American Journal of Botany 92(8): 1270–1283. 2005.

NECTAR TRAITS IN *NICOTIANA* SECTION *ALATAE* (SOLANACEAE) IN RELATION TO FLORAL TRAITS, POLLINATORS, AND MATING SYSTEM[1]

RAINEE L. KACZOROWSKI,[2] MARK C. GARDENER,[3] AND TIMOTHY P. HOLTSFORD[2,4]

[2]Department of Biological Sciences, University of Missouri–Columbia, Tucker Hall, Columbia, Missouri 65201 USA; and
[3]Department of Biological Sciences, The Open University, Walton Hall, Milton Keynes, MK7 6AA, UK

Nicotiana section *Alatae* exhibits great diversity among species in floral morphology, mating system, and predominant pollinators. As a first step towards estimating nectar's role in floral evolution, we studied nectar traits to determine whether they vary in association with predominant pollinators and mating system. Daily phenology determines when nectar becomes available to pollinators and differed between hummingbird- and moth-pollinated species. Nectar volume and concentration varied significantly among most species and pollinator groups, but were inversely correlated, so that total energy was similar among most species. In general, nectar volume was positively correlated with corolla length. The autogamous species, *N. plumbaginifolia*, had a nectar volume that matched expectations based on corolla length, but with lower concentration and total energy than predicted by corolla length, while nectar volume was lower than predicted by corolla length in the autogamous population of *N. longiflora*. Sugar and amino acid components (determined through HPLC) were similar among species, although differences did exist. The nectar of most species was sucrose-dominant, but the autogamous *N. plumbaginifolia* had nectar that contained similar proportions of sucrose, glucose, and fructose. Many nectar traits varied in association with the predominant pollinators and, in some cases, with the mating system.

Key words: amino acids; Argentina; Brazil; hummingbirds; moths; nectar; *Nicotiana*; Solanaceae.

Nectar chemistry is an important component of floral biology. Nectar drives pollination efforts by being the primary floral reward for most pollinators. Pollinators often exhibit a preference for certain types of nectar over others (Hainsworth and Wolf, 1976; Stiles, 1976; Pyke and Waser, 1981; Tamm and Gass, 1986). If pollinator preferences for certain nectar traits are strong enough, it may be possible for pollinator-mediated selection to cause ethological isolation, which has the potential to promote species divergence or maintenance during secondary contact (Grant, 1994). But pollinator roles in speciation have been called into question recently, mainly because the majority of plant–pollinator associations are generalized, while specialization is thought to be necessary to promote speciation (Ollerton, 1996; Waser et al., 1996; Waser, 1998).

Pollinators are known to respond to floral morphology (Cresswell and Galen, 1991; Schemske and Bradshaw, 1999; Ippolito, 2000; Galen and Cuba, 2001), color (Waser and Price, 1981; Jones and Reithel, 2001) and even nectar-related quantitative trait loci (Schemske and Bradshaw, 1999). Many pollinators preferentially visit particular flower types, making it possible to predict the primary pollinator of a flower by some of its distinguishing characteristics. Grant and Grant (1968) described flowers that are adapted primarily for the feeding and pollination of hummingbirds as pendulous, solitary or loosely clustered, having a thick-walled, red (or red with yellow) corolla, yielding large quantities of nectar at the base of a long, stout floral tube. ''Hummingbird flowers'' also generally lack a detectable odor (Baker, 1961; Grant, 1966; Grant and Grant, 1968; Raven, 1972). On the other hand, nocturnal moth-pollinated flowers are characterized as having corollas that are white or pale in color and as emitting a strong, sweet scent when open, which is usually in the evening or at night (Baker, 1961; Percival, 1965; Faegri and van der Pijl, 1966). Flowers adapted primarily for pollination by hawkmoths have a longer, more slender floral tube than typical ''hummingbird flowers'' (Grant and Temeles, 1992; Ippolito, 2000). Smaller perching moths are attracted to typical ''moth flowers,'' but with shorter corolla tubes that fit their proboscis length (Faegri and van der Pijl, 1966).

Nectar traits can affect pollinator behavior (Hainsworth and Wolf, 1976; Stiles, 1976; Pyke and Waser, 1981; Zimmerman, 1983; Galen and Plowright, 1985; Tamm and Gass, 1986; Cresswell and Galen, 1991; Martínez del Rio et al., 1992; Hodges, 1995; Meléndez-Ackerman et al., 1997; Schemske and Bradshaw, 1999) and, presumably, pollinator behavior can affect the evolution of nectar traits. From an outcrossing plant's perspective, flowers are most likely to be effectively pollinated when nectar reward is abundant enough to attract the pollinator, but small enough to force the pollinator to make numerous plant to plant visits (Heinrich and Raven, 1972; Heinrich, 1975; Baker, 1975). Large per-plant nectar rewards can increase pollinator visitation on a single plant, increasing the chances of geitonogamy (Galen and Plowright, 1985; Real and Rathcke, 1991; Hodges, 1995; Ferdy and Smithson, 2002). But pollinators are expected to prefer a nectar volume and concentration that optimizes foraging efficiency (Baker, 1975; Hainsworth and Wolf, 1976). Autogamous species, which are less dependent upon pollinator visitation, may evolve to produce less nectar than outcrossing species (Spira, 1980).

Many forces could affect the evolution of nectar traits, including environmental conditions, the plants' energy budget, water relations, and coevolution with nectar robbers, flori-

[1] Manuscript received 9 August 2004; revision accepted 17 May 2005.

The authors thank Mark Ellersieck for repeated assistance with statistical analyses, Allena Volskay for use of her corolla length data, Candace Galen for helpful comments on an earlier draft, and Jane Murfett for help in revising one of the final drafts. This study was supported in part by a GAANN fellowship through the U.S. Department of Education, NSF DEB 9727037, and UM Research Board 97-033.

[4] Author for correspondence (e-mail: HoltsfordT@missouri.edu)

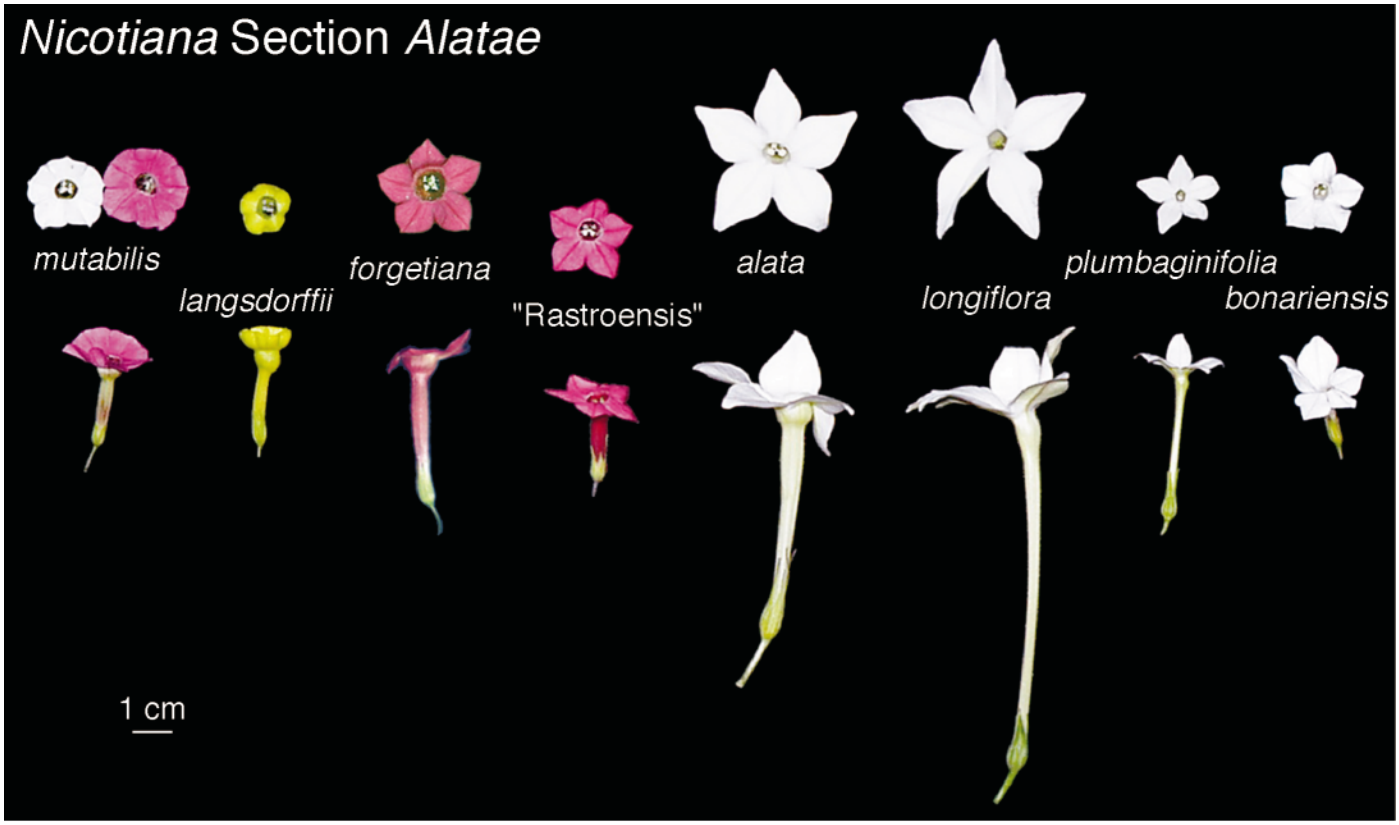

Fig. 1. The species of *Nicotiana*, section *Alatae*.

vores, and pollinators (Galen, 1999, 2000). Evolution due to any of these pressures may be constrained by lack of genetic variation in a population genetic sense, a phylogenetic sense, or due to antagonistic pleiotropy. Pollinators are unique in their evolutionary effects on plant traits because they not only contribute to plant fitness, but are also agents of gene flow and so could induce a phylogenetic split if different pollinators prefer different nectar traits (Grant, 1994). However, the occurrence of this scenario has been recently questioned because of the large number of species and guilds of pollinators that visit many flowers (Ollerton, 1996; Waser et al., 1996; Waser, 1998). We chose to study a group that seemed to be morphologically and phenologically adapted to hawkmoth, small moth, hummingbird, and autogamous pollination.

As a first step toward understanding pollinators' roles in the evolution of nectar traits, we investigated the associations between presumed pollination syndromes and several nectar traits in *Nicotiana* section *Alatae* (Fig. 1). Although it may be viewed as an oversimplification to interpret data based on pollination syndromes (Waser et al., 1996), identification of trends or differences among and between plant species with different pollinators can aid in the understanding of floral diversification mechanisms (Fenster et al., 2004). The likelihood that pollinators were important in the evolution of these species would be increased if the nectar traits were shown to vary in association with the pollinators and their preferences. Phylogenetic, quantitative genetic, and field experiments are underway separately to further test the importance of pollinator-mediated floral evolution. The primary pollinator associations in this group are hawkmoths (2 spp.), hummingbirds (4 spp.), and small settling moths (1 sp.). Autogamy is also found in one species that is derived from a hawkmoth-pollinated ancestor and still bears some hawkmoth syndrome features (Ippolito, 2000; Chase et al., 2003; J. Murfett and T. Holtsford, unpublished data) and in one population of an otherwise hawkmoth-pollinated species. We also evaluated the accessibility of nectar through daily phenology measurements for these eight *Nicotiana* species in order to determine whether nectar presentation matched periods of pollinator activity.

