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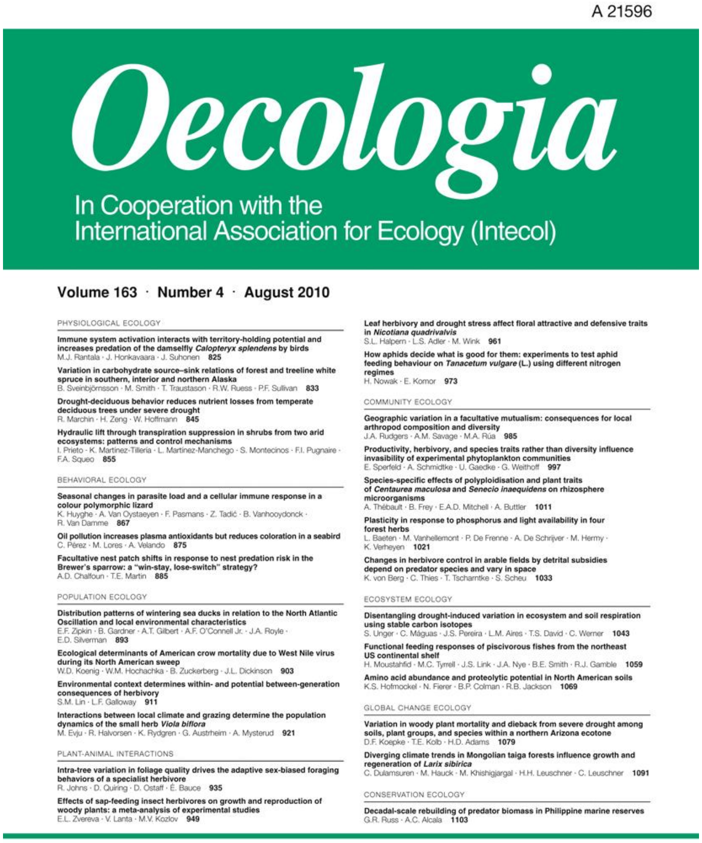

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PLANT-ANIMAL INTERACTIONS - ORIGINAL PAPER

Leaf herbivory and drought stress affect floral attractive and defensive traits in *Nicotiana quadrivalvis*

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Abstract Adaptive phenotypic plasticity allows sessile organisms such as plants to match trait expression to the particular environment they experience. Plasticity may be limited, however, by resources in the environment, by responses to prior environmental cues, or by previous interactions with other species, such as competition or herbivory. Thus, understanding the expression of plastic traits and their effects on plant performance requires evaluating trait expression in complex environments, rather than across levels of a single variable. In this study, we tested the independent and combined effects of two components of a plant's environment, herbivory and water availability, on the expression of attractive and defensive traits in *Nicotiana quadrivalvis* in the greenhouse. Damage and drought did not affect leaf nicotine concentrations but had additive and non-additive effects on floral attractive and defensive traits. Plants in the high water treatment produced larger flowers with more nectar than in the low water treatment. Leaf damage induced greater nectar volumes in the high water treatment only, suggesting that low water limited plastic responses to herbivore damage. Leaf damage also tended to induce higher nicotine concentrations in nectar, consistent with other studies showing that leaf damage can induce floral defenses. Our results suggest that there are separate and synergistic effects of leaf herbivory and drought on floral trait expression, and thus plasticity in response to complex environments may influence plant fitness via effects on floral visitation and defense.

Keywords Induced defenses · *Manduca sexta* · Nectar chemistry · Nicotine · Phenotypic plasticity

Introduction

Many plants can respond to environmental variation with changes in trait expression, or phenotypic plasticity (Schlichting 1986). Studies of phenotypic plasticity often measure responses to a single aspect of the environment, sometimes also evaluating whether the observed plasticity is adaptive (Dudley and Schmitt 1996; Baldwin 1999; Roumet et al. 1999; Buskirk and Schmidt 2000; Steinger et al. 2003; Yeh and Price 2004; Charmantier et al. 2008; Manriquez et al. 2009). In reality, however, organisms inhabit complex environments, and phenotypic plasticity to multiple environmental variables may not be predictable from the effects of each variable alone (Izaguirre et al. 2006; Gianoli et al. 2007; Valladares et al. 2007; Anten et al. 2009; Moreno et al. 2009). Nonadditive effects occur in part because the costs and benefits of phenotypic plasticity can vary with resource availability or the timing of

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environmental challenges (Alpert and Simms 2002; Valladares et al. 2007). For example, there are greater costs associated with the stem elongation response to shading in dry microhabitats than wet ones for *Impatiens capensis* (Huber et al. 2004). Nonadditive effects may also occur if simultaneously responding to multiple components of the environment limits plasticity to any individual environmental component (Weinig and Delph 2001; Haag et al. 2004; Izaguirre et al. 2006; Valladares et al. 2007; Anten et al. 2009). Thus, understanding the evolutionary ecology of plastic traits involves evaluating how their expression is affected by complex environmental variation. Although some recent studies have addressed these issues, phenotypic plasticity in complex environments is still understudied (Teplitsky et al. 2007).

