

LETTER

Reliance on pollinators predicts defensive chemistry across tobacco species

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Abstract

Defensive traits are typically studied in the context of avoiding antagonists, but may also mediate key interactions with mutualists. Plant chemical defences occur in flowers, suggesting pollinators may be agents of selection on defence. We hypothesised that floral defences would deter pollinators, and therefore, pollinators would select for lower defences in outcrossing than self-pollinating species. We measured pollinator reliance and alkaloid levels in 32 greenhouse-grown *Nicotiana* species. Using a comparative phylogenetic approach, we found significantly lower nectar, floral and leaf nicotine concentrations in outcrossing than selfing species, with a 15-fold decrease in leaf nicotine levels. Nicotine concentrations were positively correlated across tissues, suggesting that selection against floral defences could constrain the evolution of leaf defences. Thus, pollinators could shape the evolution not only of floral defences but also of defences in other tissues where herbivores have traditionally been considered the dominant agent of selection.

Keywords

Alkaloids, defence evolution, floral chemistry, herbivory, mating system, mutualism, *Nicotiana*, nicotine, pleiotropy.

Ecology Letters (2012)

INTRODUCTION

Defensive traits are ubiquitous in the animal and plant world, and the evolution of such traits is typically studied in the context of avoiding antagonists (e.g. Farrell & Mitter 1998; Becerra 2003). However, anti-predator traits may have costs if they also deter mutualists that are critical for reproduction. For example, plant floral chemical defences can benefit plants by deterring floral antagonists (McCall & Irwin 2006; Kessler *et al.* 2008), but can also deter pollinators (Detzel & Wink 1993; Adler 2000; Tadmor-Melamed *et al.* 2004; Adler & Irwin 2005; Singaravelan *et al.* 2005; Kessler & Baldwin 2007). Although the role of mutualisms in shaping the evolution of attractive traits has long been recognised (Whittall & Hodges 2007), the influence of pollinators on the evolution of defence has only been considered recently (McCall & Irwin 2006; Adler 2007; Kessler & Baldwin 2007; Armbruster *et al.* 2009; Kessler & Halitschke 2009).

Chemical defences are often found in nectar (Adler 2000). Such defences could benefit plants via a variety of antagonist- and pollinator-mediated mechanisms (reviewed in Adler 2000; Kessler *et al.* 2008); thus, nectar defences could be adaptive. Alternatively, nectar defences could be a pleiotropic consequence of defence production in other tissues (Adler 2000). For example, producing defences in one tissue that are transported throughout the plant provides a simple mechanism linking whole-plant defence concentrations via a single production site. While the underlying genetic, biochemical and developmental mechanisms linking floral and leaf chemistry are relatively understudied, current evidence suggests the possibility for independent or correlated evolution of leaf and flower defences

(reviewed in Kessler & Halitschke 2009). Intraspecific correlations between defence levels across tissues have not been examined frequently, but positive correlations have been found between leaf and nectar alkaloids in *Nicotiana tabacum* (Adler *et al.* 2006), and leaf and pollen phenolics in *Solanum peruvianum* (Kessler & Halitschke 2009). Examining how defence levels correlate across tissues will provide needed insights into the potential for constrained or independent evolution of leaf and floral defences.

Comparative phylogenetic approaches have reinvigorated work examining how traits evolve on a macroevolutionary scale. While such approaches do not elucidate underlying evolutionary processes, they are complementary to mechanistic studies of trait evolution and provide the necessary framework for testing hypotheses about how traits evolve across species. Recent applications of comparative approaches have allowed tests of decades-old theories about the evolution of plant defence (reviewed in Agrawal 2007), and provided new insights into the evolution of floral and defence traits in the context of interactions with herbivores and pollinators (Armbruster *et al.* 2009). Conceptual papers have recognised the potential for both pollinators and herbivores to shape the evolution of chemical defences (McCall & Irwin 2006; Kessler & Halitschke 2009), and highlighted the need for comparative studies examining the extent to which defence expression is correlated across floral and leaf tissues. A comparative approach is ideal to address the question of whether defence traits are correlated across tissues at a macroevolutionary scale, and if reliance on pollinators can predict the evolution of defence traits across species.

We tested the hypothesis that pollinator reliance predicts chemical defence levels and that defence concentrations were correlated

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across tissues using a phylogenetic comparative approach in the genus *Nicotiana*. Our *a priori* assumption was that pollinator deterrence due to defences would reduce plant fitness in outcrossing species (as in Adler & Irwin 2005). Thus, we predicted that outcrossing *Nicotiana* species should have lower concentrations of nectar and floral alkaloids than selfing species, which would not be under pollinator-mediated selection. Alternatively, if pollinator deterrence increases plant fitness (Kessler *et al.* 2008), we would expect higher levels of alkaloids in nectar and floral tissue of outcrossing compared with selfing species. If nectar and floral alkaloid concentrations are positively correlated with leaf alkaloid concentrations, then selection by pollinators on floral defences could constrain the evolution of leaf defences, changing our understanding of the underlying forces shaping the expression of chemical defence traits in flowering plants.

MATERIALS AND METHODS

Study system

In *Nicotiana* (Solanaceae), nicotine and other alkaloids are synthesised in the roots and transferred via the xylem to stems, leaves and flowers (Roberts & Wink 1998). Several *Nicotiana* species have alkaloids, including nicotine and anabasine, in nectar or floral scent (Detzel & Wink 1993; Euler & Baldwin 1996; Ohnmeiss & Baldwin 2000; Raguso *et al.* 2003). Although *Nicotiana* produce multiple chemical defences (Lou & Baldwin 2003; Kaplan *et al.* 2008), we focus on alkaloids due to their known presence in flowers and nectar and their strong effects on species interactions. Our study includes species known to be visited by hawkmoths, hummingbirds and bees (Raguso *et al.* 2003), all of which are deterred by *Nicotiana* alkaloids in nectar or floral scent (Detzel & Wink 1993; Singaravelan *et al.* 2005; Kessler & Baldwin 2007; Kessler *et al.* 2008), although honey bees may be attracted at low concentrations (Singaravelan *et al.* 2005). Thus, our study considers evolution in response to pollinators generally rather than specific taxa.

