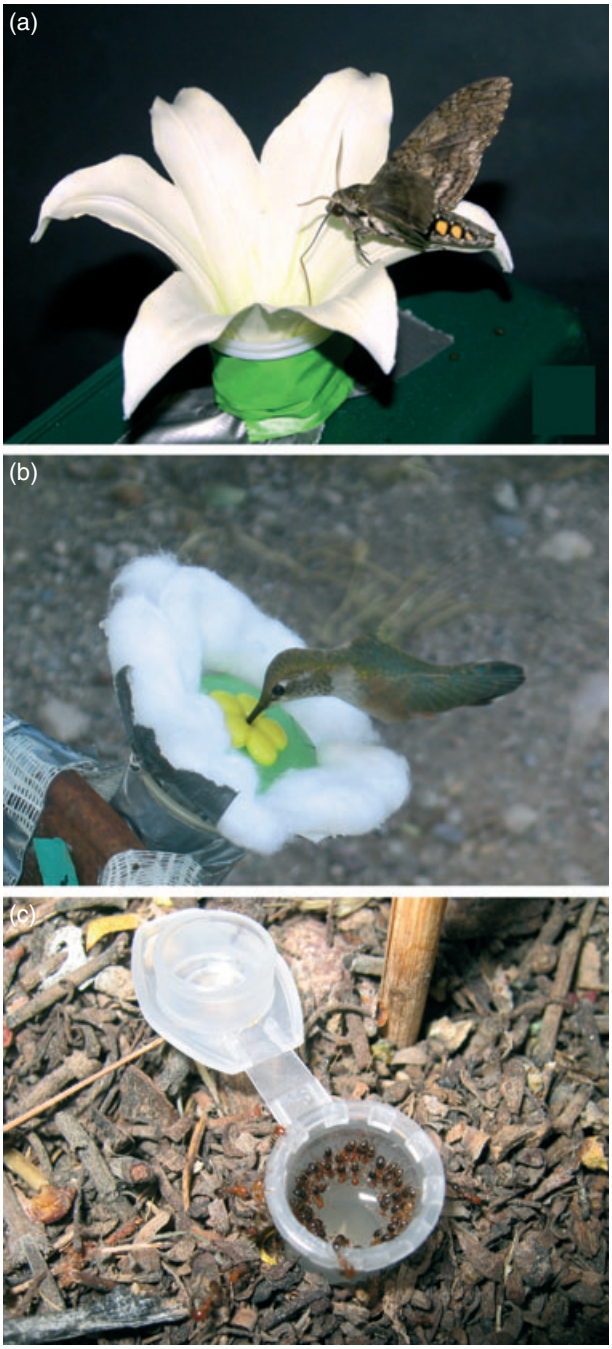

Figure 4. Flower visitors at the experimental bioassays used to collect data presented in Figure 3.
(a) *Manduca sexta*, (b) *A. alexandri*, (c) *S. xyloni*. Note the cotton fringe in (b) used to compare repellency/attractiveness of volatiles presented in the headspace versus the nectar (see Figure 5).

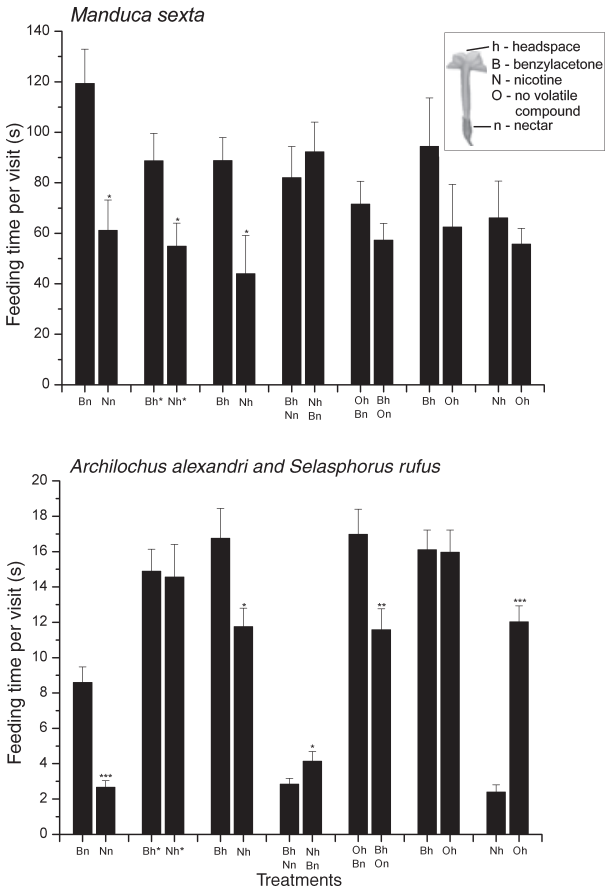

Figure 5. Influence of headspace (h) versus nectar (n) presentation of two constituents present in both the nectar and headspace of *N. attenuata* flowers.