In this study, we investigate phenotypic plasticity in a more complex environment by varying two important components of a plant's abiotic and biotic environment, water availability and damage from herbivores, respectively. We focus on floral morphology and plant chemistry, traits that often show plasticity in response to these aspects of the environment (e.g., Gershenzon 1984; Karban and Baldwin 1997; Galen 1999; Carroll et al. 2001). These traits may also influence plant fitness by attracting pollinators or defending against herbivorous insects (e.g., Marquis 1984; Hendrix 1988; Stanton and Preston 1988; Mutikainen and Delph 1996; Ashman et al. 2004). Some recent studies have investigated plasticity across complex environments in traits relevant to plant–animal interactions, focusing on the interactions between shading and induced resistance (Kurashige and Agrawal 2005; Izaguirre et al. 2006; Gianoli et al. 2007; Moreno et al. 2009) or nutrients and herbivory tolerance (e.g., Huhta et al. 2000; Wise and Abrahamson 2007). However, we still know little about how plasticity in response to complex environments affects traits relevant to both pollinators and herbivores.

Plasticity to water availability or herbivore damage alone is well-documented for both floral morphology and leaf chemistry. For example, flower size often varies along natural clines of water availability (Galen 1999; Elle 2004; Herrera 2005; Lambrecht and Dawson 2007) or among years that vary in precipitation (Campbell 1996). Experimental water additions can increase nectar production (Zimmerman 1983), and water-stressed plants tend to produce smaller flowers (Carroll et al. 2001; Caruso 2006) with less nectar (Boose 1997; Leiss and Klinkhamer 2005). Leaf damage from herbivores may also cause plants to produce smaller, less attractive flowers (Strauss et al. 1996, 1999; Lehtilä and Strauss 1997) with greater concentrations of secondary metabolites in corollas, nectar or fruits (Ohnmeiss and Baldwin 2000; Adler et al. 2006; McCall and Karban 2006). Leaf chemistry is also plastic in response to these variables; limited water or abundant nutrients can increase the constitutive concentration of secondary compounds in plant tissues (reviewed by Gershenzon 1984; Holtzer et al. 1988), while prior damage can increase the expression of mechanical or chemical defenses (i.e., induced resistance; reviewed by Karban and Baldwin 1997).

We addressed how complex environments affected floral and defensive trait expression in *Nicotiana quadrivalvis*. Based on previous studies with *Nicotiana* spp., we expect herbivory to affect both attractive and defensive traits in *N. quadrivalvis*. Leaf damage induces higher alkaloid concentrations in the vegetative tissue of several other *Nicotiana* species (reviewed by Baldwin 1999; Kaplan et al. 2008). Recent studies have also found reduced florivory (McCall and Karban 2006) or increased flower and nectar alkaloids after leaf damage (Ohnmeiss and Baldwin 2000; Adler et al. 2006), the latter typically in bolting plants. Leaf damage could induce changes in floral and nectar chemistry if the same biochemical pathways are upregulated in both kinds of tissue or if genes associated with induced resistance have pleiotropic effects, as has been suggested for constitutive resistance in other systems (Simms and Bucher 1996; Fineblum and Rausher 1997; Irwin et al. 2003). If leaf damage causes changes in resource allocation and availability, we also predict reductions in flower size. Less is known about how water influences the chemistry of attractive traits, but we predict that limited water will increase alkaloid concentrations of all tissues and decrease flower size, as has been found in other systems (Gershenzon 1984; Holtzer et al. 1988; Carroll et al. 2001; Caruso 2006). Finally, we expect synergistic effects of herbivory and water limitation on trait expression if the availability of resources constrains plasticity (Huber et al. 2004; Kurashige and Agrawal 2005). In particular, we expect water limitation to reduce the magnitude of induced defenses in response to damage, while herbivory may exacerbate the effects of drought on flower size.

To evaluate the potential for nonadditive effects of biotic and abiotic factors on attractive and defensive phenotypes, we manipulated water availability and herbivory in a greenhouse study. Specifically, we asked: what are the individual and combined effects of herbivory and water availability on the expression of attractive and defensive traits?

Materials and methods

Study system

Nicotiana quadrivalvis Pursh (=*bigelovii*; Solanaceae) is an annual allopolyploid ($n = 24$) native to western North

America that flowers from May to October (Goodspeed 1954). It grows in sandy washes and dry river beds in riparian zones at lower elevations in California and Oregon in populations ranging from dozens to hundreds of individuals (Goodspeed 1954). *Nicotiana quadrivalvis* produces relatively high leaf alkaloid concentrations (mean 7.76 mg/g), and nicotine constitutes 94% of total alkaloids (Sisson and Severson 1990). Herbivory induces production of several defenses including nicotine (Lou and Baldwin 2003). Common herbivores include the generalists *Heliothis virescens* and *Spodoptera exigua* (Noctuidae) and the specialists *Manduca sexta*, *M. quinquemaculata* (Sphingidae), *Tupiocoris notatus* (Miridae), and *Trichobaris* weevils (Curculionidae) (A. Kessler, personal communication).