Species selection

We examined 36 *Nicotiana* taxa (32 species including 2 species with 3 accessions each; 'species' hereafter) thought to vary in their reliance on pollinators, from obligate outcrossing to highly selfing (Fig. 1). Plants were grown in the greenhouse (see Table S1 in Supporting Information) with regular pesticide applications as needed (Table S2) to keep plants free from herbivores and pathogens. Measures of alkaloid concentrations should therefore represent constitutive levels. Although natural populations may often have herbivore-induced defences, there is unlikely to be one herbivore or damage level that would be ecologically relevant to all species. Assessing constitutive levels provides a baseline context for evaluating allocation to alkaloids in different tissues. Seeds from all species except *N. obtusifolia* were obtained from the USDA (Table S1). Although accession histories were unknown, outcrossing increased reproduction compared with selfing for many species (Results; Table S3), indicating genetic variation within accessions. Species were selected from across the genus, including all sections except *Nicotiana* (contains only *N. tabacum*) and *Sylvestres* (contains only *N. sylvestris*), with emphasis on diploids, for which a robust

phylogeny is available (Clarkson *et al.* 2004). We included multiple accessions or varieties of *N. rustica* and *N. obtusifolia* (Table S1) thought to vary in pollinator reliance, resulting in 36 taxa. Each accession/variety was treated as a separate experimental taxon.

Pollinator reliance

To assess reliance on pollinators for reproduction, plants of each species were assigned to outcross, self, or control treatments. The stigmas of outcrossed flowers were coated with a mix of pollen from at least two other individuals of the same species, applied with a camelhair paintbrush washed with ethanol between flowers to prevent self-pollen transfer. Pollen donor flowers were typically non-experimental plants and were never used for reliance treatments. We attempted to treat newly opened flowers that had receptive stigmas but undehiscent anthers, so that stigmas received outcross pollen before self-pollen. In some cases, anthers dehisced as soon as flowers were open, so that our treatments may represent a mixture of self and outcross pollen. We chose not to emasculate flowers as our goal was to assess the benefit of a pollinator visit for reproduction, and pollinators would typically encounter intact flowers. The stigmas of selfed flowers were coated with pollen from the same flower, and paintbrushes were rinsed between plants. Control flowers were labelled but otherwise unmanipulated. Each flower was treated only once. In most cases, each plant was assigned to only one treatment. In two cases, we subjected flowers on each plant to each treatment due to an insufficient number of plants (*N. alata*, four plants; *N. bonariensis*, five plants). This method can overestimate treatment effects as flowers on the same plant are not independent, and plants may allocate resources preferentially to outcrossed flowers. However, both species were already known to be self-incompatible (Kaczorowski *et al.* 2005), as we found (Table S3). We treated five flowers per plant, except in cases with high fruit abortion or flower loss during treatments, in which we treated up to 10 flowers per plant (*N. cordifolia*, *N. plumbaginifolia* and *N. tomentosiformis*). For each plant, we measured fruit set (fruits/treated flowers) and mean total seed weight per fruit. To measure seed weight per fruit, mature fruits were collected before dehiscence and dried at 50 °C for at least 48 h. The pooled seeds per fruit were weighed; this total seed weight was then averaged within plant. Pooled seed weight per fruit provides one response variable that reflects a combination of seed number and individual seed weight.

To quantify pollinator reliance, we used separate ANCOVAs for each species with fruit set and mean seed weight per fruit as response variables and a model including pollination treatment (fixed effect), plant biomass (covariate) and the treatment \times biomass interaction. Treatment means were compared with Tukey's HSD test. When necessary, fruit set was arcsin(square root(x)) transformed and mean seed weight was log(x) transformed to improve normality. Plants that appeared unhealthy or with fewer than two flowers treated were excluded from analysis. Based on Tukey's HSD comparisons, each species was assigned to one of three pollinator reliance categories: (1) little reliance on pollinators (highly selfing; supplemental self or outcross pollen did not significantly increase reproduction relative to unmanipulated controls), (2) intermediate reliance on pollinators (self-compatible but increased reproduction with a vector to transfer pollen; higher reproduction with supplemental self and/or outcross pollen relative to unmanipulated controls) or (3) highly reliant on pollinators (self-incompatible; zero