MATERIALS AND METHODS

Study system—*Nicotiana* section *Alatae* is a monophyletic group (Ippolito, 2000; Chase et al., 2003) comprised of seven or eight species (Fig. 1). The relationship of these species could not be completely resolved through ITS sequences, although species with the same chromosome number are more closely related (Ippolito, 2000; Chase et al., 2003). The self-incompatible species, *N. alata* Link and Otto, *N. forgetiana* hort. ex Hemsl., *N. langsdorffii* Schrank, *N. bonariensis* Lehm., the newly described *N. mutabilis* Stehmann and Semir (Stehmann et al., 2002), and the putative species ''Rastroensis'' (chromosome $n = 9$), are restricted to southeastern Brazil, Paraguay, Uruguay and eastern Argentina. The self-compatible species, *N. longiflora* Cav. and *N. plumbaginifolia* Viv. (chromosome $n = 10$), have a more extensive range. *Nicotiana longiflora* is found in northern Argentina, southern Bolivia, Paraguay and Uruguay. *Nicotiana plumbaginifolia*, which evolved from *N. longiflora* (nrITS: Ippolito, 2000; 256 ISSR bands: J. Murfett and T. Holtsford, unpublished data), is autogamous and weedy. It can be found from northwestern Argentina, north through Central America into Mexico. It has also been found on multiple Caribbean islands, as well as in India (Goodspeed, 1954).

More than 200 h of field observations over 5 years suggest that there are

TABLE 1. Populations and number of plants sampled within each species of *Nicotiana* for nectar volume and concentration data (N_1), as well as sugar and amino acid data (N_2).

Species	Population	N_1	N_2
N. mutabilis Stehmann and Semir	Quebra Cabo (1.3K), RS, Brazil	4	2
N. mutabilis Stehmann and Semir	Quebra Cabo (6.7K), RS, Brazil	9	2
N. mutabilis Stehmann and Semir	Quebra Cabo (8.1K), RS, Brazil	7	2
N. langsdorffii Schrank	Major Vieira, SC, Brazil	8	2
N. langsdorffii Schrank	Morro da Igreja, Urubici, SC, Brazil	8	2
N. langsdorffii Schrank	Papanduva, SC, Brazil	0	2
N. forgetiana hort. ex Hemsl.	Caxias do Sul, RS, Brazil	9	2
N. forgetiana hort. ex Hemsl.	Otavia, RS, Brazil	9	2
N. forgetiana hort. ex Hemsl.	São Marcos, RS, Brazil	9	2
''Rastroensis''	Bom Jardim da Serra, SC, Brazil	9	6
N. alata Link and Otto	Rio das Antas (North), RS, Brazil	9	2
N. alata Link and Otto	Rio das Antas (South), RS, Brazil	9	2
N. alata Link and Otto	Rio Pelotas, RS/SC, Brazil	9	2
N. longiflora Cav.	Calilegua, Jujuy, Argentina	8	0
N. longiflora Cav.	Jujuy, Jujuy, Argentina	3	0
N. longiflora Cav.	Universidad Nacional de Nordeste, Corrientes, Argentina	7	6
N. plumbaginifolia Viv.	Calilegua, Jujuy, Argentina	5	0
N. plumbaginifolia Viv.	USDA accession TW106, origin unknown	0	6
N. bonariensis Lehm.	Bom Jardim da Serra, SC, Brazil	7	6
N. bonariensis Lehm.	Santa Tereza (East), RS, Brazil	4	0
N. bonariensis Lehm.	Santa Tereza (Road), RS, Brazil	8	0

three different pollinator groups for these *Nicotiana* species (Ippolito et al., 2004; T. Holtsford, R. Kaczorowski, and A. Ippolito, unpublished data). Hawkmoths are the predominant (most common) pollinators of species with long-tubed, white flowers (*N. alata* and *N. longiflora*). *Nicotiana plumbaginifolia* has a similar floral morphology, suggesting hawkmoth visitation (Cocucci, 1988), but is smaller in size and autogamous. Hummingbirds are the predominant pollinators of species with short-tubed flowers of various other colors, ranging from red to pink to greenish-yellow (Rastroensis, *N. forgetiana*, *N. mutabilis*, and *N. langsdorffii*), but Halictid bees have also been seen visiting, and apparently collecting pollen from, all four species (R. Kaczorowski, unpublished data). Bumblebees have also been seen visiting, and apparently collecting nectar from, *N. langsdorffii* in one of five populations observed (T. Holtsford, unpublished data) and *N. mutabilis* in one population observed (R. Kaczorowski, unpublished data). Small perching moths, which land on the long lower limbs of *N. bonariensis*, are thought to be the predominant pollinators of this species. These moths have probosci that match the short corolla tube length very well, but few such moths (nor any other pollinator) have been observed in the field in over 30 h of observation in four populations. Florivorous beetle larvae can be common inside *N. bonariensis* flowers in some populations, but their role in pollination was not apparent (R. Kaczorowski, personal observation).

Experimental design—The 137 *Nicotiana* plants used for the nectar volume and concentration experiments were grown beginning in late August 2000 from seed collected from various populations in Brazil and Argentina. Beginning in May 2002, a new set of plants was grown for the sugar and amino acid determinations, of which 118 plants survived. Multiple populations within each species were sampled, when available (Table 1). The plants were raised in 3.8-L pots in a common greenhouse environment (14-h days at c. 24°C and 10-h nights at c. 13°C at the University of Missouri–Columbia) as part of a randomized complete block design. The design included three blocks (different benches within the same greenhouse bay), each of which contained one progeny from three different maternal plants from up to three populations within each species. The plants were watered and fertilized with Peters Pro 20-10-20 (Scotts Co., Marysville, Ohio, USA) as necessary and regularly pruned to keep plant size manageable. Data for this study were collected between February 2001 and February 2003.

Daily phenology—Daily phenology observations were made in February 2001 to determine the schedule of corolla opening for each species. On 4 consecutive days, three flowers per plant on all plants from one block were marked and checked about every 3 h (from 0800–2300 hours CST). The flower was recorded as being closed, opening, fully open, or flaccid. Because corolla opening allows the nectar to become available to pollinators, the time of day associated with corolla opening for each species was used to determine when to sample nectar.

Nectar volume and concentration—Plants from one of three blocks were sampled on any given day for one of the three relative flower ages (approximately the time of anthesis [0 h], 12, and 24 h after opening). Day-to-day variation was therefore included in the block term. For the 0 and 24-h measurements, nectar collection started in the early afternoon (about 1400 CST) with random sampling of all *N. langsdorffii* plants on that block, then *N. mutabilis*, and then *N. forgetiana*. Rastroensis was considered a variant of *N. forgetiana* at the time of this experiment and therefore randomly sampled at the same time as *N. forgetiana*. Random sampling of all nocturnal species began around dusk (approximately 1730 hours). For the 12-h measurement, sampling started in the early morning (about 0200 hours) and followed the same progression.

At least three flowers from each plant were destructively sampled for nectar volume and concentration measurements. For most species, the calyx and corolla were separated and the corolla tube gently squeezed to bring the nectar to the base of the tube, where it was collected. *Nicotiana bonariensis* gave almost no nectar when sampled this way; dissection of flowers was necessary to collect the trace amounts of nectar along the corolla tube. Therefore, nectar was resampled from all *N. bonariensis* plants on different days than the other species (a day each for 0 and 24-h measurements, 12-h measurements were not possible because reduced turgor during daylight hours complicated dissection and collection). Other species' nectar sampled with *N. bonariensis* did not differ from the results (presented later), so we presume that the *N. bonariensis* measurements are comparable. Nectar was collected with glass micropipette tubes, and the volume was recorded. Nectar samples from individual flowers on the same plant were pooled to obtain a single concentration measurement from a temperature-compensated refractometer (Leica Microsystems, Buffalo, New York, USA, but Bellingham and Stanley, Lawrenceville, Georgia, USA, with a low volume sample window, used for *N. bonariensis*). Dilutions were performed as necessary to keep the concentration readings within the range of the refractometer. The refractometers measure concentration as the percentage solids in solution (sucrose equivalents, wt/wt, keeping manufacturer's usage).

Nectar volume and concentration analysis—We used population, block, and time in a randomized complete block (RCB) split-plot design (PROC MIXED in SAS 6.12 [SAS Institute Inc., Cary, North Carolina, USA]) to analyze average volume, nectar concentration, and total energy (average nectar volume multiplied by nectar concentration, which was first converted from wt/wt to wt/vol, as suggested by Bolton et al., 1979). A second analysis, without the 12-h measurements, was also run to estimate the relationship of *N. bonariensis* to the other species. Two species were represented by only one population each, so a population nested within species design could not be used. We formulated contrasts that grouped populations within species to test for among species differences, as well as grouping species within pollinator groups to test for among pollinator group differences. Because the variances were unequal among populations and did not equalize with various transformations, the data were ranked. Because multiple analyses were being performed, a more rigorous alpha of 0.01 was chosen to reduce type II errors. To make pairwise comparisons of slopes and quadratic terms of nectar accumulation and concentration changes, we fit least square models and tested the differences of first and second-order terms using software from UMC Statistics Department (Critical SS for each contrast = Error MS $\times$ F from the RCB split-plot analysis described). *Nicotiana bonariensis* could not be included in this testing because only two time measurements were taken for this species.

Sugar and amino acid composition—Four to six plants per species, from a different set of plants, were sampled for the sugar and amino acid analysis (Table 1). All flowers were sampled during the day of anthesis to minimize effects of flower aging that have been shown to affect amino acid concentrations in nectar samples (Gottsberger et al., 1990; Petanidou et al., 1996). All species were destructively sampled because it would otherwise be impossible to collect nectar from some species without contaminating with pollen or floral tissue. The flower to be sampled was taken off the plant, held upside-down, and cut below the nectar pool (the nectar pool can be seen through the corolla). This allowed the stamens to be removed, leaving full access to the nectar pool and minimizing possible contamination with pollen, which can release free amino acids in solution (Linskens and Schrauwen, 1969). The nectar was withdrawn using 10-μL glass micropipette tubes (Drummond ''Microcaps'' [Drummond Scientific Co., Broomall, Pennsylvania, USA]). Care was taken to avoid touching the cut edges of the corolla. Each sample was aspirated into a glass chromatography vial using a microcap bulb. The samples were preserved by addition of 50 μL of 80% ethanol shortly afterwards and kept sealed with polytetrafluoroethylene push-fit tops. This formed a tight seal and prevented any loss of sample due to evaporation. On return to the laboratory, the sample vials were cooled in the refrigerator (at 4°C) to condense any vapor before opening the vial. Ten microliters of sample were removed and used for sugar analysis. The remainder was analyzed for amino acids.

The samples were prepared and the amino acids derivatized using the AccQtag protocol (Waters Corp., Milford, Massachusetts, USA; Cohen and Micheaud, 1993) in a 0.02 M borate buffer (pH 8.59). High-performance liquid chromatography (HPLC) was performed, with standards every four samples, using the following equipment: Waters 712 WISP autosampler, Waters 600 pump controller, Waters 600 HPLC pump with 510 pump-heads (Waters Corp.). Separation was achieved using a Waters Novapak C18 (15 $\times$ 0.46 cm) cartridge with guard column. The binary solvent system was a 6 : 4 acetonitrile : water mix and a (triethylamine)-phosphate (pH 5.04) buffer. Detection was via a Waters 474 scanning fluorescent detector (excitation at 295 nm and detection at 350 nm). The system was monitored and data collected using the Waters Millenium^{32} software. Chromatograms were analyzed and compared to standards for identification of individual amino acids. Standard amino acids were made up to a concentration of 100 pmol/μL. In addition to all the protein-building amino acids, standards of hydroxyproline (hyp), ornithine (orn), taurine (tau), α-aminobutyric acid (AABA) and γ-aminobutyric acid (GABA) were used. Peak areas were compared to standards to determine the concentration of individual amino acids. From these data, the total concentration of all amino acids was determined, and the proportion that each made to the total was also calculated as a percentage.

