NECTAR IN *NICOTIANA*: POLLINATOR ASSOCIATIONS, SOURCES OF VARIATION, AND EVOLUTIONARY CONSEQUENCES

A Dissertation

Presented to

The Faculty of the Graduate School

University of Missouri-Columbia

In Partial Fulfillment

Of the Requirements for the Degree

Doctor of Philosophy

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by

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MAY 2007

The undersigned, appointed by the Dean of the Graduate School, have examined the dissertation entitled

NECTAR IN *NICOTIANA*: POLLINATOR ASSOCIATIONS, SOURCES OF VARIATION, AND EVOLUTIONARY CONSEQUENCES

presented by RAINEE L KACZOROWSKI

a candidate for the degree of DOCTOR OF PHILOSOPHY

and hereby certify that in their opinion it is worthy of acceptance.

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Dr. Reginald Cocroft

Dr. Robin Kennedy

Dr. Bruce McClure

To my mother and father,
who nurtured my passion for nature and
encouraged my desire to know more about it…

ACKNOWLEDGEMENTS

First of all, I would like to thank my dissertation advisor, Tim Holtsford, for so many things. His insightful ideas were helpful to me in developing and understanding my project. In the beginning, his enthusiasm helped drive me to produce data as quickly as possible. In the end, his support and encouragement helped to push me through the difficult times. Tim has become like family to me, along with his wife, Cathy Gunther, and son, Joe. I owe so much to them.

I would also like to thank my other committee members, Candi Galen, Robin Kennedy, Rex Cocroft, and Bruce McClure, for their time, advice and helpful comments. In particular, Candi Galen always took the time to thoroughly critique manuscripts and project proposals in a way that amazed me. Robin Kennedy also deserves additional gratitude for encouraging me to pursue graduate school. Without her persuasion, I may not have accomplished all the things that coincide with attending graduate school.

I also appreciate the opportunity Robin gave me to work at the herbarium through the Ernest J. Palmer Herbarium Fellowship, which helped support my research in Brazil. I am also appreciative of Dr. John David for authorizing additional support through the Department of Biological Sciences to help fund a research assistant in Brazil. I am grateful for the funding I received through the GAANN fellowship from the Department of Education, which provided me with ample time to perform research without teaching commitments.

I have so much gratitude towards Mark Gardener and Tom Juenger, the coauthors on manuscripts from this dissertation. Mark Gardener analyzed a number of samples

through High-Performance Liquid Chromatography (HPLC) and Tom Juenger provided the SAS program to analyze the heritability data. Their assistance certainly saved me countless hours of working on these things myself. In addition, I am very appreciative of Mark Ellersieck, who provided extensive statistical advice on a number of analyses, and Ray Semlitsch provided advice on some of my experimental designs.

I would like to thank the many members of the Holtsford Lab from over the years, including Dulce Figueroa-Castro, Julie Ketner, Paul Walker, Paul Hage, Sherry Ellberg, Jacob Soule, Esther Stroh, Allena Volskay, Casey Dillman, Gordon Burleigh, Anthony Ippolito, Jana U'Ren, and Jane Murfett, for providing advice, support, and friendship. I would especially like to thank Dulce and Jane for their valuable comments on manuscripts, Allena for providing floral trait data used in my second chapter, and Anthony for his advice and support during my time in Brazil.

I am especially thankful to so many people for the many hours of help necessary to conduct the large quantitative genetic experiment. I thank Mike McMullen for allowing me to conduct my field experiment at the MU Genetics Farm, along with Chris Browne and crew for helping to set up and maintain the plot. Many people helped with planting seeds, transplanting seedlings, caring for the plants, surveying the field, bagging flowers, or taking measurements, including Julie Ketner, Dulce Figueroa-Castro, Paul Walker, Paul Hage, Sherry Ellberg, Jacob Soule, Esther Stroh, Jenni Geib, Andy Eshbacher, Leah Dudley, Cathy Gunther, and Rob Raguso.

I am also thankful for the help I received while conducting research in Brazil. I thank Morgan Peckosh, my field assistant, for his ability to come to Brazil with very little notice, for countless hours of driving around Brazil and measuring floral traits, and for

providing much needed entertainment while continuously on the road in a foreign country. I am very grateful to Daniel Tiscornia and his family for providing the “home-base” in Brazil and helping me take care of any issue that presented itself during my time there. I am thankful to all the people in Campestre da Serra, RS, that helped make it possible for me to conduct my experiments at the research station there, especially A. Alcindo Nunz, the station manager, who kept my plants watered while I was away, prepared and maintained my experimental plots, and allowed us to use half of his “home” as our research station. Marcía Vignoli-Silva provided important information and herbarium resources that helped to locate additional populations and Vinicius Renner Lampert helped locate populations and also helped with some of the measurements. I also thank Cathy Gunther for traveling to Brazil to count my seeds after they had matured and Molly Kirkconnell for her help in the field in both Brazil and Argentina. I would also like to thank my sweetheart, Chris Martin, for his help in some of the later Brazil experiments, as well as for his advice, general support and encouragement throughout the course of my graduate studies.

I would like to thank almost everyone in the Division of Biological Sciences for making my graduate experience so wonderful. I would like to give special thanks to Josh Hartley, Matt Nevels, and Alan Marshall for all of their computer assistance, Tyeece Little for helping to keep me on track with spending my GAANN funds, and especially Nila Emerich. Nila was always there to remind me of important issues, listen to my problems and offer crucial support and encouragement when it was needed most.

And finally, I thank my family and friends, who have always provided me with love, support and encouragement, and often a non-scientific outlet.