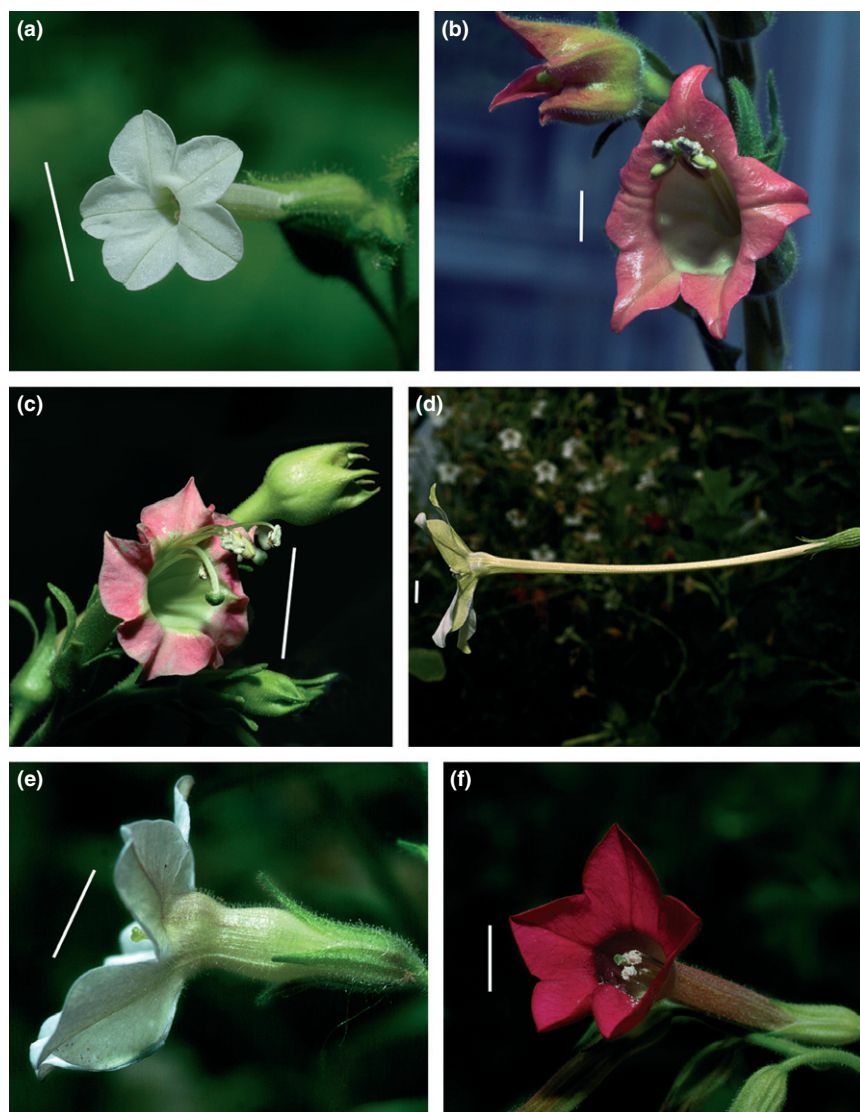


Figure 1 Examples of (a, b) selfing, (c, d) intermediate and (e, f) outcrossing species of *Nicotiana*. (a) *N. clevelandii*, (b) *N. glutinosa*, (c) *N. tomentosiformis*, (d) *N. longiflora*, (e) *N. bonariensis* and (f) *N. forgetiana*. Bar in each photo represents approximately 1 cm for scale comparison.

or nearly zero fruit set in the control and self treatments). We found that species fell naturally into these three categories, and attempts at finer-scale differentiation would be obscured by substantial random variation in seed mass due to early dehiscence and fruit set due to abortion from factors other than pollination. When results differed for fruit set and seed weight (six species that had results indicative of both selfing and intermediate reliance; Table S3), the species was considered to have intermediate reliance to reflect that benefits of pollination could be due to either increased fruit set or seed quality.

Sample collection and chemical analysis

Leaf, flower and nectar samples for chemical analysis were collected from separate plants (usually 10 per species; Table S4) than those used to assess pollinator reliance. Nectar was collected first by removing and gently pressing the corolla to produce nectar at the flower base, and collecting with glass microcapillary tubes. This

method is unlikely to contaminate samples with corolla alkaloids because alkaloids are stored in the vacuoles rather than on the tissue surface except in leaf trichomes (Wink 1993). Collection through the top corolla opening resulted in pollen contamination, and removing stamens before nectar collection could have created alkaloid exudates from the damaged tissue. Collection through the corolla base provided the cleanest sample and follows methods of similar studies (Kaczorowski *et al.* 2005). Nectar was pooled across plants to produce samples of > 200 μ L each. Collecting sufficient nectar samples often required collecting and pooling nectar over time; samples were frozen after collection. Once sufficient nectar samples were collected (usually 3–4 per species), flowers and leaves were collected. The youngest fully expanded leaves were collected by slicing the petiole with a razor. Typically leaves and whole flowers were collected simultaneously and pooled within tissue type (leaf or flower) to create three samples per tissue per species. Because we collected whole flowers including nectaries, floral alkaloid concentrations will depend on the relative proportions of different flo-

ral organs in each species and could be influenced by nectar values. Analysing each floral tissue separately was beyond the scope of this study. Nectar amounts were relatively small compared to floral tissue, and nectar concentrations were orders of magnitude below floral concentrations (see Results), so that nectar alkaloids should have little influence on floral alkaloid results. Leaves, flowers and nectar were stored at -20°C and freeze-dried prior to analysis. Leaf and flower samples were extracted in 50 mL methanol, and nectar samples were extracted in 1 mL methanol. Samples were analysed by HPLC as in Halpern *et al.* (2010); nicotine and anabasine were the two major alkaloids recovered. Concentrations were calculated based on g dry weight for leaves and flowers, and per μL for nectar.

Correlation analysis

Pearson's r correlations were calculated between (1) pollinator reliance scores and leaf, flower and nectar nicotine and anabasine concentrations, to assess relationships between reliance on pollinators and chemical defence, (2) nicotine and anabasine concentrations across tissues (e.g. leaf vs. flower nicotine), to assess correlation of defence concentrations between tissue types and (3) nicotine and anabasine concentrations within tissues (e.g. leaf anabasine vs. leaf nicotine), to assess independence of nicotine and anabasine analyses (Table 1). All traits were averaged to create one value per species prior to analyses because samples were pooled

across subsets of plants and we consider the species to be the unit of replication. Each species was considered a separate replicate (maximum $n = 36$; lower for some analyses as some traits could not be measured in some species). When species had alkaloid concentrations greater than two standard deviations above the mean for that tissue, analyses were re-run excluding those values individually. Some results changed for Pearson's correlations following exclusions, but phylogenetic analyses were robust to exclusions (Table 1). Species with high values (Table S4) were *N. benthamiana* (nectar nicotine and anabasine), *N. excelsior* (leaf nicotine), *N. nesophila* (leaf and flower nicotine), *N. noctiflora* (leaf and flower anabasine), *N. petunoides* (flower and nectar anabasine) and *N. stocktonii* (flower nicotine).