Sugars were analyzed using an Alltech Varex Mk III evaporative light scattering laser (ELSD) system (Alltech Associates, Deerfield, Illinois, USA) and HPLC. Each sample was diluted with 30 μL of eluant. Separation was achieved using an Alltech 525 pump and pulse dampener fitted with a 5-μL injection loop. The eluant system was a 3 : 1 acetonitrile/water mix with an isocratic flow of 0.5 mL/min through a Capital NH_2 Optimal narrow-bore column (5-μm particle size, 250 $\times$ 3.2 mm; Capital HPLC Ltd., West Lothian, Scotland, UK) and C18 guard cartridge (4 $\times$ 3 mm, Phenomenex, Torrence, California, USA) . Detection was via an ELSD with a gas-flow rate of 3 L/min and drift tube temperature of 105°C. Output was monitored on a Shimadzu C-RIB integrator (Shimadzu Corp., Columbia, Maryland, USA). Chromatograms were analyzed and compared to standards for identification. Peak areas were compared to standards to determine the concentration of individual sugars (sucrose, fructose, and glucose).

Sugar and amino acid analysis—Statistical analyses of nectar chemistry were carried out using Statistica (StatSoft, Tulsa, Oklahoma, USA). All concentration data were log transformed (natural logarithm), and proportion data were angular transformed (arcsine) to improve the distribution and homoscedasticity of the residuals. We used species as an independent variable in two separate multiple analysis of variance tests (MANOVA), one to analyze amino acid and another to analyze sugar data, although there could be a lack of independence. Among species differences in individual model components were analyzed subsequently using post-hoc tests (Tukey's ''honestly significantly different'' [HSD] test). Differences among pollinator groups were determined from post-hoc tests (Tukey HSD) in nested ANOVAs (separate for sugars and amino acids) in which species was nested within pollinator groups. Because multiple analyses were being performed a more rigorous alpha of 0.01 was chosen to reduce type II errors.

RESULTS

Daily phenology—*Nicotiana langsdorffii* generally opened in the early afternoon, and *N. mutabilis* opened between early and late afternoon, exhibiting diurnal anthesis (Fig. 2). *Nicotiana forgetiana* and Rastroensis generally opened in the late afternoon, exhibiting a crepuscular anthesis. The four white-flowered species (*N. alata*, *N. longiflora*, *N. plumbaginifolia*, and *N. bonariensis*) opened around dusk, exhibiting a nocturnal anthesis. *Nicotiana langsdorffii*, the species with the smallest flowers, was the earliest to begin opening and have all of its flowers fully open. It also had the greatest ability to avoid turgor loss, and thus all flowers remained open until senescence. Most flowers of *Nicotiana mutabilis* and Rastroensis also remained open after anthesis, but some lost turgor during the heat of the day. *Nicotiana forgetiana* flowers were quite susceptible to turgor loss during the day, although some usually remained open. The flowers of the nocturnal species (*N. alata*, *N. longiflora*, *N. plumbaginifolia*, and *N. bonariensis*) remained open throughout the night and lost turgor during the day. In *N. forgetiana* and the nocturnal species *N. alata*, *N. longiflora*, and *N. bonariensis*, the third- and fourth-day flowers opened earlier and stayed open longer. The flowers of *N. plumbaginifolia* remained open only two nights, while the flowers of other nocturnal species continued to open night after night, until the onset of senescence (about 4–7 days after anthesis). The brief flower life in *N. plumbaginifolia* is likely due to it being autogamous, because we observed that pollination shortened the flowering span of the other species (data not shown).

Nectar volume and concentration—The mixed model ANOVA found a significant time effect for both volume ($F_{2,64}$ = 72.68, $P < 0.0001$) and concentration ($F_{2,61}$ = 28.36, $P < 0.0001$). All of the *Nicotiana* species tested, except Rastroensis, exhibited a significant ($P < 0.01$) linear increase in nectar

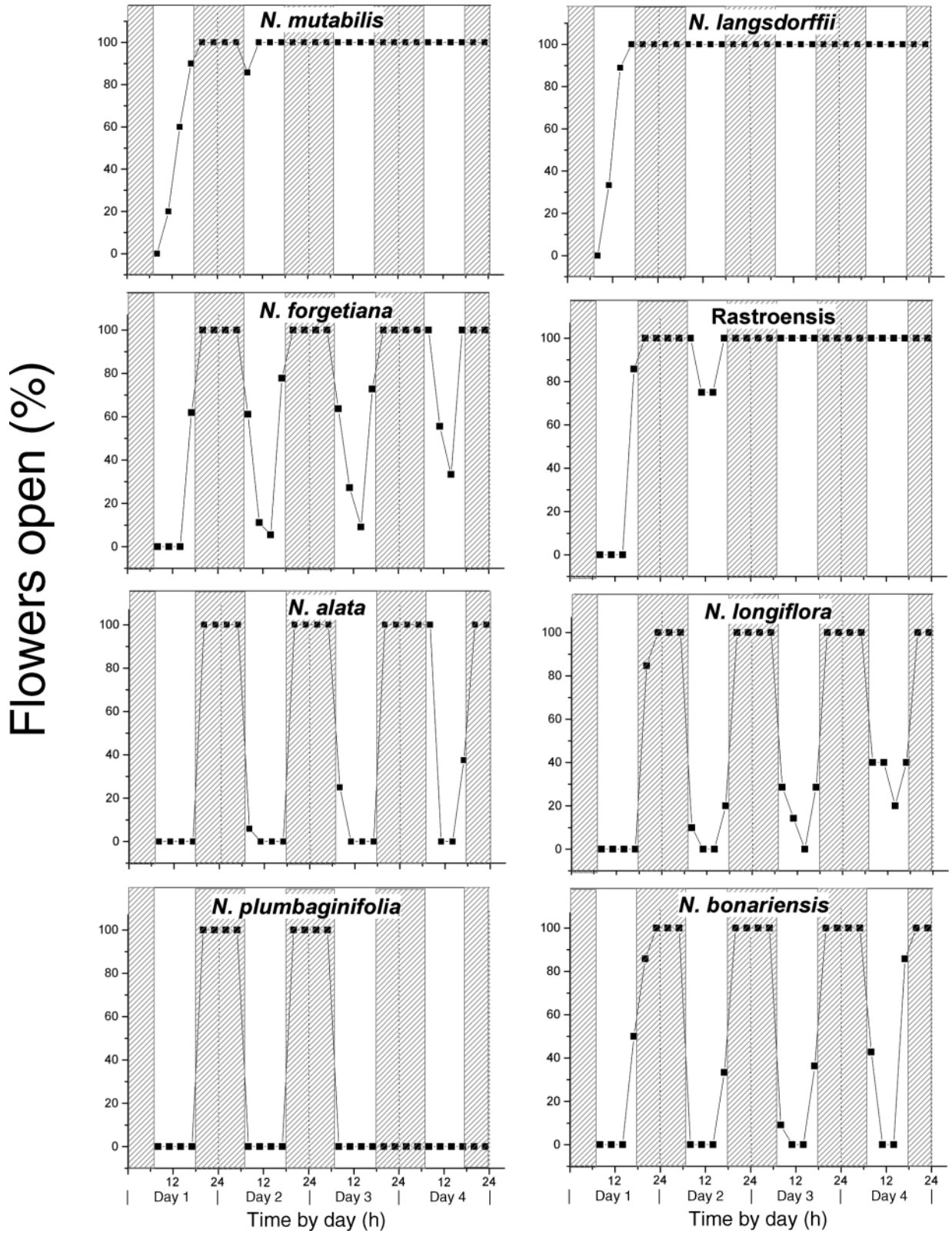

Fig. 2. Daily phenology pattern for species of *Nicotiana* sect. *Alatae*. Day 1 represents the first day the flower opens (anthesis); days 2–4 are consecutive days after anthesis. The flowers of many species close during the day. Observations were recorded for three flowers from each plant. Data points represent the percentage of flowers open at each day/time interval (only flowers that would open sometime that day were included). Shading represents the time of day that the sun was down.

TABLE 2. Changes in nectar volume and concentration after anthesis (0 h) over time for species of *Nicotiana*. The total increase was determined by subtracting the 0-h mean from the 24-h mean.

Species	N_0, N_{12}, N_{24}[a]	Nectar volume (mean ± SE, µl)				N_0, N_{12}, N_{24}[a]	Nectar concentration (mean ± SE, % solids)			
		0 h	12 h	24 h	Total increase[b]		0 h	12 h	24 h	Total increase[b]
Hummingbird-pollinated										
N. mutabilis	19, 19, 17	3.54 ± 0.66	6.63 ± 0.74	7.62 ± 0.44	4.08**L	16, 19, 17	46.47 ± 2.19	48.21 ± 1.70	58.68 ± 1.73	12.21**L/*Q
N. langsdorffii	16, 16, 15	2.87 ± 0.56	4.08 ± 0.54	6.09 ± 0.65	3.21**L	12, 15, 15	43.08 ± 1.76	50.17 ± 1.46	55.57 ± 2.36	12.48**L
N. forgetiana	26, 26, 26	4.58 ± 0.62	5.25 ± 0.55	6.68 ± 0.57	2.10**L	22, 24, 26	32.30 ± 2.72	46.00 ± 1.74	47.56 ± 2.07	15.26**L
"Rastroensis"	9, 9, 9	1.83 ± 0.78	2.07 ± 0.34	3.66 ± 0.48	1.83 ns	7, 8, 9	48.79 ± 2.73	54.67 ± 2.02	59.50 ± 2.60	10.71**L
Hawkmoth-visited										
N. alata	27, 27, 27	6.27 ± 0.45	10.27 ± 0.73	15.61 ± 1.00	9.34**L	27, 26, 27	22.40 ± 0.40	23.19 ± 0.34	23.57 ± 0.37	1.18 ns
N. longiflora	18, 18, 18	8.58 ± 0.85	13.35 ± 1.34	17.38 ± 1.51	8.80**L	17, 18, 18	21.41 ± 0.36	21.25 ± 0.21	21.42 ± 0.24	0.00 ns
N. plumbaginifolia	5, 5, 5	1.62 ± 0.85	3.13 ± 1.15	6.27 ± 0.69	4.65**L	2, 3, 5	16.38 ± 0.13	17.92 ± 0.08	19.30 ± 1.10	2.93 ns
Small-moth-pollinated										
N. bonariensis	19, 0, 19	0.27 ± 0.04		0.64 ± 0.06	0.36 n/a	18, 0, 19	41.18 ± 1.79		49.99 ± 1.79	8.81 n/a

[a] N_0 = sample size at 0 h, N_{12} = sample size at 12 h, N_{24} = sample size at 24 h.
[b] L = linearly significant, Q: quadratically significant, *: $P < 0.05$; **: $P < 0.01$, ns: not significant, n/a: not applicable (*N. bonariensis* not tested).

Fig. 3. Nectar volume and concentration as a function of flower age in species of *Nicotiana* sect. *Alatae*. Each point represents the mean for the species at each of three relative flower ages. Error bars represent one standard error. Open symbols represent hummingbird-pollinated species. Closed symbols represent hawkmoth-visited species. Crosses represent the small moth-pollinated species. The 12-h measurements for *N. bonariensis* contained substantial error; therefore, the data points were estimated and significance could not be tested. There was a significant linear increase in all slopes, except those noted as not significant (NS). Letters following species abbreviations denote species groups (a and b), where species with the same letter are not significantly different in slope (at $\alpha = 0.01$). A) Nectar volume vs. flower age for all species. The slopes of *N. plumbaginifolia* and *N. longiflora* were significantly different at $\alpha = 0.05$. The quadratic components for volume changes in *N. mutabilis* and *N. alata* were significantly different at $\alpha = 0.05$. B) Nectar concentration vs. flower age for all species. The quadratic components for concentration changes in *N. mutabilis* and *N. forgetiana* were significantly different at $\alpha = 0.01$. *Figure abbreviations*: ala = *Nicotiana alata*, bon = *N. bonariensis*, forg = *N. forgetiana*, lang = *N. langsdorffii*, long = *N. longiflora*, mut = *N. mutabilis*, plum = *N. plumbaginifolia*, Rast = putative species Rastroensis.

volume over the first 24 h after opening (Table 2, Fig. 3A; *N. bonariensis* excluded in this analysis). There was a significant linear increase in nectar concentration ($P < 0.01$) for the hummingbird-pollinated species (*N. mutabilis*, *N. langsdorffii*, *N. forgetiana*, and Rastroensis), while the hawkmoth-visited species (*N. alata*, *N. longiflora*, and *N. plumbaginifolia*) showed no significant increase in concentration over the first 24 h after opening (Table 2, Fig. 3B). *Nicotiana mutabilis* exhibited a significant quadratic component to its increase in nectar concentration ($P < 0.05$) because there was an accelerated increase 12 h after anthesis (Table 2, Fig. 3B).