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NECTAR IN *NICOTIANA*: POLLINATOR ASSOCIATIONS, SOURCES OF VARIATION, AND EVOLUTIONARY CONSEQUENCES

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ABSTRACT

Nectar is the primary floral reward offered by plants to attract pollinators. Pollinators often exhibit a preference for certain types of nectars over others. 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. To consider the possibility that *Nicotiana* nectar traits could be placed under selective pressure, it must first be determined that the traits in question exhibit variation, the variation includes a heritable component, and the variation can affect the fitness of individuals.

I determined that nectar traits exhibited a high degree of variability in the controlled environment of the greenhouse, both within and among *Nicotiana* species, and many traits varied in association with the pollination system. This variation was also demonstrated in plants growing in natural populations. However, nectar traits in naturally-growing plants can also be affected by biotic and abiotic factors (i.e., floral visitors and weather conditions). Although nectar traits often differed between the greenhouse and natural population environments, pollinator group comparisons of nectar traits from naturally-growing plants were mostly similar to those found from greenhouse-grown plants. This suggests that pollinators may have played a role in guiding the evolution of nectar traits. However, because the past cannot be definitively elucidated,

this is only correlative evidence. Heritability and fitness experiments are necessary to determine whether pollinators currently affect nectar traits.

A significant heritable component was detected for nectar volume and energy, as well as corolla tube length, in an experimental population of *Nicotiana alata*. Although phenotypic correlations were significant for all measured traits, only two correlations (corolla limb width / mouth diameter and nectar volume / energy content) had a genetic basis. However, some differences in trait means and genotype by environment interactions were detected between the novel environment in which the experiment was conducted (Missouri), and the ancestral habitat of this species (Brazil). Therefore, heritability and correlation estimates may not fully represent that which would be found in natural populations. These estimates could also change within a population over time.

I conducted an experiment in the native habitat to investigate whether increased nectar quantity can affect fitness components in *Nicotiana alata*. With the methods used, results suggest that nectar augmentation did not affect seed production in this experimental population. However, had methods, location, or time been different, results may have been different.

These experiments can elucidate potential selective pressure on nectar traits. Although significant variation and heritability in nectar traits suggest that they have the potential to respond to selection, plant fitness was not affected by increased nectar quantities with the methods used. Further studies are necessary to conclusively support or refute the possibility of pollinators' exerting selective pressure on nectar traits in *Nicotiana*.

CHAPTER 1

INTRODUCTION

Nectar is important because it is the primary floral reward offered by plants to attract pollinators and it is also the primary food source for many pollinators. Nectar variation can be ecologically important because pollinators often exhibit a preference for certain types of nectars over others (Van Riper, 1959; Hainsworth and Wolf, 1976; Stiles, 1976; Pyke and Waser, 1981; Tamm and Gass, 1986; Alm et al., 1990; Erhardt and Rusterholz, 1998), which can affect which plants are pollinated. Variation in nectar traits can also be important evolutionarily. Phenotypic variation in nectar traits can have both a genetic and environmental component. If variation of a nectar trait has a genetic component, the trait can be subjected to selective pressure if it is also heritable and affects plant fitness. If selective pressure is placed on a nectar trait through pollinator preferences for certain nectar types over others, it could result in ethological isolation, which has the potential to promote species divergence or maintenance during secondary contact (Grant, 1994).

Many pollinators are often associated with plant species that exhibit certain floral traits. For example, moth-pollinated flowers tend to be white or pale-colored and emit a strong, sweet scent when they open in the evening or at night (Baker, 1961; Percival, 1965; Faegri and van der Pilj, 1966), while hummingbird-pollinated flowers tend to be pendulous (often red in color) and lack a detectable odor (Baker, 1961; Grant, 1966;

Grant and Grant, 1968; Raven, 1972). Pollinators may also be associated with certain types of nectar.

Previous studies have found that both hummingbird- and moth-pollinated plant species tend to have sucrose as the predominant nectar sugar (Baker and Baker, 1982, 1983). Similarly, both hummingbird- and moth-pollinated plant species tend to have relatively low nectar concentrations; hummingbird-pollinated species average between 20-25% sucrose equivalents (Baker, 1975; Baker and Baker, 1983; Cruden et al., 1983), while hawkmoth-pollinated species average between 15-30% (Baker and Baker, 1983; Cruden et al., 1983; Haber and Frankie, 1989). Additionally, nectar concentration is expected to be low for pollinators that feed through narrow tubes, such as moths and other Lepidopterans, to increase the ease of nectar extraction (Baker and Baker, 1983). Plant species pollinated by Lepidopterans are also expected to have higher amino acid concentrations because they have no alternative source for proteins (Baker and Baker, 1975, 1983). However, hawkmoth-pollinated species, like hummingbird-pollinated species, tend to have a lower amino acid concentration (0.536 and 0.452 μM/ml, respectively), while small moth-pollinated flowers tend to have relatively high amino acid concentrations (1.059 μM/ml) (Baker and Baker, 1983).

Pollinator associations with nectar traits tend to be supported by most pollinator preference study results (Van Riper, 1959; Hainsworth and Wolf, 1976; Stiles, 1976; Martínez del Rio, 1990; Josens and Farina, 1997; Kelber, 2003), although several studies show that hummingbirds actually prefer higher nectar concentrations than those offered by plants they typically pollinate (Hainsworth and Wolf, 1976; Stiles, 1976; Pyke and Waser, 1981; Tamm and Gass, 1986). Nectar preferences have the potential to drive

evolutionary change if pollinators discriminate against certain nectar types. The 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 have been 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.

Although characterization of nectar traits in the greenhouse allows for an estimation of genetic differentiation among species, results may not represent what would be found in plants growing in natural populations. Environmental variation can be considerable within and among natural populations, and can contribute significantly to phenotypic variation in nectar traits from the field (Zimmerman and Pyke, 1988; Wyatt et al., 1992). Many different sources of environmental variation can be found in the field, including weather conditions and floral visitors (see Zimmerman, 1988; Rathcke, 1992). It is important to explore nectar trait variation in natural populations because that is where pollinators have the opportunity to place selective pressure on nectar traits.