(a) For hummingbirds and (b) for *M. sexta* moths: nicotine (N) and benzylacetone (B). Values are mean (+SE) seconds spent nectaring; significant differences (*$P \leqslant 0.05$, **$P \leqslant 0.001$, ***$P \leqslant 0.0001$) in feeding times for the paired treatments (see legend for all permutations of treatments) were determined by a Mann–Whitney *U*-test for tests with hummingbirds and by a Student's *t*-test for tests with moths. Each comparison consisted of 21–37 (moths) or 86–176 (hummingbirds) replicate bioassays. For nectar 0.1 mM solution and for the headspace 2 ml of 1 M solution (except * = 0.1 M) of the compound was used.

nicotine nectar of the latter ($U = 1044.5$, $P = 0.024$, $n = 107$). *Manduca sexta* did not discriminate in this comparison ($t_{21} = -0.58$, $P = 0.568$). Similar results were obtained when one treatment contained a pure sugar solution with a benzylacetone-scented headspace and the other a benzylacetone-scented nectar with no headspace [$t_{29} = -1.27$, $P = 0.213$ (hawkmoths); $U = 1142.0$, $P = 0.0009$, $n = 119$ (hummingbirds)].

In summary, hummingbirds and moths preferred flowers with benzylacetone to those with nicotine in either the nectar or the headspace. Although nectar constituents influenced both species, the moths were more strongly affected by headspace constituents. Only high concentrations of nicotine in the headspace influenced *A. alexandri* and *S. rufus*. Flower visitors clearly showed preferences by increasing nectaring times.

Correlation between visitation rate and visitation time

Analysis of the number of visitors and the time of nectaring from all trials conducted in this study reveals that in a majority of the 23 bioassays with both hummingbirds (48%) and hawkmoths (70%), longer nectaring times were associated with fewer visitations (Table S1). For example, in an hour the nicotine treatment received 112 visits compared with 64 for the benzylacetone treatment if compounds were presented in nectar. When hummingbirds are repelled by a nectar constituent, they immerse their beaks for less than a second, withdraw, 'spit' and move to the next feeding station. In contrast, when hummingbirds are attracted, nectaring bouts last longer (15–25 sec; Figure 3), resulting in fewer visitors per hour. If all trials which revealed an attractive or deterrent response to a pollinator are analyzed together, mean nectaring time and visitation frequency were inversely correlated for both pollinators [$y = -3.1827x + 1.3088$, $R^2 = 0.3818$ (hawkmoths), $F_{1,7} = 3.9$, $P = 0.0941$; $y = -0.939x - 3.6833$, $R^2 = 0.7755$, $F_{1,10} = 31.1$, $P = 0.0003$ (hummingbirds)]. For moths, the trial with *cis*-α-bergamotene was excluded from this calculation as the pattern differed from all other trials. This difference may result from the adaptation by *M. sexta* to *cis*-α-bergamotene as an oviposition deterrent as this sesquiterpene is a major compound in herbivory-induced leaf volatiles (Kessler and Baldwin, 2002). When trials in which headspace presentation treatments are included in the analysis, the negative relationship is lost [$y = -0.1134x + 25.973$, $R^2 = 0.0004$ (hawkmoths); $y = -0.002x + 0.8137$, $R^2 = 0.00006$ (hummingbirds)]. Moreover, nectar removal rates are strongly correlated with nectaring times for both pollinator guilds [$y = 0.0049x + 0.4351$, $R^2 = 0.7848$ (hawkmoths); $y = 0.0087x + 0.1701$; $R^2 = 0.8327$ (hummingbirds)] and hence plants may receive more visits per volume of nectar produced when repellents are added to the nectar.

Response of native pollinators to plants silenced for putrescine-N-methyl transferase (irPMT plants)

Nicotine-silenced irPMT plants were used to test the consequences of a lack of the toxin, nicotine, on flower visitors in native populations. No differences in nectar volume between wild-type (WT) and irPMT plants were found when inflorescences were enclosed in plastic bags overnight during the time of peak flower visitation [1.9 ± 0.1 μl (SE) (WT); 2.0 ± 0.2 μl (irPMT) paired *t*-test, $t_{11} = 0.66$; $P = 0.5228$] or enclosed in mesh bags to allow for normal evapotranspiration [1.0 ± 0.1 μl (WT) 1.1 ± 0.1 μl (irPMT); $t_{12} = 0.17$; $P = 0.8716$; Figure 6]. The removal of nectar by floral visitors in uncovered plants was significantly higher in irPMT plants