The mixed model ANOVA found a significant population effect across all species for both average nectar volume ($F_{15,30}$

= 22.33, $P < 0.0001$) and nectar concentration ($F_{15,30}$ = 71.90, $P < 0.0001$). Within species, significant population effects were found in *N. alata* for nectar concentration, and in *N. longiflora* for both average volume and nectar concentration. The Rio das Antas North population of *N. alata* had a significantly larger mean nectar concentration than the Rio Pelotas population (data not shown, df = 30, t = 2.98, P = 0.0056). The Calilegua population of *N. longiflora* was significantly lower in mean nectar concentration than the Universidad Nacional de Nordeste population (df = 30, t = 2.81, P = 0.0086). The Jujuy population of *N. longiflora*, which included several plants with smaller flowers, approximately mid-size between common *N. longiflora* and *N. plumbaginifolia*, which consistently set selfed fruit in the glasshouse, had a significantly lower mean nectar volume than the other two *N. longiflora* populations, Calilegua and Universidad Nacional de Nordeste (t = 6.60 and t = 6.47, respectively; df = 30, $P < 0.0001$).

Many among-species contrasts for nectar volume and concentration were significantly different (at $\alpha = 0.01$), although some species grouped together with similar nectar volumes or concentrations (Fig. 4; only 24-h data shown). Hawkmoth-pollinated species (*N. alata* and *N. longiflora*) tended to have more nectar at a lower concentration than the hummingbird- and small moth-pollinated species. Pollinator group contrasts found that hawkmoth-visited species were significantly different from hummingbird-pollinated species in both average nectar volume and nectar concentration based on all time measurements ($F_{1,30}$ = 118.67, $P < 0.0001$ and $F_{1,30}$ = 983.24, $P < 0.0001$, respectively). The second analysis (0 and 24 h only) found that the small moth-pollinated *N. bonariensis* had significantly lower nectar volume than the hawkmoth-visited and hummingbird-pollinated species ($F_{1,36}$ = 420.38, $P < 0.0001$ and $F_{1,36}$ = 190.58, $P < 0.0001$, respectively) and significantly greater nectar concentration than the hawkmoth-visited species ($F_{1,35}$ = 190.94, $P < 0.0001$), but not from the hummingbird-pollinated species ($F_{1,35}$ = 4.41, P = 0.0429, where $\alpha = 0.01$). Nectar volume and concentration tend to be more similar among species with the same predominant pollinator than between species with different predominant pollinators.

Temporal changes in volume and concentration—The mixed model ANOVA found no significant population by time interaction for both volume ($F_{30,64}$ = 1.19, P = 0.2746) and concentration ($F_{30,61}$ = 1.47, P = 0.1032). The temporal changes in volume and concentration were examined separately by determining the linear and quadratic differences of the functions representing the rate changes among species. Hawkmoth-pollinated *N. alata* and *N. longiflora* had significantly lower ($P < 0.01$) linear slopes for concentration change when compared to the hummingbird-pollinated species (Fig. 3B). *Nicotiana alata* and *N. longiflora* also had significantly greater linear slopes for volume change when compared to all other species tested (Fig. 3A; $P < 0.01$, except *N. longiflora* vs. *N. plumbaginifolia*: $P < 0.05$). The small moth-pollinated *N. bonariensis* was not included in this testing.

Total energy—Total energy comparisons among pollination systems showed that hummingbird-pollinated species had significantly more total energy than hawkmoth-visited species (Fig. 5; $F_{1,30}$ = 16.02, P = 0.0004), and the small moth-pollinated species had significantly less total energy than hawkmoth-visited and hummingbird-pollinated species based on all

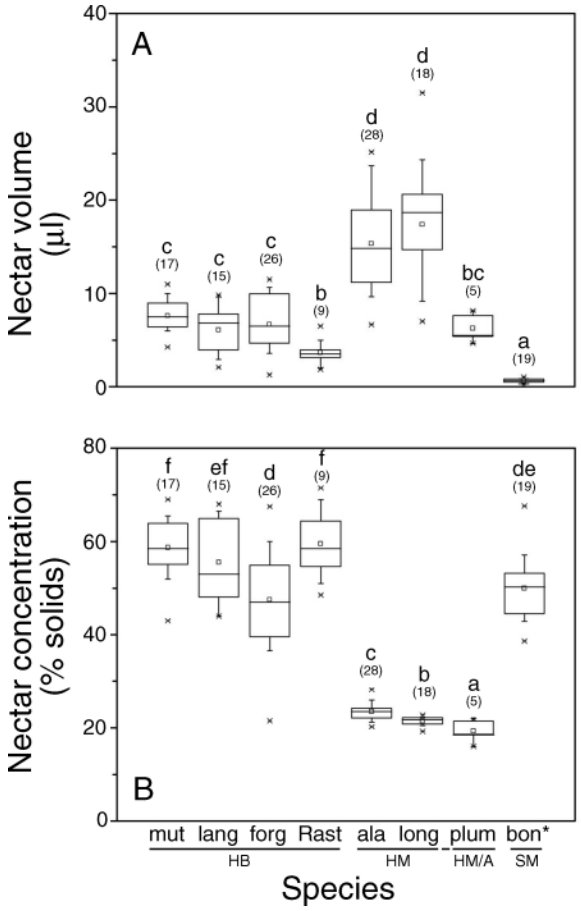

Fig. 4. Nectar volume and concentration at approximately 24 h after corolla opening. The horizontal lines of the box plot denote the 25th, 50th, and 75th percentile values. The error bars represent the 5th and 95th percentile values. The asterisks above and below the error bars denote the maximum and minimum values, respectively. The square symbol in the box represents the mean of the values. Sample sizes are given in the parentheses above each box plot. Letters above box plots denote species groups (a–d) for all time measurements, where species with the same letter are not significantly different (at $\alpha = 0.01$; significance for *N. bonariensis* determined through a separate analysis [0 and 24 h only]). A) Nectar volume values for all species. B) Nectar concentration values for all species. *Abbreviations:* A = autogamous, HB = hummingbird-pollinated, HM = hawkmoth-visited, SM = small-moth-pollinated.

time measurements ($F_{1,35}$ = 166.09, $P < 0.0001$ and $F_{1,35}$ = 278.56, $P < 0.0001$, respectively). However, there was no difference in total energy between hawkmoth and hummingbird-pollinated species when the autogamous *N. plumbaginifolia* was excluded from the hawkmoth group (df = 105, t = −0.475, P = 0.318).

Some among species contrasts for total energy were significantly different (at $\alpha = 0.01$), although many species grouped together with similar total energy (Fig. 5; only 24-h data shown). *Nicotiana mutabilis* had significantly more total energy than all other species, except *N. forgetiana*; it tends to produce more nectar than the other hummingbird-pollinated species, and the nectar is often more concentrated (Table 2). Rastroensis had significantly less total energy than the other hummingbird-pollinated species, mainly due to its lower nectar volumes (Table 2). Rastroensis had significantly lower total energy than *N. forgetiana* and significantly higher total energy

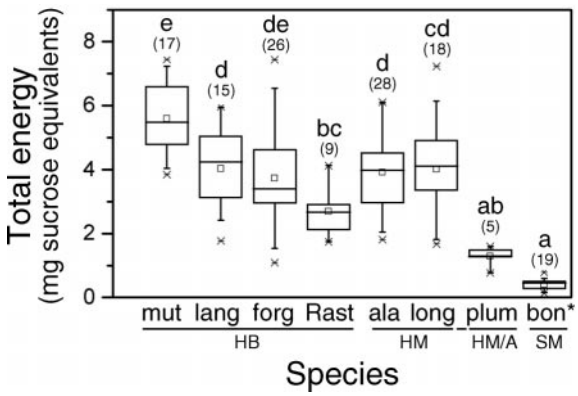

Fig. 5. Total energy at approximately 24 h after corolla opening. Total energy was calculated by multiplying the average volume for each plant by its concentration (converted to mass/volume). Sample sizes are given in the parentheses above each box plot. Letters above box plots denote species groups (a–e) for all time measurements, where species with the same letter are not significantly different (at $\alpha = 0.01$; significance for *N. bonariensis* determined through a separate analysis [0 and 24 h only]).

than *N. bonariensis*, the two species most morphologically similar, and possibly most closely related, to this putative species. The second analysis (0 and 24 h only) found *N. bonariensis* to have significantly less total energy than all species, except *N. plumbaginifolia* (Fig. 5), because it produced only trace amounts of nectar (Table 2).

There was a significant population effect ($F_{15,30} = 7.23$, $P < 0.0001$) and time effect ($F_{2,61} = 103.68$, $P < 0.0001$; data not shown) for total energy across all species, although the population by time interaction was not significant ($F_{30,61} = 1.13$, $P = 0.3383$). The only population effect within a species was found within *N. longiflora*, where the selfing Jujuy population had significantly less total energy in its nectar than both the Calilegua and Universidad Nacional de Nordeste populations (df = 30; $t = 5.09$ and $t = 5.70$, respectively; $P < 0.0001$; data not shown).

Reward scaled by flower size—To determine how total energy and its components (nectar volume and concentration) related to floral size, we examined the functional relationship between these three nectar traits and corolla length (Fig. 6). Nectar volume was positively correlated to corolla length ($r^2 = 0.8202$, $P < 0.0001$; Fig. 6A), while nectar concentration was negatively correlated to corolla length ($r^2 = 0.6834$, $P < 0.0001$; Fig. 6B). As a result, total energy was not significantly correlated with corolla length ($r^2 = 0.0238$, $P = 0.5544$; Fig. 6C). Total energy in autogamous groups was lower than in other species, except the small moth-pollinated species, which produced only trace quantities of nectar. Bud-selfing *N. plumbaginifolia* has a low concentration for its size, while the autogamous Jujuy population of *N. longiflora* has a low nectar volume for its size (Fig. 6).