Plants are self-compatible, but have higher fruit abortion in the absence of pollinators (M. Gittinger, L.S. Adler, M. Wink and G. Morse, unpublished data). Flowers are white and tubular, and most open at dusk. Plants are pollinated by the sphingid *Hyles lineata* (A. Kessler, personal communication). Flowers are also visited by adults of *M. sexta*, *M. quinquemaculata*, *H. virescens* and *S. exigua*, all of whom oviposit on tobacco and whose larvae are common herbivores (A. Kessler, Personal communication), but whose pollination efficiency has not been tested.

Experimental design and plant propagation

We manipulated water and herbivory in a 2 × 2 factorial design within 11 families of *N. quadrivalvis*. Each 'family' consisted of full-sibling seeds from 1 of 11 different parent plants, which were grown from seed obtained from the USDA (PI 555485, TW 18). All seeds from a family came from one randomly selected fruit per plant from self-pollinated flowers. Three replicate plants were grown for each family–water–herbivory combination, for a total of 132 plants (11 families × 2 water treatments × 2 herbivory treatments × 3 replicates). Seeds were germinated in plug trays filled with Metromix 360 soil (Scotts-Sierra Horticultural Products, Marysville, OH, USA) and vermiculite scattered on the surface. Seeds were germinated in March 2005 on a mist bench for 3 weeks. Beginning on April 12, they were transplanted twice into successively larger pots, ending with 3.78-l pots. Plants were randomly assigned to herbivory and water treatments, and randomly arranged on one greenhouse bench within three adjacent spatial blocks. Each block contained one replicate of each family–water–herbivory treatment combination. All plants were watered as needed until water treatments began on April 28. All plants were fertilized in twice in late June, after all measures except nectar collections were completed. On June 21, each pot received 6 g of Osmocote Classic Controlled Release Fertilizer 14-14-14 (Scotts-Sierra Horticultural Products), and on June 24, plants were treated with Peters Professional Water Soluble Fertilizer 20-20-20 General Purpose (Scotts-Sierra Horticultural Products) at a 300-ppm N rate. Plants grew under natural light only, so photoperiod increased over the season.

Water treatments

Plants were randomly assigned to high and low water treatments within family. Initially, high water plants were watered to field capacity twice daily while low water plants were watered to field capacity once every other day. One week after beginning treatments (May 5), we reduced watering to once every third day in the low water treatment. This level produced some wilting by the third day, but no mortality or signs of severe drought stress. Water treatments were then maintained for the duration of the experiment. It is difficult to precisely mimic natural drought conditions in the greenhouse, but water limitation is likely common for this species in at least some years because it grows in sandy soils with substantial seasonal and interannual variation in precipitation (data from National Oceanic and Atmospheric Administration).

Herbivory treatments

Each plant was randomly assigned to herbivory or control (no damage) treatments. Treatments were imposed beginning May 4, when plants were at the rosette stage and beginning to bolt. In studies with other *Nicotiana* species, damage to bolting plants increased alkaloids in floral structures (Ohnmeiss and Baldwin 2000; Adler et al. 2006). To impose damage, we used fifth instar *Manduca sexta* larvae (North Carolina State University Insectary, USA), which are specialists on Solanaceous plants including *N. quadrivalvis*. Leaves were damaged by placing one *M. sexta* larva sequentially on each of the three youngest expanded leaves within mesh bags; control plants received bags without larvae. Larvae and bags were removed when 90–100% of leaf material was consumed. This damage level (up to three leaves per plant) is well within the range of natural herbivory (e.g., van Dam et al. 2001). Treatments were complete by May 6 and bags were removed from all plants.

Determination of nicotine

Nicotine was quantified in leaves and nectar for each plant. At least three of the next youngest fully expanded undamaged leaves were collected from each plant 4–5 days after herbivory treatments ended, and immediately stored in plastic bags at −20°C until analysis. Leaves were then freeze dried and ground prior to analysis. Thus, our analysis of leaf induction is based on a relatively short time interval after damage.

Leaf damage could have longer-term impacts on other ecological interactions by inducing changes in floral traits (as in Lehtilä and Strauss 1999; Adler et al. 2006). After plants began to flower, nectar was collected by gently removing the corolla, squeezing the corolla tube to produce nectar at the flower base, and collecting nectar with 10- or 25-μl glass microcapillary tubes. While this method could contaminate samples with corolla alkaloids, alternative methods of collection through the top corolla opening resulted in frequent pollen contamination and incomplete nectar acquisition. Collection through the corolla base resulted in the cleanest sample and largest volume of nectar. Nectar was sampled between May 27 and September 1, and stored at −20°C until analysis. Dates of nectar collection depended on individual plant phenology; 85% of samples were collected in May, June or July. Nectar was pooled within plants to collect a minimum of 200 μl per sample and then freeze dried prior to analysis. To see whether treatments differed in the period of nectar collection for this pooled sample, we used ANOVA to test for differences among treatments in the first and last date of nectar collection, the duration of nectar collection (last date–first date), and the weighted averaged collection date (weighted by proportion of nectar in the final sample that was collected on each date). There was no effect of damage treatment, block, or any interactions on the timing of nectar collection. Plants in the high water treatment had a later weighted mean collection date (by 11 days) and a longer period of nectar collection (by an average of 17 days) that both started earlier and ended later than plants in the low water treatment.