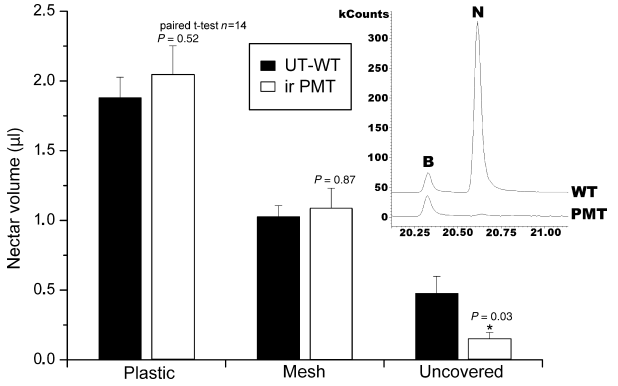

Figure 6. Nectar removal in nicotine-silenced irPMT plants.
Nicotiana attenuata wild-type plants (WT) were compared in nectar production by covering inflorescences with plastic bags (plastic), in nectar evaporation by covering plants with mesh covers (mesh), and in rates of nectar removal by the native community of flower visitors by leaving inflorescences uncovered (*$P \leq 0.05$; paired Student's *t*-test). Data shown represent mean (+SE) nectar volume per flower. The chromatogram demonstrates the absence of nicotine (N) in the line, while all other scent compounds matched concentrations found in WT plants [benzylacetone (B) as an example].

compared with WT plants ($t_{13} = -2.5$; $P = 0.0269$). irPMT plants had a mean nectar volume of only 0.2 ± 0.1 µl remaining in the morning compared with 0.5 ± 0.1 µl in WT flowers. The experiment was repeated the following year with the same results: no difference between flowers bagged in plastic [1.9 ± 0.5 µl (SE) (WT); 2.0 ± 0.4 µl (irPMT); paired *t*-test, $t_4 = 0.28$; $P = 0.7903$]; while uncovered, flowers of irPMT (mean 0.2 ± 0.1 µl) plants had more nectar removed than did uncovered flowers of WT plants (mean 0.6 ± 0.2 µl; $t_6 = -1.5$; $P = 0.1807$).

Discussion

Floral nectars are rarely pure sugar solutions used to buy pollination services from mobile floral visitors. They frequently contain a broad spectrum of metabolites, which may either influence a flower's chances of being visited by useful pollinators or repel nectar thieves or robbers (Blem and Leann, 1999; Gardener and Gillman, 2002; Stephenson, 1981). The idea that plants add toxins to their nectar to 'filter' their floral visitors is not new. Many studies report that nectar can be toxic or repellent to animals (reviewed by Adler, 2000) and describe the presence of compounds thought to be toxic. In one elegant example of a functional analysis of a nectar toxin, Adler and Irwin (2005) supplemented and diluted a nectar alkaloid to examine the consequences of altered concentrations on floral visitors. However, there have been few unbiased studies of the function of floral chemistry or morphology. Most studies start with preconceptions of which chemicals are repellents or attractants and which shapes and colors fit into particular pollination syndromes (Stebbins, 1970; Waser, 1996), opinions that are largely informed by the researchers' sensory perceptions. The observation that the most common sugar constituent of floral nectar, sucrose, which is preferred by most nectar feeders, can repel specialist nectar-feeding ants (Heil *et al.*, 2005) should counsel caution when inferring function from an anthropocentric sensory bias. The tools of molecular biology offer the possibility of removing this bias from the analysis, making it possible to genetically dissect a plant's phenotype and thereby giving researchers the tools to query the organisms that interact with the flower about the consequences of the manipulation.

By systematically characterizing the constituents of nectar, inferring how they arrived in the nectar from their distribution in different flower parts, and testing each constituent in bioassays with relevant floral visitors, we identified one metabolite of 35 (nicotine) which was consistently repellent and three compounds (methyl salicylate, *cis*-3-hexenyl benzoate and 1-hexanol) which could function as selective filters based on the responses of two groups of pollinators (hawkmoths, hummingbirds) and one nectar thief (ant) that were used in the bioassays. To determine whether the repellency of nicotine in the bioassays translated into decreased nectar removal by the natural community of floral visitors, we grew transformed plants silenced in nicotine production in native populations.

Nicotine comprised 25% of the nectar scent constituents and 1% of the floral headspace, was deterrent to the pollinating species used in the bioassays, and decreased nectar removal in nature. The presence of general deterrent compounds such as nicotine in nectar would seem counterproductive to the function of a flower. Nicotine is the most abundant alkaloid of *N. attenuata* and known to be an efficient defense against herbivores (Steppuhn *et al.*, 2004). Is its presence in nectar an unavoidable consequence of this defense mechanism, which entails nicotine synthesis in the roots and transport to the shoot in the xylem stream (Baldwin *et al.*, 1994)? Does it have a different function in the nectar?