Phylogenetic analysis

Hypotheses of phylogenetic relationships were reconstructed based on multiple chloroplast DNA markers including coding (*matK*, *ndbF*) and non-coding (*trnL* intron, *trnL-F* spacer, *trnS-G* spacer) regions (Clarkson *et al.* 2004). *Anthocercis angustifolia*, *Atropa belladonna*, *Cestrum elegans*, *Crenidium spinescens*, *Cyphanthera albicans*, *Duboisia leichhardtii*, *Grammsolen truncates*, *Mandragora officinarum* and *Symonanthus bancroftii* were used as outgroups. We implemented partition Bayesian analyses in MrBayes v3.1.2 based on four partitions: non-coding regions, first codon positions, second codon positions and

Table 1 Correlations (Pearson's r) and phylogenetic covariance for pollinator reliance and alkaloid concentrations, nicotine and anabasine correlations across tissues, and nicotine and anabasine correlations within tissues. Sample size (n) differs because some species did not flower or produce sufficient nectar. Results for comparative analyses are qualitatively the same after individually excluding possible outlier species *Nicotiana benthamiana*, *N. excelsior*, *N. nesophila*, *N. noctiflora* and *N. stocktonii*; results for Pearson's correlations are the same except where indicated. Bold indicates $P < 0.05$ for Pearson's correlations, and AIC > 2.0 for comparative analyses

	Correlation			Comparative analyses			
	N	r	P	Likelihood with no correlation	Likelihood with correlation	AIC	Covariance (R^2)
(A) Reliance on pollinators							
Nicotine							
Reliance \times leaf	30	-0.38	0.036	-311.31	-309.78	3.06	-1912.1 (0.09)
Reliance \times flower	29	-0.31*	0.096	-300.32	-297.50	5.64	-5114.3 (0.12)
Reliance \times nectar	27	-0.43	0.026	-66.18	-64.11	4.14	-1.9 (0.15)
Anabasine							
Reliance \times leaf	30	0.30	0.105	-245.07	-245.00	0.12	
Reliance \times flower	29	0.43 †	0.026	-224.02	-223.90	0.23	
Reliance \times nectar	27	0.23‡	0.249	-33.70	-3.53	0.35	
(B) Across tissue							
Nicotine							
Leaf \times flower	34	0.67	0.0001	-617.41	-607.72	19.37	1 258 503 675 (0.40)
Leaf \times nectar	31	0.41	0.024	-323.51	-321.50	4.01	2351.7 (0.07)
Flower \times nectar	31	0.53	0.002	-277.50	-269.83	15.34	28 338 064.6 (0.38)
Anabasine							
Leaf \times flower	34	0.81 §	0.0001	-464.23	-460.90	6.66	280 279 366 (0.26)
Leaf \times nectar	31	0.35	0.053	-203.88	-203.22	1.32	
Flower \times nectar	31	0.74	0.0001	-163.51	-163.32	0.39	
(C) Within tissue							
Leaf nicotine \times anabasine	36	0.15¶	0.395	-593.99	-584.70	18.57	10 054 483.5 (0.48)
Flower nicotine \times anabasine	34	-0.08	0.652	-501.92	-501.60	0.64	
Nectar nicotine \times anabasine	31	0.44 ±	0.011	-32.94	-28.20	9.50	0.135 (0.34)

* $r = -0.39$, $P < 0.05$ when *N. stocktonii* is excluded.

† $r = -0.01$, $P > 0.05$ when *N. noctiflora* is excluded.

‡ $r = 0.42$, $P < 0.05$ when *N. benthamiana* is excluded.

§ $r = 0.26$, $P > 0.05$ when *N. noctiflora* is excluded.

¶ $r = 0.58$, $P < 0.05$ when *N. noctiflora* is excluded.

± $r = -0.11$, $P > 0.05$ when *N. benthamiana* is excluded.

third codon positions. All partitions were unlinked, each partition used its own inverse Gamma model of evolution, and trees were sampled every 1000 generations. The analysis was run for 25 000 000 generations and burn-in was calculated when the standard deviation of clade split frequencies fell below 0.05 (approximately 2 750 000 generations). All trees from the burn-in were discarded. The multiple accessions of *N. rustica* and *N. obtusifolia* were added after phylogeny reconstruction as zero-length unresolved polytomies and were resolved randomly for use in Bayesian comparative analyses with internodes or terminal nodes of near-zero length (0.000001). This modification essentially treats each of these species as highly variable terminal taxa and incorporates that variation into the analysis in a statistically conservative manner. All taxa and sequence data are as in Clarkson *et al.* (2004), except that *N. maritima* was used as a placeholder for its likely sister-species *N. excelsior*, as only the latter is included in our sampled taxa while only the former was sampled by Clarkson *et al.* (2004). The final topology only differed based on pruned terminals and the Bayesian analysis allowed incorporation of phylogenetic uncertainty in subsequent comparative analyses.