Nectar and leaf samples were extracted in 1 or 50 ml methanol, respectively; an aliquot of 50 μl was used for HPLC without further evaporation in order to avoid losses. To the 50-μl aliquot, we added 25 μl of sodium acetate buffer (5.44 g in 10 ml water; adjusted to pH 4.7 with 100% acetic acid), 10 μl KCN (0.976 g in 10 ml water), 10 μl chloramine T (0.1113 g in 1 ml water), and 50 μl barbituric acid (0.1 g in 10 ml water). The solution was mixed and left standing for exactly 15 min, and then 10 μl sodium disulfite (1.9 g in 10 ml water) was added to stop the color reaction. The product was analyzed by HPLC; absorbance was measured at 505 nm. The HPLC configuration (HPLC, Beckmann system gold; Beckmann, Fullerton, USA) consisted of a HPLC pump (Beckmann 125P) connected to a photodiode array detector (Beckmann 168; wavelength 505 nm). The mobile phase-linear gradient was water–acetonitrile from 0 to 100% acetonitrile in 15 min. The column used was Merck LiChroCART RP-18 (5 μm, 250 mm, 4 mm; Merck, Darmstadt, Germany). Injection volume was 20 μl and the flow-rate was 1 ml/min. Before the next injection, the column was equilibrated for 3 min. Concentrations of authentic nicotine and anabasine were determined by calibration curves using standards at concentrations between 0.3 and 50 ng/μl.

Measuring attractive floral traits

Between May 23 and June 28, we measured corolla tube length (from petal flare to base of the ovary), corolla tube width (maximum opening at flare), corolla tube width at base (measured at top of sepals), petal lobe length (from corolla opening to outermost petal lobe tip of a haphazardly chosen petal lobe), petal lobe width (widest point of a haphazardly chosen petal lobe), stigma/anther exertion (distance between the stigma and the closest anther, with positive values for exerted stigmas and negative for exerted anthers), and nectar sugar concentration on three flowers per plant. All morphology measures were made to 0.01 mm using digital calipers, and sugar concentration was measured in Brix using a pocket refractometer. We measured the same traits on a fourth flower per plant between July 5 and 20, and also quantified nectar volume in that flower using glass microcapillary tubes following the method described above for defensive chemistry.

Statistical analysis

We used within-plant averages for early floral morphology traits and nectar sugar concentration because plants were the unit of replication. Average concentrations of nicotine were substantially greater than anabasine in both leaves (nicotine = 4,489 ± 385 μg/g, anabasine = 193 ± 16 μg/g) and nectar (nicotine = 6.95 ± 1.01 ng/μl, anabasine = 0.23 ± 0.12 ng/μl), and anabasine was not present in most nectar samples. Therefore, analyses of defensive chemistry considered only nicotine. To homogenize variances and improve distribution of residuals, we analyzed the natural log of nectar nicotine concentration, early corolla tube length, and early corolla tube width at base as well as the square root of leaf nicotine concentration.

To determine whether treatments affected plant traits, we performed MANOVA and ANOVA. Models included main effects of water treatment, herbivore treatment, block and all possible interactions. To account for maternal environmental effects, we also included family as a random effect; we anticipated little genetic variation between maternal families because all plants originated from one USDA accession. All other main effects and interactions were treated as fixed effects. We used *F* tests (constructed using the mean square error) to test the statistical significance of all effects in the full model. We tested for responses in leaf nicotine and nectar nicotine with univariate analyses alone. For floral attractive traits (morphology and nectar), we used MANOVA followed by ANOVA to identify individual traits that contributed to overall floral responses, when present. For significant interactions, we tested for differences among groups with post hoc tests of least-square means, using the Tukey–Kramer adjustment for multiple comparisons. All analyses were carried out using PROC GLM in SAS with family identified as random (PROC GLM, SAS Institute 1999).