The data presented here suggest an alternative hypothesis: that it maximizes the number of visits by nectar collectors per unit nectar produced. This hypothesis emerged from the behavior of pollinators when the deterrent was presented in the nectar along with the universally attractive compound benzylacetone. The presence of the deterrent doubled the number of visits from hummingbirds. The function of nectar is to maximize the number of visits per volume of nectar produced, not to maximize the volume of nectar removed, as becomes apparent when considering the relatively small nectar reward offered by *N. attenuata* flowers. This plant seems to follow the strategy that sugar is most efficiently utilized if it is present in sufficient quantities to attract and hold the attention of a pollinator but in small enough quantities to force pollinators to visit a large number

of flowers (Baker, 1975). Nectar production is a process that continues for the duration of the activity periods of hawk moths (dusk, midnight and again at dawn; Madden, 1945) and again for the daylight hours when hummingbirds are active (Aigner and Scott, 2002; Baker, 1961; Wells, 1959). Increasing nectar volume had the clearly deleterious consequence of increasing the oviposition rate of native hawkmoth pollinators (DK and ITB, unpublished data), as had been shown in laboratory studies with cultivated tobacco (Adler and Bronstein, 2004).

The hawkmoth and hummingbird bioassays demonstrated that nectar presentation was more important than headspace presentation, which suggests that these pollinators must taste the nectar before recognizing the deterrent. *Manduca sexta* did not discriminate between a nicotine headspace and a non-scented control, and is known not to distinguish between scented and scentless flowers bathed in the same odor plume (Raguso and Willis, 2002). Pollinators are likely to learn to associate an attractive scent with a deterrent taste and seek alternative nectar sources. However, *N. attenuata*, which grows in large monoculture stands in the post-fire environment (Baldwin *et al.*, 1994), may dominate the available sources of nectar in these habitats. Moreover, *M. sexta* may be a 'loyal' pollinator, as *N. attenuata* is the principal native host plant for the larvae of the moths in these habitats. Larvae are able to tolerate large quantities of nicotine in their diet, but prefer, and grow faster on, nicotine-free plants (Steppuhn *et al.*, 2004), and adult moths may be evaluating the quality of a potential host plant for oviposition by tasting the quality of its nectar. Given that oviposition rate increases after experimentally increasing nectar volume (Adler and Bronstein, 2004; DK and ITB, unpublished data) host plant assessment is a likely explanation. The nectar addition experiments probably diluted the nicotine content in the nectar, and hence it remains unclear whether the increase in the volume alone is responsible for the increase in oviposition rate. When comparing the responses to the complete chemical inventory of the nectar, *M. sexta* is more attracted than either the hummingbirds or the ants to *N. attenuata*'s nectar, and this may reflect its more intimate relationship with *N. attenuata*.

The hypothesis that nicotine functions to increase the number of flower visitors and thereby the outcrossing rate of flowers by decreasing the amount of nectar removed per visit will require a substantially larger effort to falsify. The experiments conducted in this study demonstrated that this bitter-tasting alkaloid plays an important role in decreasing nectar consumption, and provide incontrovertible evidence that a single compound can profoundly influence nectar removal. The study also establishes a set of experimental procedures for carrying out an unbiased functional analysis of floral traits. Future research will focus on silencing the production of specific attractants, such as benzylacetone, which, when combined with the nicotine-silenced irPMT transformant, will allow for a rigorous test of the 'push–pull' (Khan *et al.*, 2000) functional hypothesis for the secondary metabolites of floral nectar. The challenge will be to measure gene flow and seed-set in plants silenced in the production of these attractive or repellent compounds in addition to measuring nectar removal and pollinator visitation rates, assuming regulatory approval for these experiments.

Like nicotine, many of the compounds found in nectar are found in other plant parts and hence their potential function in the flower may be shaped by roles played in these other plant parts. For example, the sepals appear to contribute the monoterpenes and some of the fatty acid derivatives and nitrogenous compounds to the headspace and nectar, and these compounds are known to play a role in leaf defense. The release of monoterpenes such as limonene or fatty acid derivatives such as *cis*-3-hexenol from leaves is dramatically increased after herbivore attack; these compounds function as indirect defenses by helping natural enemies locate and attack feeding herbivores on plants (Kessler and Baldwin, 2002; Loughrin *et al.*, 1994). Terpenoids, which are also known to have antimicrobial (Himejima *et al.*, 1992) and direct antiherbivore functions (Phillips and Croteau, 1999), are commonly found in both floral odors and vegetative tissues (Dudareva and Pichersky, 2000). However, some compounds are uniquely produced by the flower, which suggests flower-related functions, such as pollinator attraction or defense against nectar robbing. Aromatic alcohols and aldehydes (benzylacetone, benzaldehyde, benzyl alcohol) were only emitted by the corolla limb and aromatic esters (methyl benzoate, methyl salicylate, ethyl benzoate) by the nectary. Compounds that are uniquely found in the nectar may have nectar-specific functions.