The comparative analyses were performed also in a Bayesian framework using the Continuous module in the program BayesTraits v1.0, with ancestral state reconstructions implemented in the beta version of BayesTraits v1.1 (Pagel & Meade 2006). Unlike comparative methods that must assume that the phylogeny is a fixed parameter, and therefore treated implicitly as a perfectly estimated entity, this method allows uncertainty in both phylogeny reconstruction and in comparative parameter estimation to be incorporated into the analyses. All outgroups were pruned from the tree for the comparative analyses, and the tree was treated as rooted based on the original Bayesian reconstruction. Analyses include all post-burn-in trees from the Bayesian phylogenetic reconstructions and Markov chain Monte Carlo (MCMC) methods to derive posterior distributions of likelihoods for comparative analyses, thereby taking into account both phylogenetic uncertainty and uncertainty in model parameter estimation. Resultant posterior distributions were evaluated based on differences in Bayes factors by comparing the marginal likelihoods of the two appropriate models (e.g. correlated vs. non-correlated, directional vs. non-directional). The marginal likelihood was approximated by calculating the harmonic means of the post-burn-in iterations and were compared using the test statistic $2(\log[\text{harmonic mean}(\text{dependent model})] - \log[\text{harmonic mean}(\text{null model})])$. We follow the traditional practice of treating a difference of 2 log harmonic mean units or more in Bayes factors as evidence that the dependent model (e.g. correlation or trajectory) is favoured over the null model (Nylander *et al.* 2004). For all analyses, we examined whether the data deviated from a constant-variance random walk model of evolution by testing whether the addition of the scaling parameters kappa, delta and/or lambda significantly increased the marginal likelihood of the data. When they did, the scaling parameter(s) were incorporated into the final comparisons. All analyses were based on 5 050 000 iterations, with the first 50 000 iterations discarded as burn-in in all analyses except for the ancestral state reconstructions in which the first 750 000 iterations were discarded, and sampling of parameters and likelihoods every 100 iterations. For all analyses of trait correlation, the marginal likelihoods of the data were calculated with the traits constrained to be correlated vs. without this constraint. For all analyses of directional

change in alkaloid content (e.g. increase in concentration of anabasine or nicotine) from the base to the tip of the phylogeny, the marginal likelihoods of the data were calculated with the traits allowed to evolve according a random walk of evolution vs. when the traits were constrained to a directional model. For the binary trait of self-compatible (reliance scores 1 and 2) vs. self-incompatible (reliance score 3), the module Multistate of BayesTraits was used to compare the marginal likelihoods of the data when transitions between the traits were constrained to be equal (no directionality) vs. when transition rates were completely free to vary (directionality permitted). We did not treat the three reliance categories separately due to low power to detect effects from insufficient replication (number of species within category).

Evaluation of alternative hypotheses

Additional plant traits were measured to evaluate whether resource allocation tradeoffs or nectar concentration levels could be underlying causes of correlations between pollinator reliance and defence concentrations. Plant size and growth rate were measured on plants used to assess pollinator reliance. Plant size was measured as fresh mass at harvest. Because plants were harvested after all fruits were collected from treated flowers, harvest mass reflects the size attained within a few weeks of flowering and can be used for gross species comparisons. Growth rate was measured as plant size divided by days between germination and harvest. Nectar sugar concentration was measured in sucrose equivalents using a pocket refractometer prior to freeze drying each vial of nectar collected for chemical analysis. All traits were measured in individuals and averaged to create one value per species for analysis.

RESULTS

We found that reliance on pollinators predicted nicotine concentrations across tissues, with the highest nicotine levels in selfing species, intermediate levels in species with intermediate reliance on pollinators and low levels in species that were entirely outcrossing (Fig. 2). Directional analyses demonstrated that self-incompatibility was consistently derived from self-compatibility (AIC > 2.0), suggesting that greater reliance on pollinators and lower defence levels are derived traits in this lineage. Correlations between reliance on pollinators and leaf, floral and nectar nicotine were all significant in both Pearson's correlations and comparative analyses (Table 1). Leaf nicotine was on average 15 times higher in selfing than outcrossing species (Table 1, Fig. 2). Pollinator reliance did not correlate with anabasine concentration in any tissue in comparative analyses (Table 1). Nicotine concentrations were positively correlated across leaves, flowers and nectar (Table 1, Fig. 3), and anabasine concentrations were correlated across leaves and flowers (Table 1).

DISCUSSION

We found that nicotine concentrations were significantly and substantially lower in outcrossing compared with selfing species, consistent with the *a priori* hypothesis that pollinator reliance predicts chemical defence concentrations in this system. Thus, pollinators may be a key agent of selection on floral chemical defences. Fur-