Results

Effect of water and herbivory on traits

Chemical traits

Across all environments, average (±SE) concentrations of nicotine were substantially greater in leaves than in nectar (leaves = 4,489 ± 389 μg/g, $n = 127$; nectar = 6.95 ± 1.03 ng/μl, $n = 110$). Damaged plants tended to have greater levels of nicotine in their nectar (7.6 ± 1.2 ng/μl vs. 6.2 ± 1.6 ng/μl), although damage effects on nectar chemistry also tended to differ among blocks (Table 1). Water availability did not significantly affect nectar nicotine concentrations (Table 1), even though nectar was collected over a longer period of time from plants in the high versus low water treatment (see "Materials and methods"). Data analysis excluding plants from which nectar was collected after July 31 had similar results, except the effect of damage on nectar nicotine was statistically significant ($F_{1,53} = 6.4$, $P = 0.01$). Leaf nicotine levels were not affected by water, damage, block, or their interactions (Table 1; Electronic supplementary material, ESM 1). Results were similar for analyses using total alkaloid concentration (anabasine + nicotine; data not shown). Maternal family affected leaf nicotine concentrations but not nectar nicotine (Table 1).

Table 1 Sources of variation in nicotine concentrations in nectar and leaves of *Nicotiana quadrivalvis* from mixed model ANOVA

Source	*df*	Nectar		Leaves	
		F	*P*	*F*	*P*
Damage (D)	1	3.73	0.057	0.00	0.955
Water (W)	1	1.95	0.166	1.17	0.281
W × D	1	1.11	0.294	1.43	0.235
Block (B)	2	1.47	0.236	0.13	0.877
D × B	2	3.03	0.053	0.40	0.672
W × B	2	0.64	0.530	0.57	0.569
W × D × B	2	0.35	0.706	0.26	0.772
Family	10	1.14	0.340	4.30	<0.0001

Response transformations were log (nectar nicotine) and square root (leaf nicotine)

Statistical significance for all factors was tested over the mean square error

Error $df = 88$ (nectar) and 105 (leaves)

Attractive traits

Both water availability and damage affected traits associated with floral attractiveness (Table 2). Water alone affected overall flower morphology in early but not late flower measurements (Tables 2, 3 and 4). Univariate ANOVA showed that this effect on early flower morphology was driven by differences in flower width; plants grown with more water had greater corolla tube diameter and petal lobe length (Fig. 1). Stigma exertion and corolla tube length were not affected by water availability. The effects of water on nectar characteristics varied among blocks and through time (Table 2; ESM 1 and 2). Earlier in the experiment (May/June), water did not affect nectar sugar concentration (Table 3); nectar volume was not measured at this time. At the later measurement time (July), plants in the low water treatment had reduced nectar volume compared to plants in the high water treatment (10.6 ± 1.4 μl vs. 14.9 ± 1.3 μl; Table 4). The effect of water on nectar volume and sugar also varied among blocks (Tables 2 and 4).

Herbivore damage did not independently induce changes in floral attractive traits (damage effect, Table 2). However, herbivore damage affected nectar volume differently in the two water treatments (water × damage interaction, Table 4). For plants in the low water treatment, damage had no effect on nectar volume. In contrast, nectar volume increased in damaged plants in the high water treatment (Fig. 2). The effects of damage on late flower morphology also differed somewhat between blocks (Table 2; ESM 1).

Both early and late floral morphology varied with maternal family (Table 2). In early flowers, these effects occurred for corolla length, petal lobe length, and stigma exertion (Table 3). For late floral measurements, the family effect only persisted in corolla tube length (Table 4).

Discussion

Plasticity in response to damage

In this experiment, phenotypic plasticity in response to herbivory was limited by the plant's abiotic environment. Evidence for these limits comes from the conditional response to leaf damage, which increased nectar volume only when water was plentiful (Fig. 2; Table 4). Floral traits commonly change in response to abiotic conditions such as water availability (Galen 1999; Carroll et al. 2001) as well as biotic interactions such as herbivory (Quesada

Table 2 Sources of variation in floral traits of *Nicotiana quadrivalvis* measured early (May–June) or late (July) in the season, analyzed with MANOVA

Source	Early floral morphology		Late floral morphology		Late nectar traits	
	Wilk's λ	P	Wilk's λ	P	Wilk's λ	P
Water (W)	0.76	<0.0001	0.89	0.11	0.86	0.005
Damage (D)	0.96	0.59	0.92	0.21	0.98	0.45
W × D	0.97	0.70	0.86	0.25	0.91	0.035
Block (B)	0.89	0.30	0.91	0.16	0.68	<0.0001
W × B	0.87	0.17	0.85	0.21	0.77	0.0007
D × B	0.91	0.45	0.80	0.048	0.94	0.35
W × D × B	0.93	0.63	0.93	0.84	0.99	0.91
Family	0.40	<0.0001	0.40	0.008	0.77	0.57

Early floral morphology traits included corolla tube length (mm), corolla tube width at flare (mm), corolla tube width at base (mm), petal lobe length and width (mm), and stigma/anther exertion (mm) measured on three flowers per plant

All these traits plus nectar volume (μl) and nectar sugar concentration (Brix) were measured on a fourth flower during the late time period

We analyzed nectar and late morphology traits separately

For early morphology only, corolla tube length and width at base were ln transformed to make variances more equitable

Table 3 Sources of variation in early floral traits of *Nicotiana quadrivalvis* in May and June from mixed model ANOVA