Sugar and amino acid composition—Pollinator groups were significantly different in total sugars; the small moth-pollinated species had significantly more sugar than the hummingbird-pollinated species, which had significantly more sugar than the hawkmoth-visited species (post-hoc Tukey HSD). There was a significant species effect found for total sugar concentration (Fig. 7; $F_{7,40} = 23.78$, $P < 0.0001$). Individual species comparisons showed few significantly different total

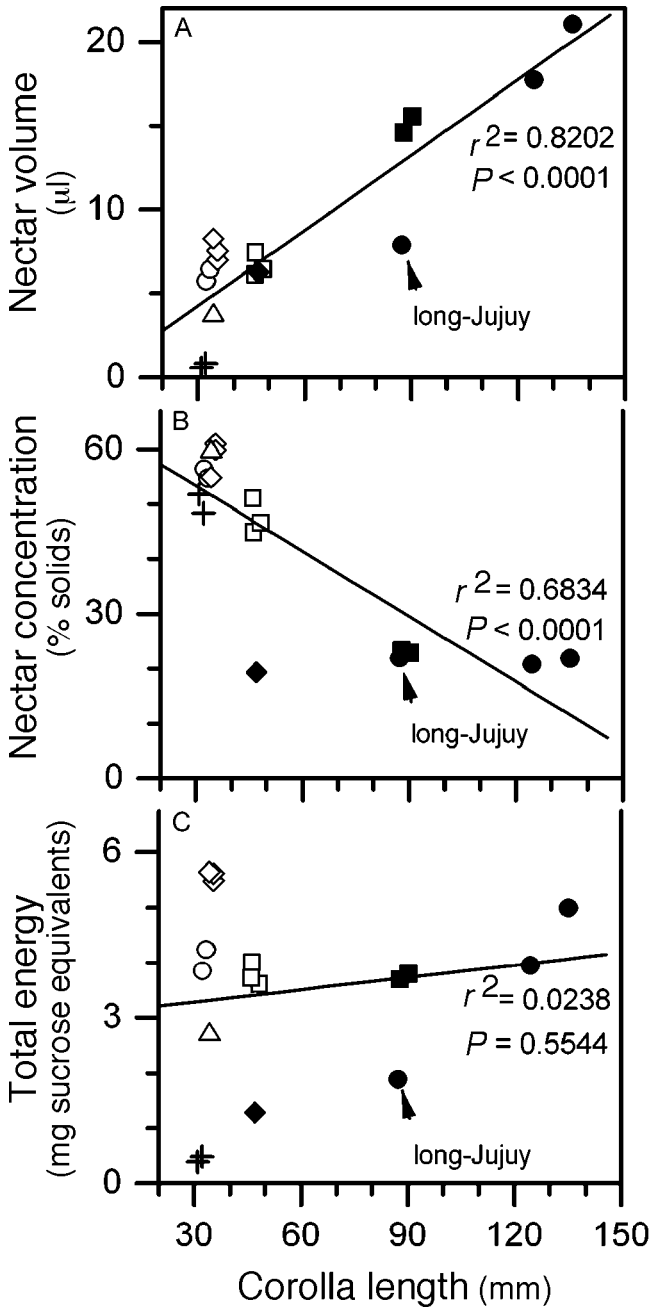

Fig. 6. Nectar volume, concentration, and energy as a function of corolla length. Species symbols correspond to those in Figs. 3 and 4, with multiple populations shown for most species. The Jujuy population of *N. longiflora*, which exhibits autogamy, is labeled. The line represents the best linear fit for each graph, with the r^2 and P values included.

sugar concentrations (at $\alpha = 0.01$), but some significant differences do exist (Fig. 7).

There was a significant species effect for the concentration of each sugar type (df = 7, 40; $F_{FRU} = 21.82$, $F_{GLU} = 8.37$, $F_{SUC} = 23.23$; $P < 0.0001$) and the proportion of each sugar type. However, Tukey HSD tests found most species comparisons were not significantly different from each other in the concentrations and proportions of each sugar type (117 of 168 overall comparisons NS at $\alpha = 0.01$). Sugar ratios (sucrose to [glucose + fructose]) ranged from 0.6 in *N. plumbaginifolia*

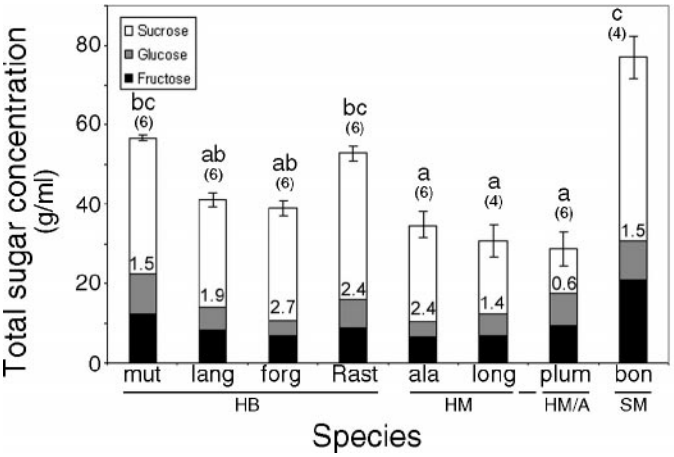

Fig. 7. Total sugar concentrations and individual sugar proportions within that total concentration. The apex of each bar represents the mean total sugar concentration. The error bars represent one standard error of the total sugar concentration. The proportion of each sugar type, relative to the total concentration, is represented within the total sugar concentration bar (sugar ratios are also included within the bar). Letters above box plots denote species groups (a–c), where species with the same letter are not significantly different (at $\alpha = 0.01$). Sample sizes are given in the parentheses.

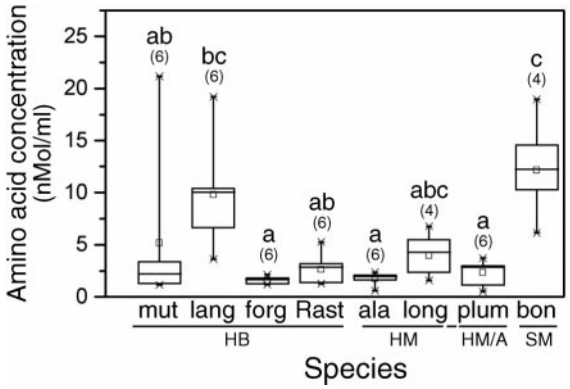

Fig. 8. Total amino acids within each species. Letters above box plots denote species groups (a–c), where species with the same letter are not significantly different (at $\alpha = 0.01$) among species. Sample sizes are given in the parentheses.

to 2.7 in *N. forgetiana* (Fig. 7). Some pollinator groups were found to be significantly different from each other in their proportions of each sugar type. Sucrose makes up the greatest proportion of the sugar concentration in all pollinator groups, but hummingbird-pollinated species had significantly greater proportions of sucrose (at $\alpha = 0.01$) than the hawkmoth-visited species, which had significantly greater proportions of glucose than the hummingbird-pollinated species, while the small moth-pollinated and hawkmoth-visited species had significantly greater proportions of fructose than the hummingbird-pollinated species.

There was a significant species effect for total amino acid concentration ($F_{7,40} = 8.806$, $P < 0.0001$), which was not significantly different among most species (by Tukey HSD at $\alpha = 0.01$), though a few significant differences were found (Fig. 8). The only significantly different pollinator group in amino acid concentration was the small moth species, *N. bonariensis*, which had significantly more amino acids than the other pollinator groups.

Some individual amino acids had a significant species effect (at $\alpha = 0.01$), while others did not (Table 3). The most common amino acid found in the species with higher nectar concentrations (*N. mutabilis*, *N. langsdorffii*, *N. forgetiana*, Rastroensis, and *N. bonariensis*) was proline (concentration means

TABLE 3. Relative proportions (% ± SE) of amino acids in nectar of each species of *Nicotiana*. Proportions above 10% are italicized, and the most predominant amino acid for each species is in bold. Heterogeneity for each amino acid among species was tested for significance by MANOVA. AABA, Hyp, Orn, and Tau contributed 0% to all species and are not included in this table. Trp also contributed 0% to all species, but is considered an essential amino acid.

Amino Acid	*mutabilis*	*langsdorffii*	*forgetiana*	"Rastroensis"	*alata*	*longiflora*	*plumbaginifolia*	*bonariensis*	*F* (df = 7, 40)	*P*
"Essentials"										
Arg	6 ± 0.9	4 ± 0.4	*13 ± 1.0*	8 ± 1.2	8 ± 0.5	4 ± 0.3	6 ± 0.8	5 ± 0.6	14.45	***
Thr	4 ± 1.0	2 ± 0.9	*15 ± 1.2*	8 ± 1.5	*10 ± 1.2*	3 ± 0.8	5 ± 1.1	5 ± 0.8	13.70	***
Phe	1 ± 0.3	2 ± 0.5	0 ± 0.0	0 ± 0.2	1 ± 0.6	7 ± 1.2	0 ± 0.1	5 ± 2.8	13.13	***
Met	0 ± 0.0	0 ± 0.0	1 ± 0.2	0 ± 0.0	0 ± 0.1	0 ± 0.1	2 ± 1.0	0 ± 0.0	9.73	***
Lys	0 ± 0.0	0 ± 0.0	0 ± 0.0	0 ± 0.1	0 ± 0.1	0 ± 0.0	0 ± 0.2	0 ± 0.0	7.74	***
His	0 ± 0.1	0 ± 0.1	0 ± 0.0	0 ± 0.0	0 ± 0.1	0 ± 0.1	0 ± 0.0	0 ± 0.1	7.23	***
Ile	1 ± 0.5	1 ± 0.5	0 ± 0.1	0 ± 0.2	1 ± 0.5	2 ± 0.4	1 ± 0.2	0 ± 0.0	5.19	**
Leu	1 ± 0.4	2 ± 0.4	0 ± 0.1	1 ± 0.5	2 ± 0.9	2 ± 0.2	1 ± 0.1	4 ± 1.8	3.53	**
Val	1 ± 0.6	2 ± 0.5	0 ± 0.1	1 ± 0.3	2 ± 0.4	1 ± 0.4	1 ± 0.2	2 ± 0.5	3.84	**
Other										
Pro	***46 ± 5.1***	***47 ± 3.2***	***45 ± 3.6***	***32 ± 2.5***	6 ± 0.8	*23 ± 4.7*	8 ± 1.3	***41 ± 6.8***	20.68	***
Ser	2 ± 0.8	3 ± 0.6	0 ± 0.2	1 ± 0.8	4 ± 1.0	1 ± 0.3	2 ± 1.0	4 ± 1.3	1.69	ns
Gly	1 ± 0.3	0 ± 0.1	1 ± 0.4	1 ± 0.6	2 ± 0.6	0 ± 0.2	1 ± 0.5	2 ± 0.7	1.38	ns
Cys	0 ± 0.0	0 ± 0.0	0 ± 0.0	0 ± 0.0	1 ± 0.4	0 ± 0.0	0 ± 0.0	0 ± 0.0	4.96	**
Tyr	0 ± 0.1	0 ± 0.0	0 ± 0.0	0 ± 0.1	0 ± 0.1	1 ± 0.1	0 ± 0.1	0 ± 0.1	3.00	ns
Ala	*16 ± 4.6*	4 ± 1.1	0 ± 0.0	8 ± 2.2	*14 ± 1.9*	3 ± 1.0	*17 ± 5.6*	*12 ± 2.0*	5.82	**
Asp	3 ± 3.1	1 ± 0.3	1 ± 0.4	7 ± 2.3	4 ± 0.9	1 ± 0.4	5 ± 2.0	1 ± 0.3	2.36	ns
Glu	2 ± 1.5	2 ± 1.4	2 ± 0.3	8 ± 2.1	5 ± 3.7	0 ± 0.3	3 ± 1.7	9 ± 0.7	4.08	**
Asn	0 ± 0.0	2 ± 1.3	0 ± 0.0	0 ± 0.0	2 ± 1.5	1 ± 0.5	0 ± 0.0	0 ± 0.0	2.90	ns
Gln	*13 ± 8.7*	*23 ± 4.1*	1 ± 0.7	6 ± 4.9	4 ± 2.2	***42 ± 4.0***	6 ± 3.2	6 ± 1.6	9.32	***
GABA	5 ± 2.0	5 ± 1.4	*20 ± 5.9*	*17 ± 3.7*	***33 ± 2.1***	7 ± 1.9	***39 ± 1.8***	5 ± 2.4	14.86	***

Note: ** = $P < 0.01$, *** = $P < 0.0001$, ns = not significant.

± SE, in nMol/mL: 1.66 ± 0.70, 4.49 0.98, 0.74 ± 0.13, 0.77 ± 0.12, and 4.53 ± 0.79, respectively). *Nicotiana longiflora* also had a substantial proportion of proline (0.99 ± 0.32 nMol/mL), but glutamine was its most abundant amino acid (1.54 ± 0.22 nMol/mL). Glutamine was also relatively abundant in *N. mutabilis* and *N. langsdorffii* (1.97 ± 1.81 nMol/mL and 2.22 ± 0.49 nMol/mL, respectively). The most predominant amino acid found in *N. alata* and *N. plumbaginifolia* was GABA (0.57 ± 0.09 nMol/mL and 0.89 ± 0.19 nMol/mL, respectively), which also contributed a substantial proportion of amino acids to *N. forgetiana* and Rastroensis (0.28 ± 0.07 nMol/ml and 0.38 ± 0.05 nMol/ml, respectively; see Table 3 for amino acid proportions).