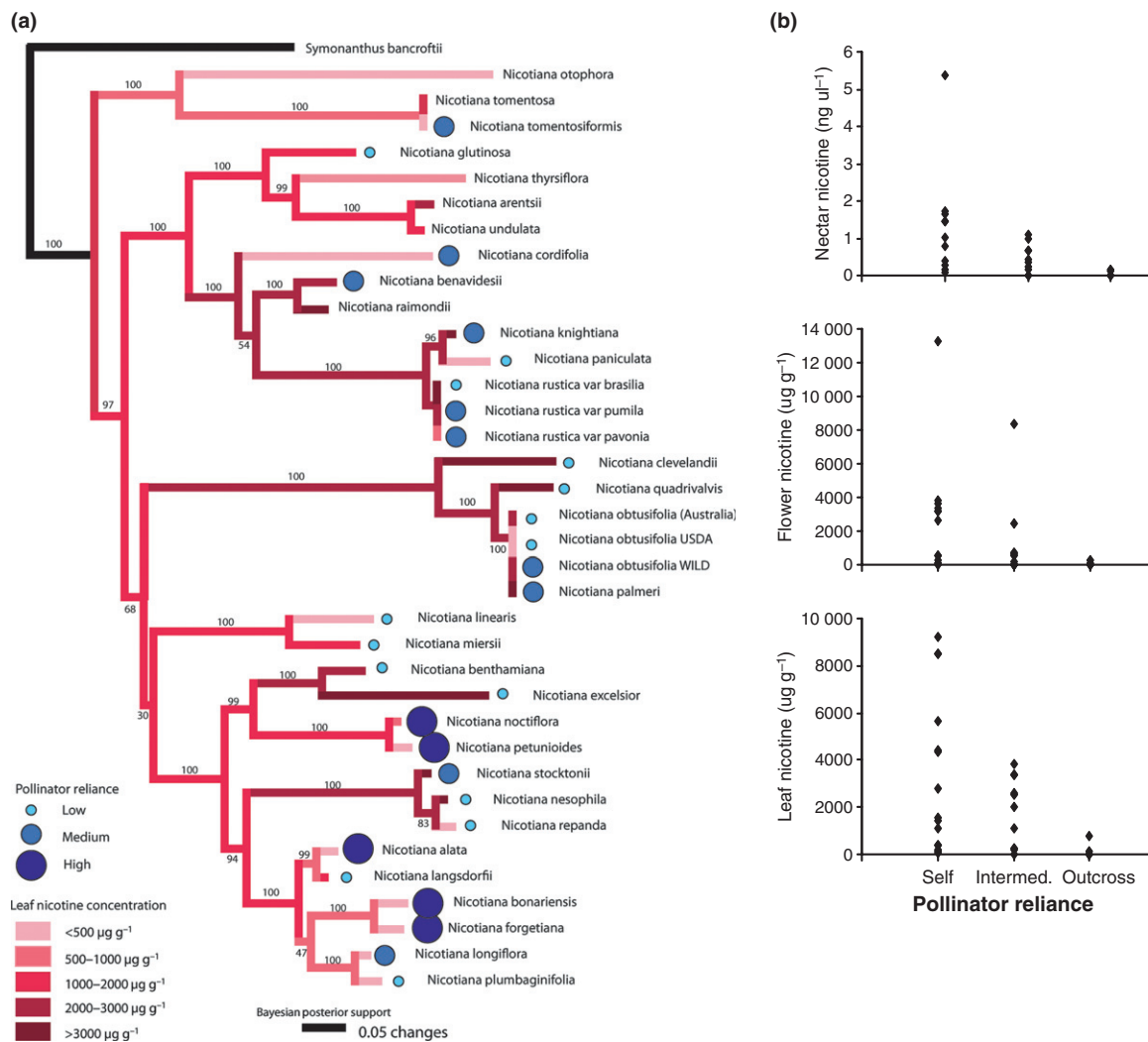


Figure 2 Relationship between nicotine levels and pollinator reliance across 36 *Nicotiana* taxa. (a) *Nicotiana* phylogeny reconstructed based on partitioned Bayesian analysis of multiple cpDNA loci, with Bayesian posterior support indicated above branches. Colour codes on branches are based on Bayesian reconstruction of leaf nicotine levels. Pollinator reliance is displayed only for the terminal taxa. These traits are inversely correlated when controlling for phylogenetic influence. (b) Relationship between pollinator reliance and nicotine concentrations in nectar ($N = 27$), flowers ($N = 29$) and leaves ($N = 30$). Each point represents one *Nicotiana* taxon. Pollinator reliance was determined as selfing (little reliance on pollinators; 15 taxa), intermediate reliance on pollinators (10 taxa) or outcrossing (high reliance on pollinators; five taxa).

thermore, alkaloid levels were positively correlated across leaves, flowers and nectar. These results suggest there may be substantial genetic covariance between alkaloid concentrations across floral and leaf tissues, and thus that selection on floral alkaloids could cause correlated selection on leaf alkaloids.

Our results are consistent with our *a priori* hypothesis that species that rely on pollinators will have lower levels of floral chemical defences, presumably via negative selection due to pollinator deterrence. Only three previous studies have experimentally manipulated nectar chemical defences and examined consequences for plant reproduction in the field. All studies found that nectar defences deterred pollinators (at high concentrations only for one compound), but deterrence reduced plant reproduction or outcross pollen receipt in *Gelsemium sempervirens* and *Polemonium viscosum*, respectively, while there were benefits of deterrence in *N. attenuata* (Adler & Irwin 2005; Kessler *et al.* 2008; Galen *et al.* 2011). In general, nectar chemical defences deter pollinators (Detzel & Wink 1993; Adler 2000; Liu *et al.* 2004; Tadmor-Melamed *et al.* 2004;

Adler & Irwin 2005; Singaravelan *et al.* 2005; Kessler & Baldwin 2007), but some specialised pollinators are unaffected (Stephenson 1982; Johnson *et al.* 2006), and honey bees may be attracted at low concentrations (Detzel & Wink 1993; Liu *et al.* 2004; Adler & Irwin 2005; Singaravelan *et al.* 2005). Although pollinator deterrence may often reduce reproduction in pollen-limited plants, in some cases deterrence could benefit plants by reducing within-plant pollen transfer (Irwin & Adler 2008) or nectar consumption per visit (Kessler *et al.* 2008), or increasing distance travelled between plants (Adler & Irwin 2005). The results of our study suggest that pollinator deterrence due to floral nicotine exerts negative selection on this trait, providing support for the hypothesis that pollinator deterrence generally reduces plant reproduction. However, both comparative and experimental studies should be conducted in more systems to examine the generality of this result.