Source	*df*	Ln corolla tube diameter, base (mm)		Corolla tube diameter, flare (mm)		Ln corolla tube length (mm)		Petal lobe length (mm)		Stigma exertion (mm)		Sugar concentration (Brix)	
		F^{a}	P	F^{a}	P	F^{a}	P	F^{a}	P	F^{b}	P	F^{a}	P
Water (W)	1	6.66	**0.01**	6.26	**0.01**	2.78	0.10	10.6	**0.002**	0.21	0.65	0.06	0.80
Damage (D)	1	0.02	0.88	0.47	0.49	2.53	0.11	1.64	0.20	0.83	0.36	0.01	0.91
W × D	1	0.09	0.77	0.53	0.47	1.44	0.23	1.88	0.17	1.10	0.30	0.05	0.83
Block (B)	2	0.11	0.89	0.18	0.84	1.52	0.22	2.10	0.13	0.45	0.64	0.02	0.98
W × B	2	0.44	0.65	1.75	0.18	1.28	0.28	1.71	0.19	0.07	0.93	0.07	0.93
D × B	2	0.42	0.66	1.93	0.15	0.59	0.56	2.95	0.06	0.73	0.49	0.74	0.48
W × D × B	2	0.59	0.56	0.25	0.78	0.45	0.64	1.15	0.32	1.39	0.25	0.06	0.94
Family	10	0.96	0.48	0.77	0.66	4.16	**<0.0001**	2.30	**0.02**	2.63	**0.007**	1.57	0.13

Bold indicates $P < 0.05$. Morphology traits were average of measurements from three flowers/plant

[a] Error $df = 105$

[b] Error $df = 96$

et al. 1995; Strauss et al. 1996; Krupnick et al. 1999). Reports of conditional responses, however, are rarer.

Conditional changes in nectar volume in response to herbivore damage could represent a constraint on the expression of plasticity, where the degree of plasticity in nectar volume is limited by resources. Limits to plasticity have been observed in damaged plants that subsequently face abiotic stresses (reviewed by Valladares et al. 2007). Similarly, morphological changes in response to shading (the shade avoidance syndrome) can reduce the degree of plasticity to subsequent herbivory (Kurashige and Agrawal 2005; Izaguirre et al. 2006; Moreno et al. 2009). In this experiment, the herbivore-induced increase in nectar volume that occurred only with ample water indicates that synergistic effects of the biotic and abiotic environment ultimately determined the expression of phenotypic traits. Thus, this study supports the need to study phenotypic plasticity in complex environments (Teplitsky et al. 2007). It also supports the argument that understanding how selection acts on attractive and defensive traits (e.g., via herbivore or pollinator preference) requires characterizing the strength or direction of selection on those traits in multiple environments (Agrawal et al. 2007).

Nectar nicotine concentrations also increased in response to leaf herbivory, although this effect was marginal when late-flowering plants were included (Table 1). Our measure of nicotine induction in nectar represents long-term responses to early leaf damage, and may be conservative, because acquiring a large enough sample for chemical analysis required collecting and pooling multiple

Table 4 Sources of variation in late floral traits of *Nicotiana quadrivalvis* in July from mixed model ANOVA

Source	*df*	Corolla tube diameter, base (mm)		Corolla tube diameter, flare (mm)		Corolla tube length (mm)		Petal lobe length (mm)		Stigma exertion (mm)		Sugar concentration (Brix)		Nectar volume (μl)	
		F^a	P	F^a	P	F^a	P	F^a	P	F^a	P	F^b	P	F^a	P
Water (W)	1	5.02	**0.028**	1.33	0.25	3.49	0.06	2.38	0.13	3.53	0.06	3.25	0.08	7.00	**0.01**
Damage (D)	1	0.52	0.47	0.01	0.93	1.62	0.21	4.01	**0.048**	0.50	0.48	0.97	0.38	1.48	0.23
W × D	1	1.43	0.24	0.22	0.64	2.43	0.12	0.10	0.75	0.00	0.95	1.67	0.20	4.14	**0.045**
Block (B)	2	0.81	0.45	0.51	0.60	0.78	0.46	1.57	0.21	1.82	0.17	14.8	**<0.0001**	4.07	**0.021**
W × B	2	2.78	0.07	1.42	0.25	0.03	0.97	0.16	0.85	2.24	0.11	8.27	**0.0006**	1.20	0.31
D × B	2	1.83	0.17	0.02	0.98	2.36	0.10	0.02	0.98	2.80	0.07	1.23	0.30	0.53	0.59
W × D × B	2	0.37	0.69	0.02	0.98	0.63	0.54	1.27	0.29	0.74	0.48	0.42	0.66	0.06	0.95
Family	10	0.86	0.57	1.30	0.25	2.73	**0.006**	0.75	0.68	1.44	0.18	0.83	0.60	1.27	0.26