Variation in nectar traits must also be heritable to affect evolution. Relatively few studies to date have explored the heritability of nectar traits (see Mitchell, 2004). And only two studies have investigated the heritability of nectar traits in the field, where environmental variation can mask underlying genetic components (Campbell, 1996; Leiss et al., 2004). Because pollinators would exert selection in natural populations, it is important to determine whether significant heritability can be detected in the presence of considerable environmental variation.

Floral morphology traits can be important for the access of nectar by pollinators (Campbell et al., 1996; Lange et al., 2000; Ree, 2005; Darrault and Schlindwein, 2005) and may also contribute to the apparency of flowers to pollinators. Phenotypic correlations are commonly found between nectar traits and floral morphology traits (Plowright, 1987; Duffield et al., 1993; Mitchell and Shaw, 1993; Campbell, 1996; Davis, 1997; Klinkhamer and van der Veen-van Wijk, 1999). These correlations are often assumed to be adaptive; however, these assumptions depend on underlying genetics. A trait can respond to the selection of another trait if they are genetically correlated.

Genetic correlations and heritability estimates are likely to differ in different environments, as environmental variation can affect these estimates. Certain genotypes may respond differently to different environmental conditions. Genotype by environment interactions can affect the rate and direction of evolution (Via and Lande, 1985).

In addition to significant variation and heritability, nectar traits must also affect plant fitness to allow evolutionary change to occur. Some studies have linked nectar traits and plant fitness. Nectar production is an important nectar trait that has been repeatedly linked to pollinator behavior. High nectar production can increase the frequency or duration of pollinator visitation to individual flowers, inflorescences, or plants (Pedersen, 1953; Thomson and Plowright, 1980; Zimmerman, 1983; Galen and Plowright, 1985; Real and Rathcke, 1991; Mitchell and Waser, 1992; Mitchell, 1993; Hodges, 1995; Burd, 1995; Nassar et al., 1997; Cresswell, 1999; Manetas and Petropoulou, 2000; Kudo, 2003). Pollinator behaviors such as these have often been associated with increases in plant fitness, through increased pollen removal and/or deposition (Thomson and Plowright, 1980; Galen and Plowright, 1985; Thomson, 1986; Mitchell, 1983; Pleasants and

Chaplin, 1983; Harder, 1990; Galen, 1992; Hodges, 1995) or increased seed set (Pedersen, 1953; Zimmerman, 1983; Real and Rathcke, 1991; Manetas and Petropoulou, 2000). However, a number of studies have failed to find a correlation between nectar and plant fitness, despite a correlation between nectar and pollinator behavior (Mitchell and Waser, 1992; Cresswell, 1999; Kudo, 2003).

If nectar traits have a significant effect on plant fitness, in addition to having significant variation and heritability, it would suggest that these traits in *Nicotiana* have the potential to evolve. This may also suggest that current nectar traits have evolved from past selective pressure on nectar traits.

INTRODUCTION TO THE STUDY SYSTEM

Nicotiana, Section *Alatae*, is an excellent study system for evolutionary questions because it is a monophyletic group (Ippolito, 2000, Chase et al., 2003; Knapp et al., 2004) comprised of seven or eight species with highly diverse floral traits, in addition to different pollinators and mating systems. However, the relationship of these species has not been completely resolved, although species with the same chromosome number are more closely related (Ippolito, 2000, Chase et al., 2003; Knapp et al., 2004). *Nicotiana longiflora* and *N. plumbaginifolia* (chromosome $n = 10$) are self-compatible species. *Nicotiana longiflora* is typically a facultative outcrosser, but some populations exhibit some degree of autogamy (self-fertilization), while *N. plumbaginifolia* is fully autogamous. The remaining species, *N. alata, N. forgetiana, N. langsdorffii, N. mutabilis, N. bonariensis,* and putative new species "Rastroensis" (chromosome $n = 9$), are self-incompatible.

More than 200 hours of field observations over four years suggest that there are only three kinds of primary pollinators for these *Nicotiana* species (Holtsford and Ippolito, pers. comm.). Hawkmoths are the predominant pollinators of species with long-tubed, white flowers (*N. alata* and *N. longiflora*). The most common hawkmoths observed were *Agrius cingulata* and *Eumorpha labruscae* (Sphingidae). *Nicotiana plumbaginifolia* has a similar floral morphology and may be visited by hawkmoths (Cocucci, 1988), but is smaller in size and autogamous. Hummingbirds (most commonly *Chlorostibulon aureoventris* [Trochilidae]) 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 been seen visiting these species. Bumblebees have also been observed visiting *N. langsdorffii* in the field with limited frequency. Small perching moths (Noctuidae) are thought to be the primary pollinators of the species with short-tubed, white flowers (*N. bonariensis*), but few have actually been observed in the field.

Although *Nicotiana* section *Alatae* apparently has only three types of primary pollinators, it is unclear how many pollinator shifts occurred within this section because the phylogeny is not yet resolved (Ippolito, 2000, Chase et al., 2003; Knapp et al., 2004). It would be preferred to take the phylogenetic relationship of each species into account when drawing conclusions about the adaptive nature of nectar traits. When the phylogeny for *Nicotiana* section *Alatae* is resolved, nectar characters can be mapped onto the phylogeny (Maddison, 1991; Cunningham et al., 1998; Martins, 1999; Pagel, 1999) to determine whether nectar trait changes were associated with pollinator shifts

In the following chapters, I investigate the likelihood that nectar traits in *Nicotiana* evolved in response to pollinators (chapters 2 and 3) and could continue to evolve under selective pressure by pollinators (chapters 4 and 5). For chapter 2, I investigated whether nectar traits of greenhouse-grown plants were variable within and among all species of *Nicotiana* section *Alatae*. I also examined whether nectar traits varied in association with primary pollinators and mating system. In chapter 3, I investigated how natural environmental variation contributed to phenotypic variation in nectar traits of naturally-growing plants of all but two species of *Nicotiana* section *Alatae*. In addition to identifying sources of environmental variation in natural populations, I also compared nectar traits from plants grown naturally to plants grown in the greenhouse. In chapter 4, I examined whether nectar traits in *Nicotiana alata* were significantly heritable and whether correlations between and among nectar and floral morphology traits had a significant genetic component. In chapter 5, I investigated whether *N. alata* plant fitness could be affected by nectar augmentation.