A principal objective of this study was to determine if individual nectar constituents could selectively attract or repel particular floral visitors. Just as host shifts in herbivorous insects are thought to be caused by reduced sensitivity to repellent compounds (Schoonhoven *et al.*, 1998), so may shifts in pollinators be similarly mediated. The bioassay organisms had similar responses to almost a third (31%) of the floral compounds and *M. sexta*'s response to 81% of the compounds was largely positive or neutral. Key compounds that attract just one group of pollinators may be particularly important in the evolution of nectar traits (Lau and Galloway, 2004) but only a few compounds attracted only one floral visitor. 1-Hexanol attracted the nectar-robbing ants and repelled the pollinating moths and hummingbirds and thus appears to be a maladapted constituent of floral nectar. However, plants could realize a fitness benefit if this compound were deployed in extrafloral nectar, which in many plants functions to recruit protective ants. Aromatic esters (methyl benzoate, methyl salicylate) may represent key compounds for moth-pollinated plants, as *M. sexta* was uniquely attracted by these and structurally similar compounds. Morgan and Lyon (1928) reported that amyl salicy-

late applied to artificial flowers attracted *M. sexta*, which is known to show strong electroantennogram responses to methyl salicylate (Fraser *et al.*, 2003). The aromatic esters of *N. attenuata* are mainly derived from the nectary, as has also been shown for methyl benzoate production in *Oenothera primiveris* (Raguso, 2004); flavoring the nectar with these structures may make these flowers attractive to hawkmoths. The aromatic alcohols and ketones (benzaldehyde, benzylacetone), which together made up 60% of the constituents in the floral headspace according to peak area and almost 20% of the nectar constituents, were attractive in all bioassays. Both pollinators were attracted by benzylacetone, and if a plant were to be able to attract one pollinator species rather than the other, additional metabolites would be required. Benzaldehyde and *trans*-caryophyllene were only attractive to hummingbirds, and the monoterpenes, limonene and linalool, were uniquely deterrent. Few monoterpenes were detected in *N. attenuata* nectar, all in low concentrations and all with little effect on the behavior of hawkmoths and ants. Hummingbirds may find these compounds repellent because they are adapted to non-scented flowers (Knudsen *et al.*, 2004), but increasing monoterpene concentrations may be a way for a plant to favor hawkmoths over hummingbirds as pollinators. Altogether the nectar of *N. attenuata* with all its constituents has the potential to filter flower visitors, favoring some and deterring others.

Regardless of their origins, all nectar constituents have the potential to influence the role of floral nectar, which is presumably to maximize outcrossing rates. This potential role can only be determined by experimentally manipulating the constituents and examining the consequences for the plants' Darwinian fitness. Since flowers may have functions other than to mediate outcrossing during sexual reproduction, particularly for a self-compatible plant such as *N. attenuata* (Sime and Baldwin, 2003), the analysis should remain open to these other potential roles. Flowers, for example, may attract natural enemies and reduce herbivore loads on plants (Sabellis *et al.*, 2005). Just as the manufacturers of soft drinks protect their formulas and strive for constancy so as not to lose market share, only altering their recipes in accordance with the dictates of global sales, plants will evolve and incorporate ingredients into their nectar recipes in response to the dictates of their Darwinian fitness. The challenge for plant biologists is to design experiments which will uncover potential functions without observer bias. Nectar, which was thought to be nature's soft drink, may not be so soft after all.

Experimental procedures

Plant material and transformation

Nicotiana attenuata Torr. ex Watson (synonymous with *Nicotiana torreyana* Nelson and Macbr.; Solanaceae) grown from field-collected seeds (Baldwin, 1998) and inbred for 17 generations in the glasshouse were used for transformation and all experiments. Seed germination and the *Agrobacterium tumifaciens* (strain LBA 4404)-mediated transformation procedure is described in Krügel *et al.* (2002). In order to silence the expression of the two *N. attenuata pmt* genes, plants were transformed with pRESC5PMT, which contain a gene fragment of *pmt1* (which has 95% identity to *pmt2*) twice in an inverted orientation separated by intron 3 of the *Flaveria trinervia* gene *pyruvate orthophosphate dikinase* (*pdk*). Seeds from the T_4 generation of a homozygous line harboring a single IRpmt insert that was fully characterized in Steppuhn *et al.* (2004) and shown to have drastically reduced transcripts of both PMT genes and to produce no detectable quantities of nicotine but otherwise have a WT growth phenotype were used in this study. irPMT plants were analyzed for the presence and quantity of floral volatiles by collecting volatiles from inflorescences for 8 h (Halitschke *et al.*, 2000). All compounds found in the floral scent of WT plants, with the expected exception of nicotine (Student's *t*-test, $t_{14} = -4.86$, $P < 0.001$), could be detected in statistically equivalent amounts in the floral scent of transformed plants (limonene $t_{14} = 0.44$, $P = 0.67$; terpinolene $t_{14} = -0.54$, $P = 0.60$; *cis*-3-hexenol $t_{14} = -1.67$, $P = 0.12$; *cis*-α-bergamotene $t_{14} = 0.45$, $P = 0.66$; unknown sesquiterpene $t_{14} = 0.48$, $P = 0.65$; methyl salicylate $t_{14} = 1.33$, $P = 0.21$; benzylacetone $t_{14} = 0.77$, $P = 0.46$). We also examined nectar metabolites. All compounds found in WT nectar, again with the exception of nicotine, were detected in the transformants in amounts comparable to those found in WT plants. Sugar concentrations in nectar did not differ among lines (Student's *t*-test, $t_{24} = 0.32$, $P = 0.75$).