DISCUSSION

The species of *Nicotiana* section *Alatae* showed variation in daily phenology and several nectar traits that corresponded to their presumed pollination syndromes. Species within pollinator groups are not necessarily independent of each other because they share phylogenetic history. The phylogeny for *Nicotiana* section *Alatae* has not yet been fully resolved. It is possible that differences are due to shared history, rather than the pollination syndrome. This should be kept in mind while interpreting the results of all post-hoc tests performed on presumed pollination system.

Daily phenology—Daily phenology dictates the availability of nectar to effective pollinators. The timing of anthesis usually coincides with the time of day that the pollinators are actively foraging (Percival, 1974). Thus, one can expect diurnal plant species to have diurnal pollinators (hummingbirds), while nocturnal plant species have nocturnal pollinators (moths). *Nicotiana forgetiana* had nectar traits similar to the other hummingbird-pollinated species, but its phenology differed only slightly from the moth-pollinated species. Hummingbird activity is at a peak in the evening when most *N. forgetiana* flowers are open, but another possible reason for *N. forgetiana*'s phenology might be linked to its phylogenetic history. Although the ancestral pollination syndrome is unclear in section *Alatae*, the next most closely related group is section *Suaveolens* (Ippolito, 2000; Chase et al., 2003), a clade whose white, scented flowers suggest moth pollination. That, coupled with biogeography (*N. forgetiana*'s range seems to be encompassed by that of *N. alata*), suggests that *N. forgetiana* has evolved from a hawkmoth-pollinated ancestor, which was likely *N. alata* itself or its immediate progenitor, and has retained a similar daily phenology. Although the daily phenology for each species was recorded for a relatively small number of flowers during one time of the year, personal observations suggest that the patterns hold throughout the year in the greenhouse and during the flowering season in natural populations (R. Kaczorowski, A. Ippolito, and T. Holtsford, unpublished data).

Nectar volume and concentration—Nectar volume, concentration, and the rates of their increase over 24 h varied according to the presumed pollination system. The hawkmoth-pollinated species (*N. alata* and *N. longiflora*) had a lower nectar concentration than the other species, and their nectar concentration did not increase significantly over the first 24 h after anthesis. Hummingbird-pollinated species exhibited a significant increase in nectar concentration over the first 24 h after anthesis (Table 2, Fig. 3B). Evaporation may play a small part in increasing nectar concentrations (Plowright, 1987), but the increases were more likely due to continued sugar production throughout the day because nectar volumes were also increasing. Larger flowers tend to have more nectar (Fig. 6A), perhaps because of pleiotropic size effects, e.g., larger nectar glands and more space to hold more nectar. The differences in nectar concentration between hawkmoth-, hummingbird-, and the small-moth-pollinated species could be associated with differences in corolla size, which may be an artifact of phylogenetic history and constraints, not necessarily pollinator preferences. However, the lower total energy in autogamous lineages, scaled by flower size, is very suggestive of evolution of nectar traits in association with the evolution of pollination system (Fig. 6, see below).

Much of the literature claims hummingbird-pollinated species have relatively dilute nectar (20–25% sucrose equivalents; Baker, 1975; Baker and Baker, 1982; Cruden et al., 1983). However, the hummingbird-pollinated species in this study had nectar concentration means ranging from 47–60% solids (individual plant concentrations ranged from 20–70% solids), approximately 24 h after anthesis. Even shortly after anthesis (0 h), means ranged from 32–49% solids. The high values may be a result of more accommodating greenhouse conditions, but preliminary field experiments found nectar concentrations in Rastroensis to be as high as 57% solids, which is still much greater than the nectar concentrations typically found in hummingbird-pollinated flowers. The higher nectar concentrations could be the result of pollinator selection, since hummingbirds have been shown to prefer more concentrated nectar (Hainsworth and Wolf, 1976; Stiles, 1976; Pyke and Waser, 1981), although they were found to discriminate against nectar over 55% solids (Tamm and Gass, 1986). There is also the possibility that bees are playing a larger role in pollination and selection than expected. Bees prefer very concentrated nectar and probably require it to ensure that foraging will be energetically profitable (Bolton and Feinsinger, 1978). Bees are not expected to have a large role in the pollination of these species due to the low frequency of observed visits. However, more observations and pollinator efficiency experiments are necessary to examine this possibility.

The hawkmoth-pollinated species had nectar concentration means ranging from 19 to 24% solids (individual plant concentrations ranged from 18 to 28% solids), approximately 24 h after anthesis. These estimates fit well within the range of nectar concentrations typical of hawkmoth-pollinated species (15–30%, Baker and Baker, 1982; Cruden et al., 1983; Haber and Frankie, 1989). However, this range is lower than the estimated concentrations (30–40%) that should maximize sucrose intake rates for hawkmoths (Josens and Farina, 2001). Lower nectar concentrations will be less viscous and therefore more easily extracted, which is assumed to be necessary for Lepidoptera feeding through probosci (Baker and Baker, 1982). Nevertheless, diurnal hawkmoths showed no difference in preference between solutions ranging from 20 to 50% sucrose (Josens and Farina, 1997), and changes in viscosity per se do not significantly alter hawkmoth behavior (Josens and Farina, 2001).

The relatively high nectar concentration for the small moth-pollinated *N. bonariensis* (Table 2) is surprising because these moths also feed with a proboscis and therefore should favor less concentrated, less viscous nectar. Bees are not likely to have a role in the pollination of *N. bonariensis* because its flowers are white and nocturnal, although residual nectar in

nocturnal flowers of *Silene alba* has been found to encourage bee visitation early the next day (Young, 2002). Beetle larvae can often be found inside *N. bonariensis* flowers, though it is not clear how they affect pollination. Field results suggest that *N. bonariensis* nectar concentrations may be lower in a natural habitat setting, but population differences are significant (mean at anthesis ± SE; Santa Tereza: 33.55% ± 1.77, $N = 53$; Morro da Igreja: 12.14% ± 1.14, $N = 18$).

Total energy—Total energy was equivalent between the hawkmoth- and hummingbird-pollinated species, but was much lower in the autogamous groups and the small moth-pollinated species (Fig. 5). The nectar of hawkmoth- and hummingbird-pollinated species was equivalent in total energy per flower; perhaps there is a trade-off constraining the amount of total energy per flower, but autogamy may release that constraint (Fig. 6).

Autogamy may facilitate the evolution of reduced nectar volumes, and thus total energy, because of the decreased need for pollinator attraction. The two autogamous groups in this analysis produce less total reward than expected based on floral size. The average nectar volume found in *N. plumbaginifolia* was close to the expected value for its corolla length (Fig. 6A), but its nectar concentration was more similar to the other hawkmoth-visited species, which was well below the expected value for its corolla length (Fig. 6B). However, the Jujuy population of *N. longiflora* (represented by three progeny from a single maternal plant, all exhibiting autogamy) produced less nectar than expected for its corolla length.

Energy values were estimated for individual flowers, rather than for the whole plant. It is possible that differences in energy per flower could be balanced across species through differences in the number of available flowers, though this was not measured. Total energy represents the total amount of sugar equivalents found in a flower (the product of nectar volume and concentration), while the total sugar concentration indicates the amount of sugar found per unit volume of nectar. Different plants (see Table 1) and different techniques were used to obtain the total sugar concentration (wt/vol) data (HPLC) than those used to obtain the average nectar volume and nectar concentration (wt/wt) results (refractometer). When the weight/weight values were converted to weight/volume, it was apparent that the two measurements differed significantly in each species (t tests at $\alpha = 0.05$). Refractometers are calibrated for percentage sucrose concentrations, but there are other dissolved solids in nectar (including fructose and glucose) that can alter the refractive index from that expected with a pure sucrose solution, producing a concentration value that often does not represent the most accurate sugar concentration (Inouye et al., 1980). The HPLC method was selective for individual sugars and therefore is more accurate in determining the total sugar concentration than the refractometer. A certain degree of error can be expected for the total energy measurements as well, because they were determined from refractometer readings, but using the refractometer values allowed us to determine total energy for individual flowers measured within each population of each species. Only six samples per species were analyzed with HPLC, restricting total energy to only one data point per species or population. Therefore our statistical power would be greatly compromised if we used the HPLC data to determine total energy.

Sugar and amino acid composition—*Sugar concentration*—The small moth-pollinated species, *N. bonariensis*, had a significantly higher sugar concentration than the hummingbird-pollinated species, which had a significantly higher sugar concentration than the hawkmoth-visited species (post-hoc Tukey HSD on HPLC data; see also Fig. 7). The small moth-pollinated *N. bonariensis* was not significantly different from the hummingbird-pollinated species in nectar concentration as determined by refractometer readings ($P = 0.0429$, $\alpha = 0.01$).

Sugar components—All of the *Nicotiana* species studied here had sucrose-dominant nectar [sucrose to (glucose + fructose) > 1.0; (Baker and Baker, 1982, 1983)] except *N. plumbaginifolia*, which had a sucrose-rich nectar [sucrose to (glucose + fructose) > 0.5], but was relatively close to having a more balanced nectar (Fig. 7). Nevertheless, there were significant differences among sugar components that varied according to presumed pollination syndrome. Hummingbird-pollinated species had significantly greater proportions of sucrose than the hawkmoth-visited species, which had significantly greater proportions of glucose than the hummingbird-pollinated species, while the small moth-pollinated and hawkmoth-visited species had significantly greater proportions of fructose than the hummingbird-pollinated species. Hummingbirds prefer sucrose to other sugar types (Hainsworth and Wolf, 1976; Stiles, 1976), and at least one hawkmoth species has been shown to prefer sucrose as well (Kelber, 2003). Sugar type may also be a highly conserved character, as signs of phylogenetic constraint can be found in some families (Baker and Baker, 1982, 1983). Therefore it is possible that the similarity in sugar types among these *Nicotiana* species is due to some degree of phylogenetic constraint. However, the abundance of sucrose in these species may also be due to their morphology, as concealed nectaries tend to be associated with sucrose-dominated nectar (Percival, 1961; Gottsberger et al., 1984).

Amino acid concentration—There was significant variation in total amino acid concentration among some species (Fig. 8). Pollinator groups did not have significantly different amino acid concentrations, with the exception that *N. bonariensis*, the only small-moth-pollinated species, had a significantly greater amino acid concentration than the other pollinator groups. This finding coincides with data compiled by Baker and Baker (1982), showing that perching moth-pollinated flowers have significantly more amino acids than hummingbird- or hawkmoth-pollinated flowers. The reason for this pattern is presumably due to the lack of alternative protein sources for moths. Because hummingbirds augment their nectar diet with insects (Wagner, 1946; Baker and Baker, 1982; Brice and Grau, 1991), a lower amino acid concentration might be expected in the species they typically pollinate. Hummingbirds have even been shown to prefer nectar with lower amino acid concentrations (Hainsworth and Wolf, 1976). Hawkmoths, on the other hand, also lack an alternative protein source, and therefore the lower amino acid concentration found in flowers they typically pollinate is somewhat surprising. It is likely, though, that they may accumulate sufficient amino acids because they collect relatively large quantities of nectar each night (Baker and Baker, 1982). Also, hawkmoths have a relatively short lifespan (up to 3 weeks in *Manduca sexta*) and use reserves stored during larval growth and may not need to build up protein as an adult (Ziegler, 1991). Amino acid concentration could also be affected by fertilizer treatments in the greenhouse (Gardener and Gillman, 2001b). Therefore, it is possible that the amino acid concentrations found in this study would be different from

those of plants in situ. However, barring strong species by fertilizer interaction, we expect the deviations would be consistent across species. It is also possible that the pollinators would dislodge pollen into the nectar while collecting it, which would significantly increase the amino acid concentration (Baker and Baker, 1982).