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CHAPTER 2

NECTAR TRAITS IN *NICOTIANA* SECTION *ALATAE* IN RELATION TO FLORAL TRAITS, POLLINATORS, AND MATING SYSTEM

ABSTRACT

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, I 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

INTRODUCTION

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, florivores, 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). I 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, I 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. I 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; Knapp et al., 2004) 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; Knapp et al., 2004). 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 three different pollinator groups for these *Nicotiana* species (Ippolito et al., 2004; T. Holtsford, R. Kaczorowski, and A. Ippolito, unpublished data). Hawkmoths (Sphingidae) are the predominant 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 (Trochilidae) 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 3.785 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 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 I 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. 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).

Nectar volume and concentration analysis

I used population, block, and time in a randomized complete block (RCB) split-plot design (PROC MIXED in SAS 6.12 [SAS Institute Inc., Cary, NC, 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. I 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, I 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 × 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.

Reward scaled by flower size

Measurements for nectar and floral morphology were taken on different plants from the same populations (for methods used for floral morphology measurements see Volskay, 2002). Therefore correlations between nectar and floral morphology traits were

determined through linear regressions based on population means (in Origin 6.0, Microcal Software Inc., Northhampton, MA, USA).

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"). 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.; 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., Milford, MA, USA). Separation was achieved using a Waters Novapak C18 (15 × 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 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 × 3.2 mm) and C18 guard cartridge (4 × 3 mm). 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. 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 Inc., Tulsa, OK, 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. I 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" 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 I 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 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 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 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, I 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 sugar concentrations (at α = 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 α = 0.01). Sugar ratios (sucrose to [glucose + fructose]) ranged from 0.6 in *N. plumbaginifolia* 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 α = 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 α = 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 ± 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; Knapp et al., 2004), 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 I 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, I 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 *Imaptiens 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 ten 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 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.

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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

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.

		Nectar volume (mean ± SE, µl)			
Species	N_0, N_{12}, N_{24} [a]	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
N. langsdorffii	16, 16, 15	2.87 ± 0.56	4.08 ± 0.54	6.09 ± 0.65	3.21 **L
N. forgetiana	26, 26, 26	4.58 ± 0.62	5.25 ± 0.55	6.68 ± 0.57	2.10 **L
"Rastroensis"	9, 9, 9	1.83 ± 0.78	2.07 ± 0.34	3.66 ± 0.48	1.83 ns
Hawkmoth-visited					
N. alata	27, 27, 27	6.27 ± 0.45	10.27 ± 0.73	15.61 ± 1.00	9.34 **L
N. longiflora	18, 18, 18	8.58 ± 0.85	13.35 ± 1.34	17.38 ± 1.51	8.80 **L
N. plumbaginifolia	5, 5, 5	1.62 ± 0.85	3.13 ± 1.15	6.27 ± 0.69	4.65 **L
Small-moth-pollinated					
N. bonariensis	19, 0, 19	0.27 ± 0.04		0.64 ± 0.06	0.36 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)

Table 2. Continued.

Species	N_0, N_{12}, N_{24} [a]	Nectar concentration (mean ± SE, % solids) 0 h	12 h	24 h	Total increase [b]
Hummingbird-pollinated					
N. mutabilis	16, 19, 17	46.47 ± 2.19	48.21 ± 1.70	58.68 ± 1.73	12.21 **L/*Q
N. langsdorffii	12, 15, 15	43.08 ± 1.76	50.17 ± 1.46	55.57 ± 2.36	12.48 **L
N. forgetiana	22, 24, 26	32.30 ± 2.72	46.00 ± 1.74	47.56 ± 2.07	15.26 **L
"Rastroensis"	7, 8, 9	48.79 ± 2.73	54.67 ± 2.02	59.50 ± 2.60	10.71 **L
Hawkmoth-visited					
N. alata	27, 26, 27	22.40 ± 0.40	23.19 ± 0.34	23.57 ± 0.37	1.18 ns
N. longiflora	17, 18, 18	21.41 ± 0.36	21.25 ± 0.21	21.42 ± 0.24	0.00 ns
N. plumbaginifolia	2, 3, 5	16.38 ± 0.13	17.92 ± 0.08	19.30 ± 1.10	2.93 ns
Small-moth-pollinated					
N. bonariensis	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)