IRpmt plants and WT plants were transplanted at the rosette stage into a native population that grew in an area that had burned in 2003 and contained >200 000 plants during the 2004 growing season. Plants were bagged to exclude floral visitors during flowering-stage growth except for the experimental period, after which all transplanted plants were removed from the population. The release was regulated by USDA APHIS notification number 04-344-06n and compliance with 7 CFR 340.3 (c) was met by collecting seed capsules from neighboring plants that could have been pollinated by the transformed plants. In 2006, irPMT and WT plants were planted into a field plantation at the Lytle Ranch Preserve, Santa Clara, UT, as regulated by USDA APHIS notification number 06-003-08n.

IRpmt nectar removal experiment

Fourteen pairs of same-size and same-stage IRpmt and WT plants growing 1–4 m apart were selected. Stems of all plants were trimmed just after elongation to create plants with similar branching patterns and growth form. During the experimental period, one inflorescence from every plant was bagged in either a translucent plastic bag (Plastibrand, Wertheim, Germany) or a mesh bag (Breather plant bags '80 × 20 × 24'; Kleen Test Products, Brown Deer, WI, USA), or left uncovered at 18:00. In the morning between 05:00 and 07:00, the nectar volume and sugar concentration of two flowers per branch were measured and the average of both was used as a biological replicate for each treatment. Nectar was collected by inserting a clean 25 μl glass capillary into the corolla tube until it reached the base of the nectaries. With practice, a complete nectar sample could be obtained by holding the capillary with one hand and the corolla tube with the other and removing the tube against the counter pressure of the inserted capillary. Nectar volume was measured in milliliters in the capillary and converted into microliters from calibrated volume samples. The sugar concentration was measured with a portable refractometer (Optech, Sliedrecht, the Netherlands) with a range from 0% to 32% and a

resolution of 0.2%, by blowing the nectar out of the capillary directly on the measuring surface, which was cleaned after every measurement with distilled water. Flowers enclosed in plastic and mesh bags were used to determine if there were differences in nectar production and evaporation among genotypes. Since nectar in *N. attenuata* flowers is produced from dusk until midnight (DK and ITB, unpublished data), a phenology which supplies nectar between the two main activity periods of the two groups of pollinators for this plant, moths (Madden, 1945) and hummingbirds (Aigner and Scott, 2002), we measured volume and sugar concentration shortly after flowers discontinued their nectar production at 04:00. In these plant populations, Sphingidae moths and Noctuidae moths were the most frequent visitors to *N. attenuata* flowers after hummingbirds. Nectar-thieving carpenter bees did not influence the experiment in 2005 because they occurred only in small numbers, but in 2006 they were more abundant and all flowers visited by carpenter bees were excluded from the analysis.

Flower headspace analysis

Inflorescences with at least six flowers were covered with translucent plastic bags and the headspace volatiles were trapped on 12.7 ± 0.2 mg of 100-mesh activated charcoal secured in borosilicate glass Pasteur pipettes with fine pore polyester packing foam. Air was pulled from the bags through the traps at 400–500 ml min^{-1} in an open-flow trapping design (Halitschke *et al.*, 2000). Traps were stored at −20°C until extraction. Compounds were eluted from the traps with 250 μl of CH_2Cl_2 and 1 μl aliquots were analyzed by capillary gas chromatography (GC) with a Varian Star 3400cx GC and a Varian Saturn 2000 MS (Palo Alto, CA, USA) with a fused-silica column of 0.25 μm stationary phase thickness (DB-WAX; J & W Scientific, Folsom, CA, USA). The GC was programmed as follows: injector held at 225°C, initial column temperature at 45°C held for 6 min, and subsequently ramped to 10°C min^{-1} to 180°C and with 20°C min^{-1} to 230°C, held for 5 min and finally to 20°C min^{-1} to 250°C, held for 5 min. Compounds were identified by comparing retention times and mass spectra with those of authentic standards and by comparing mass spectra with spectra of a Saturn database (Halitschke *et al.*, 2000). Fragrance standards were from Sigma-Aldrich (Darmstadt, Germany) and *cis*-α-bergamotene was purified by HPLC using oil of *Opoponax chironium* (Halitschke *et al.*, 2000). Peak areas were integrated with the Varian software (MS Workstation 6.41) and expressed as percentages of total emission per sample.