Amino acid components—Amino acid complements have been shown to vary little (on a presence or absence basis) within a species (Baker and Baker, 1977, 1982; Gardener and Gillman, 2001a). Nevertheless, Gottsberger et al. (1984) found that many species vary in amino acid complements across different samples of the same species. Lanza et al. (1995) found variation between different populations of *Impatiens campensis*, as well as within a single population. Gardener and Gillman (2001a) found that amino acid concentrations were much more variable than the relative proportions of amino acids within a species. In multiple cases, at least one sample per species differed in whether a particular amino acid was present or absent (data not shown). The possibility of contamination by pollen or damaged cell contents cannot be ruled out, although much care was taken to avoid any form of contamination.

Amino acid complements are thought to be important in determining the taste of the nectar to the pollinator (Baker and Baker, 1977, 1982; Gardener and Gillman, 2002), although sugars are much more abundant than amino acids and most likely dominate the taste of nectar. Shiraishi and Kuwabra (1970) found that amino acids could be categorized into four classes based on their effects on chemosensory cells of two fly species: no effect (asn, gln, ala, cys, gly, ser, thr, tyr), general inhibitory (arg, asp, glu, his, lys), salt cell stimulatory (pro and hyp), and sugar cell stimulatory (ile, leu, met, phe, trp, val). The nectar of each species contains at least some amino acid from each of these categories. The effects of amino acids have not been investigated in other insects or hummingbirds and may differ from the effects found on flies. Abundant amino acids were not restricted to any one biochemical category. There were no species that contained all 10 of the essential amino acids (see Table 3) for insects (Haydak, 1970; Dadd, 1973) or birds (Brue, 1994).

Baker and Baker (1982) found alanine and arginine to be the most common amino acid found in the nectar of species they examined. Arginine was found in all species in this study and contributed a substantial proportion of amino acids to *N. forgetiana* nectar. Although alanine was found in relatively large proportions in a few species, it was absent from *N. forgetiana* nectar. Glutamine was predominant in *N. longiflora* nectar, but was also abundant in *N. mutabilis* and *N. langsdorffii* nectar. GABA, a neurotransmitter derived from glutamate, was predominant in *N. alata* and *N. plumbaginifolia*, but was also abundant in the nectar of *N. forgetiana* and Rastroensis. Proline was the predominant amino acid found in the hummingbird- and small-moth-pollinated species, but also contributed a substantial proportion to *N. longiflora* nectar. Baker (1978) found proline to be a very common amino acid in nectar, present in 87% of the species in his study, but some authors argue that the abundance of proline is only an indication of pollen contamination (Linskens and Schrauwen, 1969; Gottsberger et al., 1984). It is interesting that the species in our study that have a predominance of proline are those with shorter and wider corolla tubes, offering a slightly better chance for pollen to fall into the corolla tube as the flowers move. However, contaminated samples would probably be detectable because specific profiles are produced by pollen, which were not detected in these samples (M. Gardener, unpublished data). It is also possible that the destructive sampling of flowers altered the amino acid content, even if contact with damaged cells was avoided (Gottsberger et al., 1984). Tryptophan was not present in any of the species in this study, which is surprising because it is considered an essential amino acid for both insects and birds (Haydak, 1970; Dadd, 1973; Brue, 1994). AABA was also not detected in any sample of any of the species, and there were several other amino acids that were found in very small amounts (see Table 3).

Evolution of nectar rewards—This study shows that there are significant differences in nectar traits between different species associated with different presumed pollination systems (see Table 4). Hummingbird-pollinated species had relatively low nectar volumes with high sugar and low amino acid concentrations. Hummingbirds prefer relatively high sugar (Hainsworth and Wolf, 1976; Stiles, 1976; Pyke and Waser, 1981) and low amino acid concentrations (Hainsworth and Wolf, 1976), which is supported by our results. Hawkmoth-pollinated species had relatively high nectar volumes with low sugar and amino acid concentrations. Although there is a paucity of hawkmoth preference tests, our results support previous findings that hawkmoth-pollinated species tend to have relatively low sugar and amino acid concentrations (Baker and Baker, 1982). The small-moth-pollinated species had an extremely low nectar volume, but very high sugar and amino acid concentrations. Although the high sugar concentration contradicts assumptions that Lepidoptera should prefer less-concentrated nectar for ease of extraction, the high amino acid concentration supports previous findings (Baker and Baker, 1982). The autogamous groups had lower total energy than expected, through lower concentration (*N. plumbaginifolia*) or less nectar (Jujuy population of *N. longiflora*), than their flower size would predict. Autogamous species often exhibit smaller flower sizes and lower nectar volumes (Spira, 1980), also suggested by our results.

Factors other than pollinator associations are also important to the observed values of these nectar traits. Environmental variation can alter nectar production and composition (Zimmerman, 1988; Pacini, 2003), but this was accounted for in the experimental design of this study. Floral size probably has a large role in determining nectar volume and concentration. Nectar volume tends to increase, while concentration decreases, with increasing floral size (Fig. 6). Total energy in hawkmoth vs. hummingbird species could be constrained, but if so, that constraint appears to have been released in the autogamous lineages. Nectar traits are sometimes associated with taxonomic families (Baker and Baker, 1982, 1983), suggesting phylogenetic constraints had a role in nectar evolution. A more-resolved phylogeny is needed to determine the likelihood that *Nicotiana* section *Alatae* exhibits phylogenetic constraints in nectar traits, although the group may be too small to provide enough independent comparisons among pollination systems.

Many of the nectar traits examined in this study vary in association with the presumed predominant pollinator. The variation in these traits makes it possible for pollinators to select for or discriminate against certain types of nectar. Some studies have demonstrated pollinator preference or discrimination for certain nectar (Hainsworth and Wolf, 1976; Stiles, 1976; Pyke and Waser, 1981; Zimmerman, 1983; Galen and

TABLE 4. Results of floral traits by species. Numerical data is presented as mean ± SE, except for sugar ratios. Results for the Jujuy population of *N. longiflora* are presented separately when possible.

	Nicotiana species							
Floral or nectar characteristics	*N. mutabilis*	*N. langsdorffii*	*N. forgetiana*	"Rastroensis"	*N. alata*	*N. longiflora*	*N. longiflora* (Jujuy)	*N. plumbaginifolia*	*N. bonariensis*
Pollination syndrome	hummingbird	hummingbird	hummingbird	hummingbird	hawkmoth	hawkmoth	hawkmoth	hawkmoth	small moth
Mating system	SI	SI	SI	SI	SI	SC	SC/auto-gamous	SC/auto-gamous	SI
Typical color	white into pink	greenish-yellow	red	magenta	white	white	white	white	white
Daily phenology	diurnal	diurnal	crepuscular	crepuscular	nocturnal	nocturnal	nocturnal	nocturnal	nocturnal
Corolla length (mm)	34.7 ± 0.4	32.9 ± 0.4	46.9 ± 0.6	34.3 ± 0.8	89.1 ± 1.8	129.2 ± 1.7	87.3 ± 0.5	47.1 ± 4.4	31.7 ± 0.7
Volume at 24 h (μl)	7.6 ± 0.4	6.1 ± 0.7	6.7 ± 0.6	3.7 ± 0.5	15.6 ± 1.0	19.3 ± 1.3	7.9 ± 0.7	6.3 ± 0.7	0.6 ± 0.1
Concentration at 24 h (% solids)	58.7 ± 1.7	55.6 ± 2.4	47.6 ± 2.1	59.5 ± 2.6	23.6 ± 0.4	21.3 ± 0.3	22 ± 0.1	19.3 ± 1.1	50.0 ± 1.8
Total energy at 24 h (mg sucrose equivalents)	5.6 ± 0.3	4.0 ± 0.3	3.7 ± 0.3	1.8 ± 0.3	4.0 ± 0.2	4.4 ± 0.3	1.9 ± 0.2	2.8 ± 0.5	0.4 ± 0.04
Sugar ratio (sucrose/glucose + fructose)[a]	1.5	1.9	2.7	2.4	2.4	1.4	—	0.6	1.5
Sugar concentration (g/ml)	56.5 ± 0.6	41.1 ± 1.8	38.9 ± 1.9	52.7 ± 2.0	34.7 ± 3.2	30.7 ± 4.1	—	28.8 ± 4.2	77.2 ± 5.3
Amino acid concentration (nMol/ml)	5.2 ± 3.2	9.8 ± 2.2	1.6 ± 0.2	2.6 ± 0.6	1.8 ± 0.3	4.0 ± 0.8	—	2.3 ± 0.5	12.2 ± 1.8
Predominant amino acid	proline	proline	proline	proline	GABA	glutamine	—	GABA	proline

Note: SC = self-compatible, SI = self-incompatible.

Plowright, 1985; Tamm and Gass, 1986; Cresswell and Galen, 1991; Martínez del Rio et al., 1992; Hodges, 1995; Meléndez-Ackerman et al., 1997; Schemske and Bradshaw, 1999), making pollinator-mediated selection a real possibility. The nectar trait variation in *Nicotiana* section *Alatae* could be a result of past selection pressures from pollinators, but nectar traits could have evolved in association with other floral traits with or without the aid of pollinators. More pollinator preference tests, especially with moths, are necessary to determine whether preferences truly match the nectar traits offered in the plant species they pollinate. Eco-genetic experiments are also needed to determine whether those preferences are strong enough to affect evolution.

LITERATURE CITED

BAKER, H. G. 1961. The adaptation of flowering plants to nocturnal and crepuscular pollinators. *The Quarterly Review of Biology* 36: 64–73.

BAKER, H. G. 1975. Sugar concentrations in nectars from hummingbird flowers. *Biotropica* 7: 37–41.

BAKER, H. G. 1977. Non-sugar chemical constituents of nectar. *Apidologie* 8: 349–356.

BAKER, H. G. 1978. Chemical aspects of the pollination biology of woody plants in the tropics. *In* P. B. Tomlinson and M. H. Zimmerman [eds.], Tropical trees as living systems, 57–82. Cambridge University Press, New York, New York, USA.

BAKER, H. G., AND I. BAKER. 1977. Interspecific constancy of floral nectar amino acid complements. *Botanical Gazette* 138: 183–191.

BAKER, H. G., AND I. BAKER. 1982. Chemical constituents of nectar in relation to pollination mechanisms and phylogeny. *In* H. M. Nitecki [ed.], Biochemical aspects of evolutionary biology, 131–171. University of Chicago Press, Chicago, Illinois, USA.

BAKER, H. G., AND I. BAKER. 1983. Floral nectar sugar constituents in relation to pollinator type. *In* C. E. Jones and R. J. Little [eds.], Handbook of experimental pollination biology, 117–140. Scientific and Academic Editions, New York, New York, USA.

BOLTON, A. B., AND P. FEINSINGER. 1978. Why do hummingbird flowers secrete dilute nectar? *Biotropica* 10: 307–308.

BOLTON, A. B., P. FEINSINGER, H. G. BAKER, AND I. BAKER. 1979. On the calculation of sugar concentration in flower nectar. *Oecologia* 41: 301–304.

BRICE, A. T., AND C. R. GRAU. 1991. Protein requirements of Costa's hummingbirds *Calypte costae*. *Physiological Zoology* 64: 611–626.

BRUE, R. N. 1994. Nutrition. *In* B. Ritchie, G. Harrison, and L. Harrison [eds.], Avian medicine: principles and application, 63–95. Wingers Publishing, Lake Worth, Florida, USA.

CHASE, M. W., S. KNAPP, A. V. COX, J. J. CLARKSON, Y. BUTSKO, J. JOSEPH, V. SAVOLAINEN, AND A. S. PAROKONNY. 2003. Molecular systematics, GISH and the origin of hybrid taxa in *Nicotiana* (Solanaceae). *Annals of Botany* 92: 107–127.