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	*N. mutabilis*	*N. langsdorffii*	*N. forgetiana*	"Rastroensis"	*N. alata*
<u>"Essentials"</u>					
Arg	6 ± 0.9	4 ± 0.4	*13* ± *1.0*	8 ± 1.2	8 ± 0.5
Thr	4 ± 1.0	2 ± 0.9	*15* ± *1.2*	8 ± 1.5	*10* ± *1.2*
Phe	1 ± 0.3	2 ± 0.5	0 ± 0.0	0 ± 0.2	1 ± 0.6
Met	0 ± 0.0	0 ± 0.0	1 ± 0.2	0 ± 0.0	0 ± 0.1
Lys	0 ± 0.0	0 ± 0.0	0 ± 0.0	0 ± 0.1	0 ± 0.1
His	0 ± 0.1	0 ± 0.1	0 ± 0.0	0 ± 0.0	0 ± 0.1
Ile	1 ± 0.5	1 ± 0.5	0 ± 0.1	0 ± 0.2	1 ± 0.5
Leu	1 ± 0.4	2 ± 0.4	0 ± 0.1	1 ± 0.5	2 ± 0.9
Val	1 ± 0.6	2 ± 0.5	0 ± 0.1	1 ± 0.3	2 ± 0.4
<u>Other</u>					
Pro	***46*** ± ***5.1***	***47*** ± ***3.2***	***45*** ± ***3.6***	***32*** ± ***2.5***	6 ± 0.8
Ser	2 ± 0.8	3 ± 0.6	0 ± 0.2	1 ± 0.8	4 ± 1.0
Gly	1 ± 0.3	0 ± 0.1	1 ± 0.4	1 ± 0.6	2 ± 0.6
Cys	0 ± 0.0	0 ± 0.0	0 ± 0.0	0 ± 0.0	1 ± 0.4
Tyr	0 ± 0.1	0 ± 0.0	0 ± 0.0	0 ± 0.1	0 ± 0.1
Ala	*16* ± *4.6*	4 ± 1.1	0 ± 0.0	8 ± 2.2	*14* ± *1.9*
Asp	3 ± 3.1	1 ± 0.3	1 ± 0.4	7 ± 2.3	4 ± 0.9
Glu	2 ± 1.5	2 ± 1.4	2 ± 0.3	8 ± 2.1	5 ± 3.7
Asn	0 ± 0.0	2 ± 1.3	0 ± 0.0	0 ± 0.0	2 ± 1.5
Gln	*13* ± *8.7*	*23* ± *4.1*	1 ± 0.7	6 ± 4.9	4 ± 2.2
GABA	5 ± 2.0	5 ± 1.4	*20* ± *5.9*	*17* ± *3.7*	***33*** ± ***2.1***

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

Table 3. Continued.

Amino Acid	*N. longiflora*	*N. plumbaginifolia*	*N. bonariensis*	*F*(df=7,40)	*P*
"Essentials"					
Arg	4 ± 0.3	6 ± 0.8	5 ± 0.6	14.45	***
Thr	3 ± 0.8	5 ± 1.1	5 ± 0.8	13.70	***
Phe	7 ± 1.2	0 ± 0.1	5 ± 2.8	13.13	***
Met	0 ± 0.1	2 ± 1.0	0 ± 0.0	9.73	***
Lys	0 ± 0.0	0 ± 0.2	0 ± 0.0	7.74	***
His	0 ± 0.1	0 ± 0.0	0 ± 0.1	7.23	***
Ile	2 ± 0.4	1 ± 0.2	0 ± 0.0	5.19	**
Leu	2 ± 0.2	1 ± 0.1	4 ± 1.8	3.53	**
Val	1 ± 0.4	1 ± 0.2	2 ± 0.5	3.84	**
Other					
Pro	*23* ± *4.7*	8 ± 1.3	***41*** ± ***6.8***	20.68	***
Ser	1 ± 0.3	2 ± 1.0	4 ± 1.3	1.69	ns
Gly	0 ± 0.2	1 ± 0.5	2 ± 0.7	1.38	ns
Cys	0 ± 0.0	0 ± 0.0	0 ± 0.0	4.96	**
Tyr	1 ± 0.1	0 ± 0.1	0 ± 0.1	3.00	ns
Ala	3 ± 1.0	*17* ± *5.6*	*12* ± *2.0*	5.82	**
Asp	1 ± 0.4	5 ± 2.0	1 ± 0.3	2.36	ns
Glu	0 ± 0.3	3 ± 1.7	9 ± 0.7	4.08	**
Asn	1 ± 0.5	0 ± *0.0*	0 ± 0.0	2.90	ns
Gln	***42*** ± ***4.0***	6 ± *3.2*	6 ± 1.6	9.32	***
GABA	7 ± 1.9	***39*** ± ***1.8***	5 ± 2.4	14.86	***

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

Table 4 . Results of floral traits by species of *Nicotiana*. 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"
Pollination syndrome	hummingbird	hummingbird	hummingbird	hummingbird
Mating system	SI	SI	SI	SI
Typical color	white into pink	greenish-yellow	red	magenta
Daily phenology	diurnal	diurnal	crepuscular	crepuscular
Corolla length (mm)	34.7 ± 0.4	32.9 ± 0.4	46.9 ± 0.6	34.3 ± 0.8
Volume at 24 h (µl)	7.6 ± 0.4	6.1 ± 0.7	6.7 ± 0.6	3.7 ± 0.5
Concentration at 24 h (% solids)	58.7 ± 1.7	55.6 ± 2.4	47.6 ± 2.1	59.5 ± 2.6
Total energy at 24 h (mg sucrose equivalents)	5.6 ± 0.3	4.0 ± 0.3	3.7 ± 0.3	1.8 ± 0.3
Sugar ratio (sucrose/glucose+fructose)[a]	1.5	1.9	2.7	2.4
Sugar concentration (g/ml)	56.5 ± 0.6	41.1 ± 1.8	38.9 ± 1.9	52.7 ± 2.0
Amino acid concentration (nMol/ml)	5.2 ± 3.2	9.8 ± 2.2	1.6 ± 0.2	2.6 ± 0.6
Predominant amino acid	proline	proline	proline	proline

[a] Sugar ratios above 0.5 are considered sucrose-rich, above 1.0 are considered sucrose-dominant (Baker and Baker, 1982, 1983).

[b] SI: Self-incompatible, SC: Self-compatible

Table 4. Continued.