Analysis of nectar volatiles

For the nectar analysis, bulk collections of 150 μl nectar per sample collected from ~100 flowers between 05:00 and 07:00 were used. The nectar sample was combined with 2 μl of a 2 ng μl^{-1} tetralin standard solution in a 1.5-ml GC vial with a 3-mm stir bar and crimp-sealed. Solid-phase microextraction (Mills, 2000) fibers (100 μm polylydimethylsiloxane coating; Supelco, Bellefont, PA, USA) were exposed to equilibrated headspace air for 90 min while the nectar sample was heated to 60°C and stirred. The equilibrated SPME fibers were inserted into a GC-mass spectrometry (MS) injector port and volatiles were analyzed as described for the floral headspace samples. Standard curves of benzylacetone [$y = (3 \times 10^8)x$ [mM] − 59 345; $R^2 = 1.0$], nicotine [$y = (2 \times 10^7)x$ [mM] − 363 609; $R^2 = 0.90$], methyl salicylate [$y = (1 \times 10^9)x$ [mM] + 930 223; $R^2 = 1.0$] and *cis*-3-hexenol [$y = (1 \times 10^7)x$[mM] + 17 744; $R^2 = 1.0$] were determined from solutions spanning the range of natural variation (0.1–0.001 mM).

Analysis of flower parts

Flowers were dissected with clean razorblades into six parts: corolla limb, corolla tube, gynecium plus nectary (Figure 2), anthers, stigma plus style and sepals. The combined parts of two flowers were used for each analysis. Flower parts were collected and dissected at the same time in the field at dusk or in the glasshouse at 20:00, transferred directly into a vial containing 1 ml distilled water, crimped and stored at room temperature. After 12 h, plant material was removed and the vial was crimped again and stored at −20°C until analysis by SPME. Fibers were placed directly into solution for 30 min at room temperature and injected into the GC-MS under the conditions described above. All compounds detected in the analysis of individual flower parts, with the exception of ethyl benzoate in the nectaries and four compounds from the sepals, were detected in the nectar or the floral headspace of intact flowers, thereby excluding the possibility that wounding elicited the release of certain volatiles. For quantification, the different flower parts were weighed before being inserted into the vial, and tetralin, as an internal standard, was added so that the mass specific accumulation of a given compound could be compared among the different flower parts.

Bioassays

Three groups of nectar visitors (two pollinators and one nectar thief) from the native habitat of *N. attenuata* were used for the bioassays. The moth, *M. sexta*, is one of the main pollinators of *N. attenuata* in Utah; its larvae are also one of the main herbivores of the plant. The hummingbirds, *A. alexandri* and *S. rufus*, are known to collect nectar from *N. attenuata* (Aigner and Scott, 2002; Wells, 1959), but have numerous other nectar sources. The fire ant, *S. xyloni*, was chosen to represent a potential nectar thief and is known to obtain nectar from a variety of plants, including *Datura* flowers in *N. attenuata* habitats. When excised *N. attenuata* flowers are presented to these ants, *S. xyloni* readily collect nectar. During some experiments, two other ant species, *Dorymyrmex insanus* and *Tapinoma sessile*, were found in the test arenas. These ants were also counted because they are also known to collect nectar, showed the same preferences as did *S. xyloni*, and always represented a minor (5%) number of the recruited individuals.

These species were used in bioassays to determine their preferences for three different sugars and 16 volatile compounds that occur in nectar and flower headspace. Experiments with hummingbirds and ants were conducted with native populations at the Lytle Ranch Preserve, Santa Clara, UT, USA in April and August 2004. Bioassays with moths were conducted in the laboratory in Jena, Germany, with a colony of *M. sexta*. Because all species showed a strong concentration-independent preference for sucrose over fructose and glucose for all experiments, we used a 12.5% sucrose solution as a control stock solution and added the volatile constituents at 0.1 mM to this stock solution for bioassays. Bioassays were conducted with nectar secondary metabolites at this standard concentration, which is the concentration of nicotine, the main secondary metabolite found in the nectar headspace of field-collected nectar samples.

In experiments with hummingbirds and moths, the mean nectaring time for the pure sugar solution was determined before and after the presentation of each test solution, and this value was set as the zero. The difference in nectaring times between treatment and control solutions reflected the deterrence (negative values) or attractiveness (positive values) of the secondary metabolite to the flower visitor. For ant bioassays, the procedure was the same, except we counted the number of ants that were recruited to control and test solutions during a test period.

For some bioassays with moths and hummingbirds, replication of treatments with particular compounds was required after some weeks or months due to low pollinator activity or density. We obtained equivalent results in these replicates, demonstrating that the assays produced data that were not influenced by monthly variation in pollinator preferences.

Preferences of hummingbirds

The bioassays were conducted when the hummingbirds were migrating between their winter and summer ranges (April–June 2004), which meant that the individuals changed daily, preventing the establishment of territorial behavior at feeding stations. All experimental measures were conducted during the two main activity periods, morning (08:00–09:30) and evenings (16:30–19:30), and during a smaller activity window at noon (11:30–12:30). Birds were provisioned with a 12.5% sucrose solution from three-port feeders, and test birds were self-recruited to the experimental arenas in groups of 15–40 individuals.