Nicotine and anabasine concentrations were positively correlated across tissues, suggesting that pleiotropy or shared genetic pathways could constrain the independent evolution of these traits in

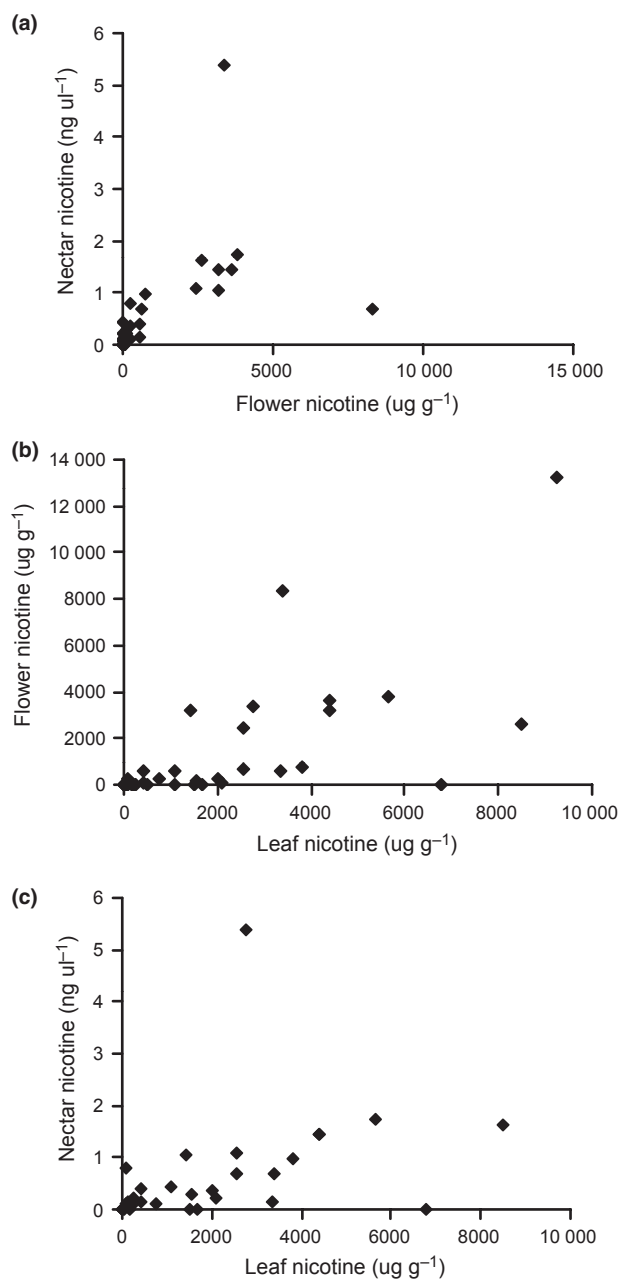


Figure 3 Correlations between nicotine concentrations across plant tissues. (a) Flowers and nectar ($N = 31$), (b) flowers and leaves ($N = 34$) and (c) leaves and nectar ($N = 31$). Each point represents one *Nicotiana* taxon. Note different units for nectar concentration compared with flowers and leaves.

response to floral or leaf interactions. In *Nicotiana*, alkaloids are synthesised in the roots and transferred via the xylem to stems, leaves and flowers (Roberts & Wink 1998). Thus, genes that influence alkaloid production in roots could affect whole-plant concentrations. For example, RNAi constructs that silenced expression of the *Napmt1/2* genes in transgenic *N. attenuata* reduced nicotine accumulation throughout the plant, including in nectar (Kessler *et al.* 2008). However, genes that regulate transport in different tissues could decouple trait expression; alkaloid levels are often highest in young or damaged leaves, indicating the potential for compartmentalisation of defences across tissues. We examined correlations amongst

constitutive defence levels, but leaf damage can induce floral defences in several systems (reviewed in Kessler & Halitschke 2009) and floral volatiles that deter pollinators (Kessler *et al.* 2011). Induced defences across tissues could provide a mechanism for decoupling leaf and floral traits, and such phenotypic plasticity could be under selection to maximise benefits and minimise costs of defence. Examining the evolution of inducibility in a comparative framework would provide insights into the ability of plants to compartmentalise responses to local and tissue-specific interactions.

Although our results are consistent with the hypothesis that selection by pollinators shapes the evolution of chemical defences, other hypotheses could also explain our results, including resource allocation tradeoffs and correlations between nectar traits. If faster-growing species produce lower alkaloid concentrations due to resource allocation patterns (Coley *et al.* 1985) and if growth rate affects reliance on pollinators, then our results could occur without invoking direct selection by pollinators on defences. However, we found no correlation between growth rate or plant size and pollinator reliance or alkaloid concentrations (Pearson's correlations; $n = 27-29$, $|r| < 0.27$, $P > 0.16$ for all), indicating that selection for selfing due to short growing seasons or small plant size (Goodwillie *et al.* 2005) should not influence alkaloid concentrations. Second, if nectar alkaloid concentrations reflect sugar concentration, correlations between reliance on pollinators and nectar nicotine could reflect pollinator response to sugars rather than alkaloids. We found no correlation between nectar sugar and concentrations of either alkaloid (Pearson's correlations; $n = 29$, $r < |0.16|$, $P > 0.4$ for both). Thus, our evidence suggests that neither resource allocation trade-offs nor nectar concentration levels drive the patterns we found.

Although we cannot discount the possibility that selection by herbivores caused our patterns, our results are more consistent with the *a priori* hypothesis that pollinators drive defence evolution. Florivory occurs in *Nicotiana* (Kessler *et al.* 2008) and could cause the observed patterns if florivore pressure is higher in selfing species. Although traits affecting florivory are largely unknown (McCall & Irwin 2006), more apparent flowers may be more likely to be damaged (Gomez 2003) which should select for higher defences in outcrossing rather than selfing species. Leaf herbivores could select for both high defences and reproduction via selfing if damage deters pollinators (e.g. Lehtila & Strauss 1997). However, directional analyses indicated that self-incompatibility was consistently derived from self-compatibility (Fig. 2), suggesting the evolution of lower rather than higher defences. Herbivores could also drive differential defence evolution based on mating system if there is higher herbivory on selfed than outcrossed species. However, there is a trend for greater herbivory in outcrossing rather than selfing species (Levin 1975), and inbreeding or asexual reproduction is often associated with reduced resistance to herbivory (Du *et al.* 2008; Johnson *et al.* 2009), the opposite of our pattern. These studies do not negate the potential generality of our results, as our hypothesis is relevant only for systems with floral chemical defences. Comparative approaches document patterns rather than discerning underlying mechanisms, and we assert that demonstrating the linkage between pollinator reliance and chemical defence evolution creates exciting new research directions regardless of whether pollinators or herbivores were the underlying driver.