Bold indicates $P < 0.05$

[a] Error $df = 83$

[b] Error $df = 73$

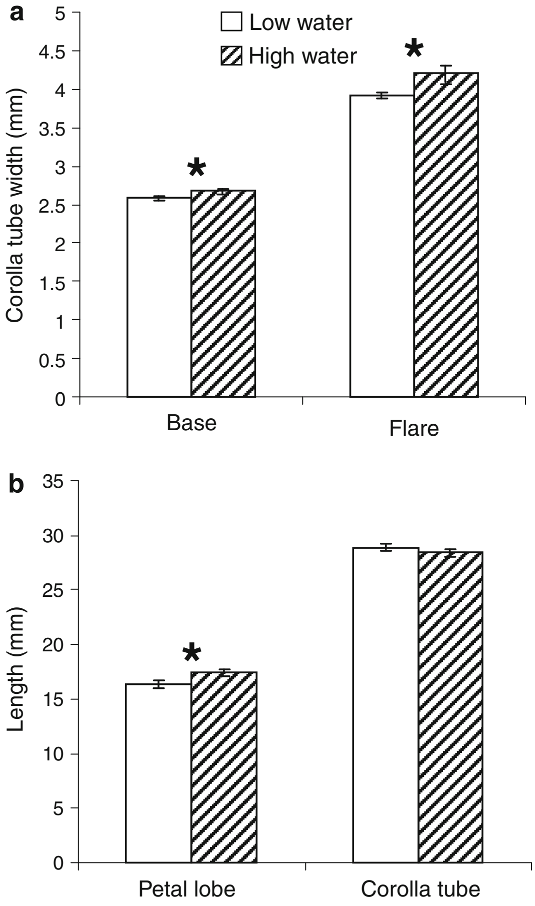

Fig. 1 Effects of water availability on May/June flower size of *Nicotiana quadrivalvis* grown in the greenhouse. **a** Mean corolla tube width at the base of the flower (*bottom*) and the flare (*top*). **b** Mean petal lobe and corolla tube lengths. Traits with *asterisks* differ significantly between water treatments as determined by ANOVA. *Error bars* ±1 SE

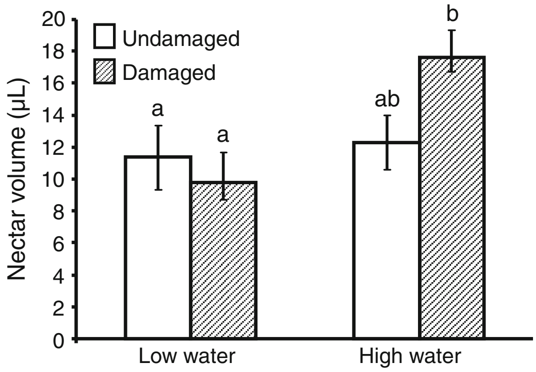

Fig. 2 Effects of water availability and prior leaf damage on mean (±1 SE) nectar volume of *Nicotiana quadrivalvis* grown in the greenhouse ($n = 105$). The interaction between water and damage treatments was significant ($F_{1,20.7} = 4.35$, $P = 0.0495$). *Bars* with *different letters* differ at the $P < 0.05$ level, based on post hoc comparisons of least square means, with the Tukey–Kramer adjustment for multiple tests

samples for each plant over several weeks. Given this, it is remarkable that we detected stronger effects of leaf damage on nectar nicotine than on leaf nicotine, which was sampled once per plant during the time frame of greatest induced responses in other *Nicotiana* species (e.g., Baldwin 1988). Our results are consistent with other studies finding that leaf damage to bolting *Nicotiana* plants induced higher nicotine concentrations in flowers but not leaves (Ohnmeiss and Baldwin 2000; Adler et al. 2006). Thus, herbivore damage to pre-reproductive plants may have long-lasting consequences for interactions with nectar-feeders, including pollinators, nectar-robbers, and/or nectar-feeding herbivores. Long-term chemical changes with damage have also been observed in trees, where systemic responses to damage can alter leaf defensive chemistry later in the season (Stevens and Lindroth 2005) or in subsequent years (e.g., Tuomi et al. 1984; Haukioja and Neuvonen 1985).

The tendency for induction of nectar alkaloids was not simply a by-product of greater alkaloid production in other plant tissues or of plants being pot-bound. Leaf nicotine levels did not change in response to damage, and leaf and nectar nicotine concentrations were not correlated (Pearson $r = 0.01$, $P = 0.95$, $n = 95$). In this experiment, differences in nicotine induction between leaves and nectar could arise from ontogeny (i.e., differences in inducibility among plant organs at various points in development), from tissue-specific responses to treatments, or both. Although concerns about the inability of pot-grown plants to increase leaf nicotine concentrations in response to damage have been raised (i.e., in *Nicotiana sylvestris*; Baldwin 2007), another congener, *N. tabacum*, can induce leaf nicotine when grown in pots (Kaplan et al. 2008). Furthermore, our results parallel those of similar experiments in reproductive *N. sylvestris* and *N. tabacum*, in which leaf damage induced floral or nectar but not leaf alkaloids (Ohnmeiss and Baldwin 2000; Adler et al. 2006).