COCUCCI, A. A. 1988. Polinización en Solanáceas Neotropicales. Ph.D. dissertation, Universidad Nacional de Córdoba, Facultad de Ciencias Exactas Físicas y Naturales, Argentina.

COHEN, S. A., AND D. P. MICHEAUD. 1993. Synthesis of a fluorescent derivatization reagent, 6-aminoquionyl-N-hydroxysuccinimidyl carbamate and its application for the analysis of hydrolysate amino acids via high performance liquid chromatography. *Analytical Biochemistry* 211: 279–287.

CRESSWELL, J. E., AND C. GALEN. 1991. Frequency-dependent selection and adaptive surfaces for floral character combinations: the pollination of *Polemonium viscosum*. *American Naturalist* 138: 1342–1353.

CRUDEN, R. W., S. M. HERMANN, AND S. PETERSON. 1983. Patterns of nectar production and plant-pollinator coevolution. *In* B. Bentley and T. Elias [eds.], The biology of nectaries, 80–125. Columbia University Press, New York, New York, USA.

DADD, R. H. 1973. Insect nutrition: current developments and metabolic implications. *Annual Review of Entomology* 18: 381–420.

FAEGRI, K., AND L. VAN DER PIJL. 1966. The principles of pollination ecology. Pergamon Press, London, UK.

FENSTER, C. B., W. S. ARMBRUSTER, P. WILSON, M. R. DUDASH, AND J. D. THOMPSON. 2004. Pollination syndromes and floral specialization. *Annual Review of Ecology, Evolution, and Systematics* 35: 375–403.

FERDY, J.-B., AND A. SMITHSON. 2002. Geitonogamy in rewarding and unrewarding inflorescences: modelling pollen transfer on actual foraging sequences. *Evolutionary Ecology* 16: 155–175.

GALEN, C. 1999. Flowers and enemies: predation by nectar-thieving ants in relation to variation in floral form of an alpine wildflower, *Polemonium viscosum*. *Oikos* 85: 426–434.

GALEN, C. 2000. High and dry: drought stress, sex-allocation trade-offs, and selection on flower size in the alpine wildflower *Polemonium viscosum* (Polemoniaceae). *American Naturalist* 156: 72–83.

GALEN, C., AND J. CUBA. 2001. Down the tube: pollinators, predators, and the evolution of flower shape in the alpine skypilot, *Polemonium viscosum*. *Evolution* 55: 1963–1971.

GALEN, C., AND R. C. PLOWRIGHT. 1985. The effects of nectar level and flower development on pollen carry-over in inflorescences of fireweed (*Epilobium angustifolium*) (Onagraceae). *Canadian Journal of Botany* 63: 488–491.

GARDENER, M. C., AND M. P. GILLMAN. 2001a. Analyzing variability in nectar amino acids: composition is less variable than concentration. *Journal of Chemical Ecology* 27: 2545–2558.

GARDENER, M. C., AND M. P. GILLMAN. 2001b. The effects of soil fertilizer on amino acids in the floral nectar of corncockle, *Agrostemma githago* L. (Caryophyllaceae). *Oikos* 92: 101–106.

GARDENER, M. C., AND M. P. GILLMAN. 2002. The taste of nectar—a neglected area of pollination. *Oikos* 98: 552–557.

GOODSPEED, T. H. 1954. The genus *Nicotiana*. Chronica Botanica, Waltham, Massachusetts, USA.

GOTTSBERGER, G., T. ARNOLD, AND H. F. LINSKENS. 1990. Variation in floral nectar amino acids with aging of flowers, pollen contamination, and flower damage. *Israel Journal of Botany* 39: 167–176.

GOTTSBERGER, G., J. SCHRAUWEN, AND H. F. LINSKENS. 1984. Amino acids and sugars in nectar, and their putative evolutionary significance. *Plant Systematics and Evolution* 145: 55–77.

GRANT, K. 1966. A hypothesis concerning the prevalence of red coloration in California hummingbird flowers. *American Naturalist* 100: 85–97.

GRANT, V. 1994. Modes and origins of mechanical and ethological isolation in angiosperms. *Proceedings of the National Academy of Sciences, USA* 91: 3–10.

GRANT, K., AND V. GRANT. 1968. Hummingbirds and their flowers. Columbia University Press, New York, New York, USA.

GRANT, V., AND E. J. TEMELES. 1992. Foraging ability of Rufous hummingbirds on hummingbird flowers and hawkmoth flowers. *Proceedings of the National Academy of Sciences, USA* 89: 9400–9404.

HABER, W. A., AND G. W. FRANKIE. 1989. A tropical hawkmoth community: Costa Rican dry forest Sphingidae. *Biotropica* 21: 155–172.

HAYDAK, M. H. 1970. Honey bee nutrition. *Annual Review of Entomology* 15: 143–156.

HAINSWORTH, F. R., AND L. L. WOLF. 1976. Nectar characteristics and food selection by hummingbirds. *Oecologia* 25: 101–113.

HEINRICH, B. 1975. Energetics of pollination. *Annual Review of Ecology and Systematics* 6: 139–170.

HEINRICH, B., AND P. H. RAVEN. 1972. Energetics and pollination ecology. *Science* 176: 597–602.

HODGES, S. A. 1995. The influence of nectar production on hawkmoth behavior, self-pollination, and seed production in *Mirabilis multiflora* (Nyctaginaceae). *American Journal of Botany* 82: 197–204.

INOUYE, D. W., N. D. FAVRE, J. A. LANUM, D. M. LEVINE, J. B. MEYERS, M. S. ROBERTS, F. C. TSAO, AND Y.-Y. WANG. 1980. The effects of nonsugar nectar constituents on estimates of nectar energy content. *Ecology* 61: 992–996.

IPPOLITO, A. 2000. Systematics, floral evolution and speciation in *Nicotiana*. Ph.D. dissertation, University of Missouri–Columbia, Columbia, Missouri, USA.

IPPOLITO, A., G. W. FERNANDES, AND T. P. HOLTSFORD. 2004. Pollinator preferences for *Nicotiana alata*, *N. forgetiana*, and their F1 hybrids. *Evolution* 58: 2634–2644.

JONES, K. N., AND J. S. REITHEL. 2001. Pollinator-mediated selection on a flower color polymorphism in experimental populations of *Antirrhinum* (Scrophulariaceae). *American Journal of Botany* 88: 447–454.

JOSENS, R. B., AND W. M. FARINA. 1997. Selective choice of sucrose solution concentration by the hovering hawk moth *Macroglossum stellatarum*. *Journal of Insect Behavior* 10: 631–637.

JOSENS, R. B., AND W. M. FARINA. 2001. Nectar feeding by the hovering hawk moth *Macroglossum stellatarum*: intake rate as a function of viscosity and concentration of sucrose solutions. *Journal of Comparative Physiology A* 187: 661–665.

KELBER, A. 2003. Sugar preferences and feeding strategies in the hawkmoth *Macroglossum stellatarum*. *Journal of Comparative Physiology A* 189: 661–666.

LANZA, J., G. C. SMITH, S. SACK, AND A. CASH. 1995. Variation in nectar volume and composition of *Impatiens capensis* at the individual, plant, and population levels. *Oecologia* 102: 113–119.

LINSKENS, H. F., AND J. SCHRAUWEN. 1969. The release of free amino acids from germinating pollen. *Acta Botanica Neerlandica* 18: 605–614.

MARTÍNEZ DEL RIO, C., H. G. BAKER, AND I. BAKER. 1992. Ecological and evolutionary implications of digestive processes: bird preferences and the sugar constituents of floral nectar and fruit pulp. *Experientia* 48: 544–551.

MELÉNDEZ-ACKERMAN, E., D. R. CAMPBELL, AND N. M. WASER. 1997. Hummingbird behavior and mechanisms of selection on flower color in *Ipomopsis*. *Ecology* 78: 2532–2541.

OLLERTON, J. 1996. Reconciling ecological processes with phylogenetic patterns: the apparent paradox of plant–pollinator systems. *Journal of Ecology* 84: 767–769.

PACINI, E., M. NEPI, AND J. L. VESPRINI. 2003. Nectar biodiversity: a short review. *Systematics and Evolution* 238: 7–21.

PERCIVAL, M. S. 1961. Types of nectar in angiosperms. *New Phytologist* 60: 235–281.

PERCIVAL, M. S. 1965. Floral biology. Pergamon Press, London, UK.

PERCIVAL, M. 1974. Floral ecology of coastal scrub in southeast Jamaica. *Biotropica* 6: 104–129.

PETANIDOU, T., A. J. VAN LAERE, AND E. SMETS. 1996. Change in floral nectar components from fresh to senescent flowers of *Capparis spinosa* (Capparidaceae), a nocturnally flowering Mediterranean shrub. *Plant Systematics and Evolution* 199: 79–92.

PLOWRIGHT, R. C. 1987. Corolla depth and nectar concentration: an experimental study. *Canadian Journal of Botany* 65: 1011–1013.

PYKE, G. H., AND N. M. WASER. 1981. The production of dilute nectars by hummingbird and honeyeater flowers. *Biotropica* 13: 260–270.

RAVEN, P. H. 1972. Why are bird-visited flowers predominantly red? *Evolution* 26: 674.

REAL, L. A., AND B. J. RATHCKE. 1991. Individual variation in nectar production and its effect on fitness in *Kalmia latifolia*. *Ecology* 72: 149–155.

SCHEMSKE, D. W., AND H. D. BRADSHAW JR. 1999. Pollinator preference and the evolution of floral traits in monkeyflowers (*Mimulus*). *Proceedings of the National Academy of Sciences, USA* 96: 11910–11915.

SHIRAISHI, A., AND M. KUWABRA. 1970. The effects of amino acids on the labellar hair chemosensory cells of the fly. *Journal of General Physiology* 56: 768–782.

SPIRA, T. P. 1980. Floral parameters, breeding system and pollinator type in *Trichostema* (Labiatae). *American Journal of Botany* 67: 278–284.

STEHMANN, J. R., J. SEMIR, AND A. IPPOLITO. 2002. *Nicotiana mutabilis* (Solanaceae), a new species from southern Brazil. *Kew Bulletin* 57: 639–646.

STILES, F. G. 1976. Taste preferences, color preferences, and flower choice in hummingbirds. *The Condor* 78: 10–26.

TAMM, S., AND C. L. GASS. 1986. Energy intake rates and nectar concentration preferences by hummingbirds. *Oecologia* 70: 20–23.

WAGNER, H. O. 1946. Food and feeding habits of Mexican hummingbirds. *Wilson Bulletin* 58: 69–132.

WASER, N. M. 1998. Pollination, angiosperm speciation, and the nature of species boundaries. *Oikos* 81: 198–201.

WASER, N. M., L. CHITTKA, M. V. PRICE, N. M. WILLIAMS, AND J. OLLERTON. 1996. Generalization in pollination systems, and why it matters. *Ecology* 77: 1043–1060.

WASER, N. M., AND M. V. PRICE. 1981. Pollinator choice and stabilizing selection for flower color in *Delphinium nelsonii*. *Evolution* 35: 376–390.

YOUNG, H. J. 2002. Diurnal and nocturnal pollination of *Silene alba* (Caryophyllaceae). *American Journal of Botany* 89: 433–440.

ZIEGLER, R. 1991. Changes in lipid and carbohydrate metabolism during starvation in adult *Manduca sexta*. *Journal of Comparative Physiology B* 161: 125–131.

ZIMMERMAN, M. 1983. Plant reproduction and optimal foraging: experimental nectar manipulations in *Delphinium nelsonii*. *Oikos* 41: 57–63.

ZIMMERMAN, M. 1988. Nectar production, flowering phenology, and strategies for pollination. *In* J. L. Doust and L. L. Doust [eds.], Plant reproductive ecology: patterns and strategies, 157–178. Oxford University Press, Oxford, UK.