Floral or nectar characteristics	*Nicotiana* species				
	N. alata	*N. longiflora*	*N. longiflora* (Jujuy)	*N. plumbaginifolia*	*N. bonariensis*
Pollination syndrome	hawkmoth	hawkmoth	hawkmoth	hawkmoth	small moth
Mating system	SI	SC	SC/autogamous[b]	SC/autogamous[b]	SI
Typical color	white	white	white	white	white
Daily phenology	nocturnal	nocturnal	nocturnal	nocturnal	nocturnal
Corolla length (mm)	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)	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)	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)	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]	2.4	1.4	—	0.6	1.5
Sugar concentration (g/ml)	34.7 ± 3.2	30.7 ± 4.1	—	28.8 ± 4.2	77.2 ± 5.3
Amino acid concentration (nMol/ml)	1.8 ± 0.3	4.0 ± 0.8	—	2.3 ± 0.5	12.2 ± 1.8
Predominant amino acid	GABA	glutamine	—	GABA	proline

[a] Sugar ratios above 0.5 are considered sucrose-rich, above 1.0 are considered sucrose-dominant (Baker and Baker, 1982, 1983).

[b] SI: Self-incompatible, SC: Self-compatible

Figure 1. The species of *Nicotiana*, Section *Alatae*.

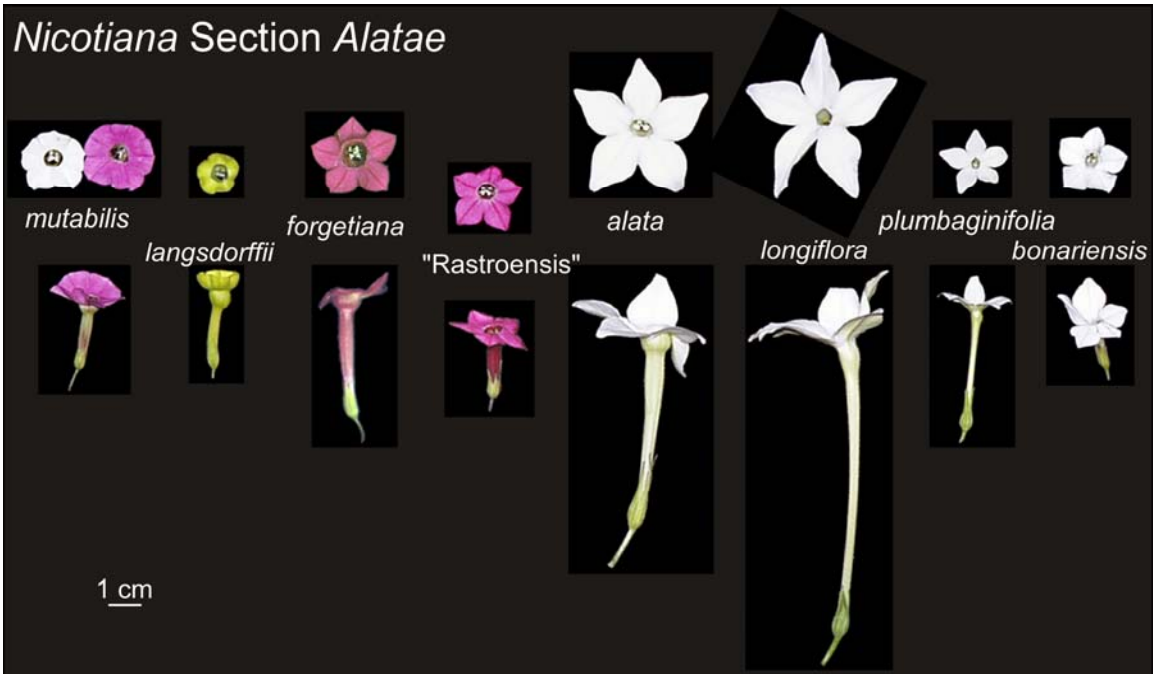
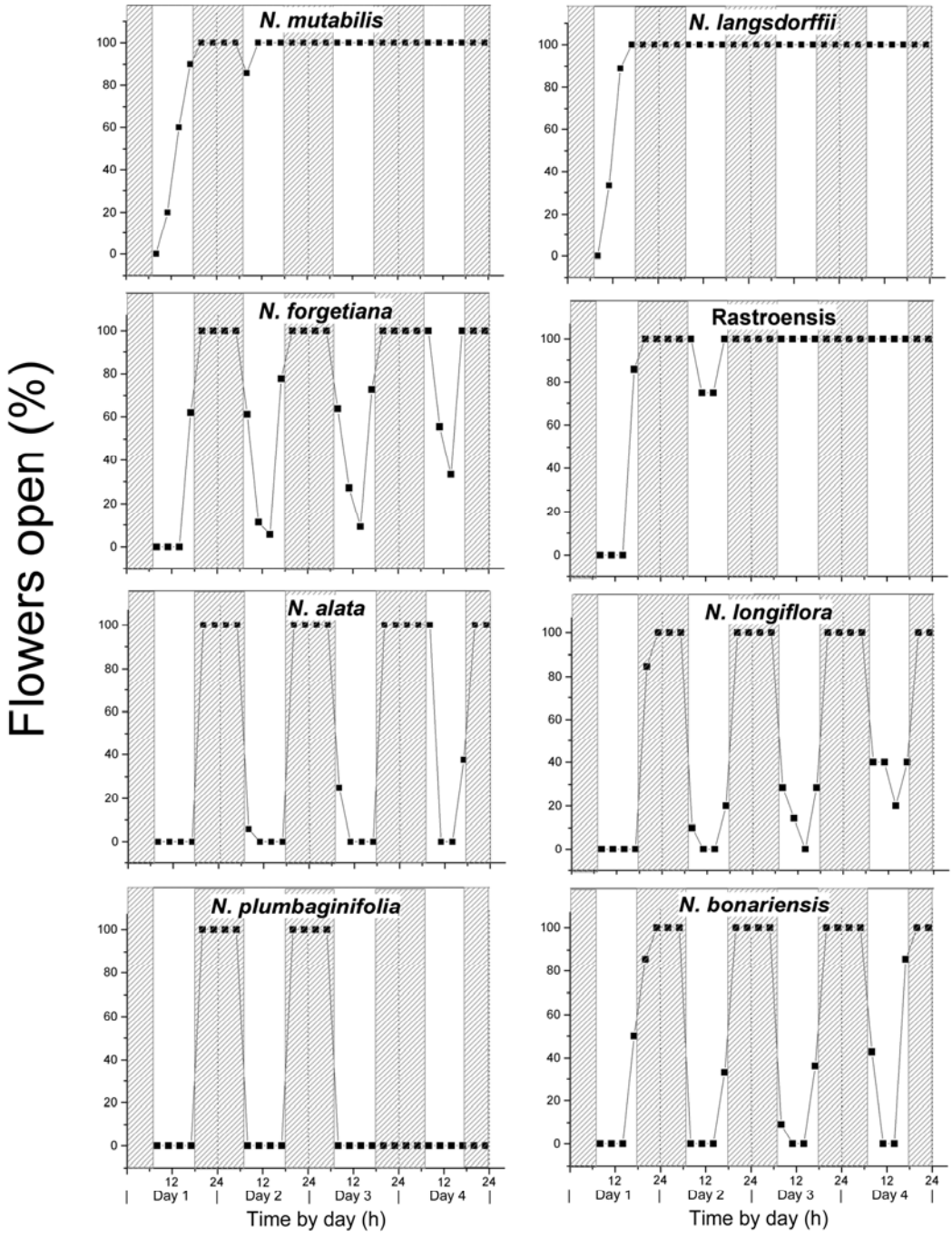
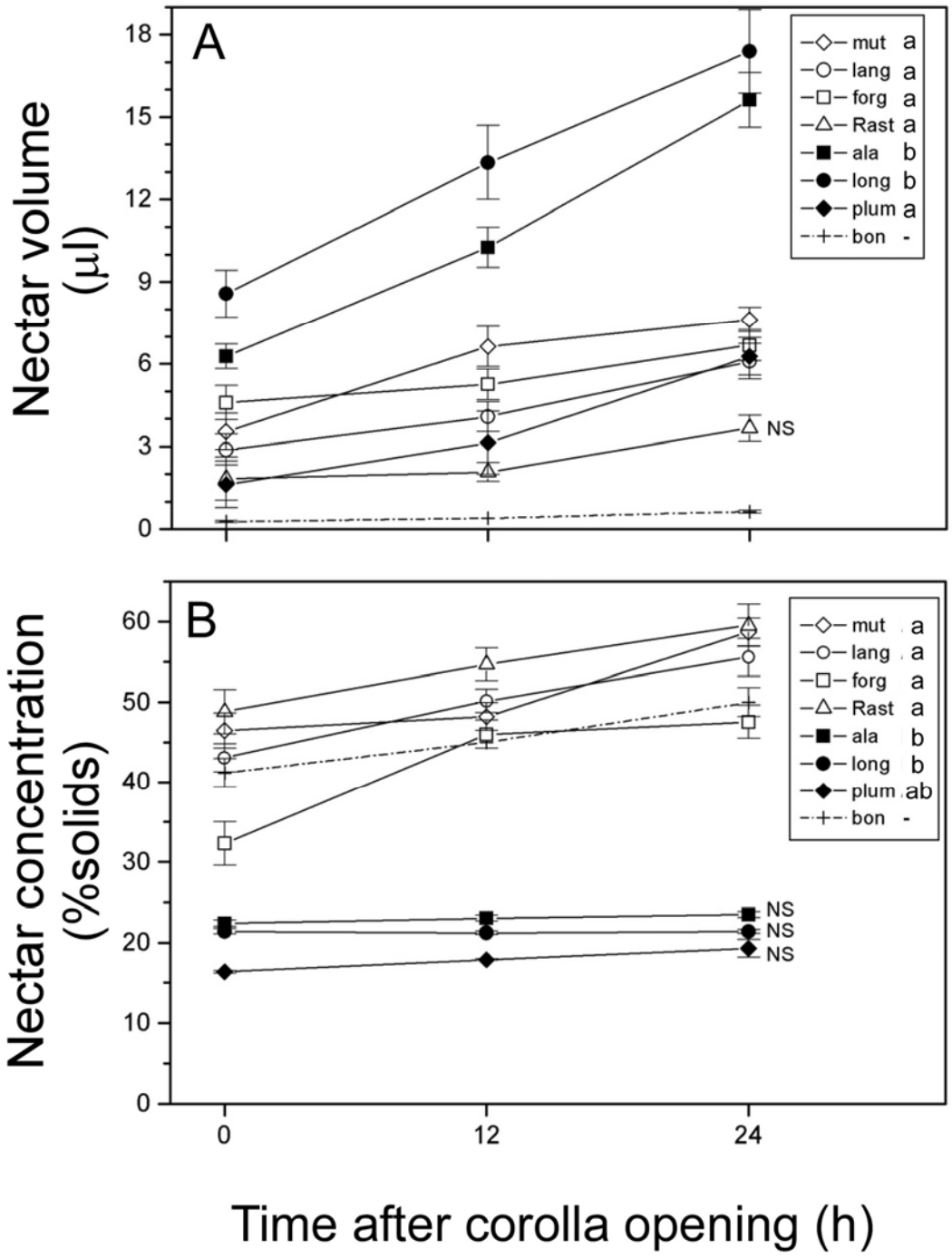

Nicotiana Section Alatae
mutabilis
langsdorffii
forgetiana
"Rastroensis"
alata
longiflora
plumbaginifolia
bonariensis
1 cm

Figure 2. Daily phenology pattern for the *Nicotiana* Section *Alatae* species. 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.