Plasticity in nectar volume and nicotine could be adaptive if these traits influence herbivore or pollinator behavior in ways that increase plant fitness. Greater nectar volume could influence pollinator behavior directly by increasing visitation rate (Hodges 1995). In some cases, increasing nectar volume can compensate for the decreased pollinator visitation that otherwise occurs with herbivore damage (Krupnick et al. 1999). Nectar volume could also influence pollinators via effects on the concentration of sugar or alkaloids. In this experiment, for example, sugar tended to be less concentrated in plants in the high water treatment compared to the low water treatment (19.6 ± 0.5% vs. 23.4 ± 0.7%, respectively; Table 4). In addition, there was a negative phenotypic correlation between nectar volume and nectar nicotine levels (Pearson $r = -0.22$, $P = 0.03$, $n = 98$). Higher nicotine levels in nectar (due to lower nectar volume or induced nectar nicotine) could increase plant fitness by altering the behavior of pollinators to maximize benefits of legitimate pollinators (Kessler and Baldwin 2006; Irwin and Adler 2008; Kessler et al. 2008) or to reduce visits from less desirable floral visitors such as nectar-thieves (Stephenson 1981), nectar-robbers (Adler and Irwin 2005; Kessler et al. 2008), florivores (Kessler et al. 2008), or poor pollinators (Johnson et al. 2006; Kessler and Baldwin 2006). In natural habitats, the net fitness effect of responses to drought and herbivory may depend on the community of floral interactors and the degree of pollen limitation. Regardless of the direction of fitness effects, the existence of inducible nectar characteristics suggests that leaf herbivory could alter selection on floral traits in this system.

Plasticity in response to water availability

Water availability affected floral characteristics but not leaf chemistry in this experiment. Compared to water-limited plants, plants in the high water treatment produced larger flowers that had a greater volume of nectar with a lower sugar concentration. These differences could arise in part from effects of water availability on ontogeny; environmental stressors can both delay development and alter developmental trajectories (i.e., Moriuchi and Winn 2005). Such differences in floral traits could affect pollinator attraction and plant fitness (Stanton and Preston 1988; Campbell et al. 1991; Johnson et al. 1995; Boose 1997). For example, larger flowers or floral displays are often more attractive to both pollinators and floral enemies (e.g., Stanton and Preston 1988; Campbell et al. 1991; Johnson et al. 1995; Carroll et al. 2001), although the strength of the preference for larger size can change across environments (Galen 1999; Totland 2001). Larger flowers may impose a cost, however, as water loss through corollas can be extensive (Galen et al. 1999). Therefore, producing smaller flowers in water-limited environments could improve overall plant water status and photosynthetic capacity (Galen 1999; Elle 2004) and thus the plant's ability to provision seeds.

In contrast, water did not affect constitutive or induced levels of alkaloids in nectar or leaves. These results differ from patterns observed in other systems where water stress results in higher concentrations of secondary metabolites in plant tissues or secretions (reviewed by Gershenzon 1984; Holtzer et al. 1988; Koricheva et al. 1998). Because other plant traits differed in the low water treatment, it is unlikely that our water treatments were insufficient to influence plant physiology. Other resources may simply be more important in determining alkaloid concentrations in this species. For example, nitrogen additions strongly increase alkaloid concentrations in leaves and nectar of other *Nicotiana* species (Lammarre 1979; Lynds and Baldwin 1998; Adler et al. 2006). *Nicotiana quadrivalvis*, along with most other *Nicotiana* native to North America, grow in relatively dry environments of the western part of the continent (Goodspeed 1954). As a result, these species may be relatively well adapted to cope with water stress. This hypothesis, however, should be explored with other *Nicotiana* species.

Phenotypic variation in nectar chemistry

Nectar nicotine levels varied substantially in this experiment (0–79 ng/μl). Few studies have examined intraspecific variation in nectar secondary compounds even though such variation may affect plant–animal interactions and plant fitness. For example, Kessler et al. (2008) used transgenic *Nicotiana attenuata* plants with RNA interference constructs that blocked nicotine production at the whole-plant level to manipulate nectar nicotine concentrations in the field. Transgenic plants that produced flowers without nicotine in their nectar had changes in pollinator behavior, increased attacks by florivores and nectar-robbers, and overall lower fitness than controls. Continuous variation in nectar nicotine levels may have similar effects and merits further study.

Conclusions

In this study, floral traits responded to both water stress and herbivory with substantial, and sometimes conditional, phenotypic plasticity. These results emphasize the importance of studying plastic responses in complex environments. Understanding how selection acts on both these traits and their plasticity will require incorporating these phenotypic responses into field studies that determine the effect of these traits on fitness. In particular, the growing body of literature exploring how traits mediate multiple plant–animal interactions (reviewed by Morris et al. 2007) may provide an incomplete picture of the evolutionary ecology if the abiotic environment is not considered.

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