A circular (48-cm diameter) experimental arena located 86 cm from the ground with six equally spaced feeding stations (30 ml containers: P100; Solo Cup, Highland Park, IL, USA) was fitted with a yellow star-shaped lid with a 3-mm hole (Figure 4b). Volatiles were dissolved at 0.1 mM in 10 ml of 12.5% sucrose solution, and treatment and control solutions were used in alternate feeding stations. The arena was rotated after 30 min of observation to prevent the formation of spatial memories among the test birds. Each test period lasted 1 h and the duration (in seconds) of each nectaring bout (time when the birds' beaks were immersed in the test solutions) at each feeding station was recorded. Repellent solutions elicited very short nectaring times followed by spitting behavior. To analyze responses to volatiles presented in the headspace of the nectar independently of their occurrence in the nectar, feeding stations were surrounded by cotton batting (Figure 4b) which was impregnated with 2 ml of 0.1 mM aqueous solutions of the volatiles. Different combinations of volatiles presented only in the headspace, only in the nectar or in both were tested.

Bioassays with different compounds were always separated by at least 3 h, during which the birds had free access to sucrose solutions in the three-port feeders. These between-test intervals were essential to minimize the possibility that test birds would remember the location of particular attractive or repellent test solutions. Tests were conducted when fewer than 10 birds were actively visiting the experimental arena. The presence of >10 individuals prevented the individuals from moving from one feeding station to the next in their characteristic trap-lining behavior; moreover, the birds would engage in socially interactive behavior between nectaring bouts if too many were present. Experiments were discontinued if single birds showed territorial behavior at the test arena by attacking other birds.

Preferences of moths

The laboratory colony was established from eggs collected from a native population at the Lytle Preserve, UT, and maintained for approximately a year. Bioassays were conducted in a rearing ($1.5 \times 1.5 \times 1.8$ m) cage (Bell, 1975) at the end of a 16-h day. A shaded 15-W lamp provided illumination for observations, which were made through a Plexiglas window in the rearing cage to avoid influencing feeding behavior. A minimum of 20 (up to 51) moths were included in the experiments and three generations of moths were used for all observations. A circular experimental arena, similar to that used in the hummingbird bioassays, was used. Large (11×14 cm) plastic lily flowers (Klee Gartenland, Jena, Germany) were trimmed to 7 cm and fitted on top of 30-ml beakers (Figure 4a). The arena with six equally spaced feeding stations, with treatment and control stations in alternating order, was placed 40 cm above the ground. In the center of the arena, a flowering *N. attenuata* plant was placed to provide a bouquet of plant volatiles to stimulate feeding (Raguso and Willis, 2002) and to prohibit headspace volatiles from influencing feeding preferences. Observations lasted for 1.5–2 h for each compound and only one compound was tested per day. Nectaring bouts, flower visits with proboscis extension, and nectaring were measured by recording the amount of time moths had their tongues in the test solutions. No more than five moths searched for nectar at the same time at the arena. A single moth had at most one to three feeding events during the 2-h observation period. After a prolonged feeding period, moths typically rested or started to lay eggs. More than half of the total volume removed per night (8 h) was removed within the first 90 min after the start of the dark period. To determine the effect of headspace volatiles, we attached cotton batting to the corolla limbs of the artificial flowers and impregnated the batting with the test solutions. The batting was located on the adaxial surface of the corolla, so that the moths could not come into direct contact with the batting when nectaring.

Preferences of ants

A circular experimental arena with four pairs of control and treatment feeding stations (1.5-ml plastic vial; Plastibrand) was placed into the soil at the Lytle field station. Pairs of feeding stations were separated by 25 cm, and within pairs feeding stations were separated by 15 cm. Each experiment was repeated four times in different arenas (separated by at least 20 m) for the same compound, so that each compound was tested with 16 pairs of feeding stations. Feeding stations were placed directly in the soil so that the opening was at the soil surface, thereby providing free access to each solution (Figure 4c). After 3 h, the number of ants feeding at each feeding station was recorded.

Statistical procedures

For all experiments that met the requirements for a *t*-test, we used either unpaired (hawkmoth bioassays) or paired Student's *t*-test (ant bioassays, nectar volume). Due to the strong responses of hummingbirds to deterrent compounds, the data for some hummingbird assays were not normally distributed. Therefore we used the non-parametric Mann–Whitney *U*-test for the analysis of hummingbird experiments.

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Supplementary Material

The following supplementary material is available for this article online:

Table S1 Visitations per trial of pollinators compared to mean nectaring time [s]. Included are all trials testing pure 12.5% sucrose

solutions (C) against the same solution containing 0.1 mM of a single volatile compound (T) (Figure 3) and trials were headspace (h) versus nectar (n) presentation of two constituents [nicotine (N) and benzylacetone (B)] was tested (Figure 5).
This material is available as part of the online article from http://www.blackwell-synergy.com.

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