We did find a relationship between reliance on pollinators and life history traits (based on Goodspeed 1954). Annual species were

most likely to be highly selfing (Table S3), consistent with patterns in a wide range of plants (Barrett *et al.* 1996). However, within life history categories that had sufficient replicates across reliance categories (annuals and 'annual-perennials'), Pearson's correlations between pollinator reliance and leaf, floral and nectar nicotine were all still negative (although no longer statistically significant due to much lower sample sizes), ranging from $r = -0.13$ to -0.326 (except $r = 0.014$ for floral nicotine and reliance in annual-perennials; this was also the weakest correlation in the full data set; Table 1). This suggests that the negative relationship between chemical defence and pollinator reliance is robust across life history strategy. Recent theories of plant defence syndromes have built on Grime's (1977) paradigm of classifying species as ruderal, competitive or stress tolerators, and incorporated predictions about the value of 'defence' vs. 'escape' via fast growth (Kursar & Coley 2003). These theories predict that fast-growing, short-lived species, such as annuals, should invest in growth rather than defence and a strategy of 'escaping' herbivores, which is counter to the patterns we found. An alternative hypothesis consistent with our data is that perennial species are more able to tolerate herbivory than annuals via induced sequestration of nutrients (Orians *et al.* 2011) or other mechanisms, and thus can rely on tolerance rather than investment in defence. Future work in a larger comparative framework would provide more power to dissect relationships between reliance on pollinators, traits mediating herbivory and life history.

Pollinators could respond to nectar or whole-flower nicotine concentrations. Although nectar alkaloid concentrations were several orders of magnitude below concentrations in leaves and flowers (Table S4), studies with multiple *Nicotiana* species have shown that pollinators including sunbirds, hawkmoths, and hummingbirds can discriminate between or are affected by naturally occurring nectar alkaloid levels (Tadmor-Melamed *et al.* 2004; Kessler & Baldwin 2007; Kessler *et al.* 2008). Thus, nectar alkaloids alone may affect pollinator preference. In addition, floral alkaloid concentrations were substantially higher than nectar concentrations and of similar magnitude to leaves. Nicotine is volatile and has been detected in the floral headspace of *Nicotiana* species (Euler & Baldwin 1996; Raguso *et al.* 2003; Kessler & Baldwin 2007). Volatile nicotine could arise from nectar or other floral tissue. Thus, nicotine could affect floral visitors not only via nectar consumption but also through affecting floral scent that deters pollinators before consuming nectar.

Nicotine but not anabasine fit our predictions for pollinator-mediated selection on chemical defence. There are several explanations for these contrasting results. First, anabasine may not be as deterrent to pollinators as nicotine (but see Singaravelan *et al.* 2005). Anabasine is deterrent to some non-native pollinators (Tadmor-Melamed *et al.* 2004; Singaravelan *et al.* 2005), but effects on native species are unknown. Second, nicotine was detected in nearly all taxa, while anabasine was absent or in trace amounts from more than one-third of species. When present, anabasine concentrations were substantially lower than nicotine (Table S4). The low frequency and concentration of anabasine compared with nicotine may result in less statistical power to detect effects, or the concentrations may be below thresholds detectable by pollinators. Third, anabasine is much less volatile than nicotine. Because alkaloid concentrations were 2–3 orders of magnitude higher in flowers than nectar, the primary mechanism for influencing pollinator preference may be via floral scent rather than nectar

consumption. If this is the case, then we would expect our predictions to hold for nicotine but not anabasine.

Our ability to understand and predict the evolution of defences may be enhanced by including interactions with mutualists such as pollinators as well as herbivore antagonists. We found that outcrossing species, which rely on pollinators for reproduction, had lower nicotine concentrations in all tissues compared with selfing species, with a 15-fold difference in leaf nicotine levels. Because floral chemical defences are found in many plant families with varied mating systems (Adler 2000), our hypothesis represents a mechanism of selection on chemical defence that is relevant to a diversity of species. Correlated defence concentrations across tissues suggest that selection by pollinators could shape the evolution not only of floral chemical defences but also chemical defences in vegetative tissues where herbivores have traditionally been considered the driving agent of selection.

ACKNOWLEDGEMENTS

We thank J. Barron, K. Benoit, K. Bouley and B. Sloop for research assistance; A. Backhaus, T. Beauchesne, M. Distl, C. Joyner and D. Wiley for greenhouse support and technical assistance; J. J. Clarkson for providing DNA sequence data; P. S. Warren for assistance with figures; F. M. Adler for photographs; D. Zaitlin for seeds; V. Sisson for seeds and tobacco consultation; and R. E. Irwin, M. Johnson, R. Raguso, S. McArt, S. Y. Strauss, J. Whittall, members of the Adler lab and three anonymous reviewers for comments on the manuscript. Research was funded by NSF DEB-0211480/0514398 (LSA) and associated REU for MGS. Any opinions, findings, and conclusions or recommendations expressed in this material are those of the authors and do not necessarily reflect the views of the National Science Foundation.

AUTHOR CONTRIBUTIONS

LSA designed the study and wrote the manuscript with assistance from MGS; MGS conducted the experiment, analysed data and wrote the manuscript with assistance from LSA; MW conducted chemical analysis; GEM conducted phylogenetic and comparative analyses and wrote associated text. All authors discussed the results and commented on the manuscript.

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Editor, Ted Turlings

Manuscript received 10 May 2012

First decision made 13 June 2012

Manuscript accepted 26 June 2012