Flowers open (%)
N. mutabilis
N. langsdorffii
N. forgetiana
Rastroensis
N. alata
N. longiflora
N. plumbaginifolia
N. bonariensis
0
20
40
60
80
100
12
24
Day 1
Day 2
Day 3
Day 4
Time by day (h)

Figures 3. Nectar volume and concentration as a function of flower age. 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-hour 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 versus 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 versus 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.)


A
Nectar volume (µl)
18
15
12
9
6
3
0
mut a
lang a
forg a
Rast a
ala b
long b
plum a
bon -
NS
B
Nectar concentration (%solids)
60
50
40
30
20
10
0
mut a
lang a
forg a
Rast a
ala b
long b
plum ab
bon -
NS
NS
NS
0
12
24
Time after corolla opening (h)

Figures 4. Nectar volume and concentration at approximately 24 hours 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 numbers 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 hour only]). A) Nectar volume values for all species. B) Nectar concentration values for all species. (*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, HB = hummingbird-pollinated, HM = hawkmoth-visited, self = autogamous selfer, SM = small moth-pollinated)

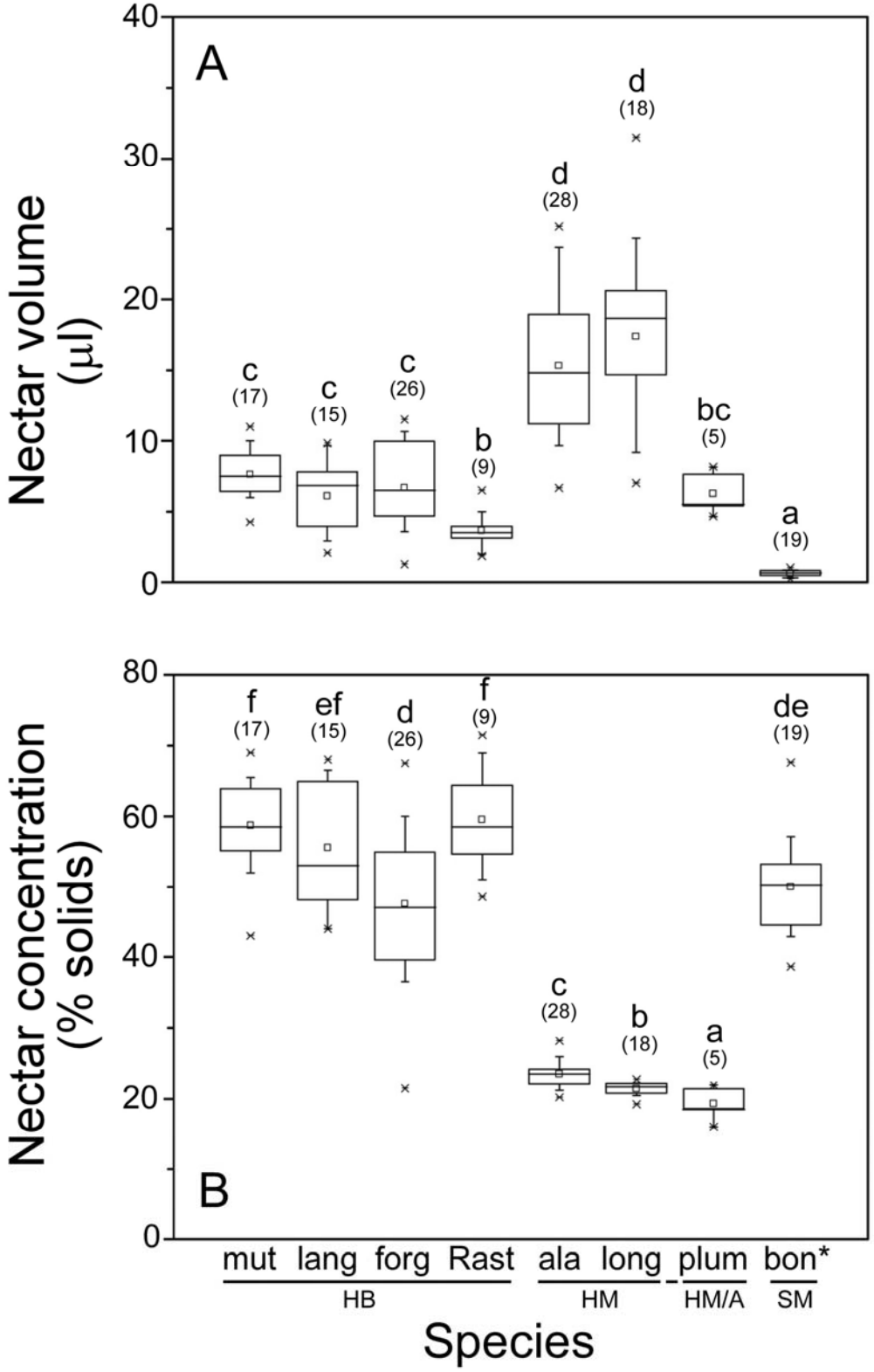

A
Nectar volume (µl)
0
10
20
30
40
c (17)
c (15)
c (26)
b (9)
d (28)
d (18)
bc (5)
a (19)
B
Nectar concentration (% solids)
0
20
40
60
80
f (17)
ef (15)
d (26)
f (9)
c (28)
b (18)
a (5)
de (19)
mut
lang
forg
Rast
ala
long
plum
bon*
HB
HM
HM/A
SM
Species

Figure 5. Total energy at approximately 24 hours after corolla opening. Total energy was calculated by multiplying the average volume for each plant by its concentration (converted to wt/vol.). 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 hour only]). (*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.)

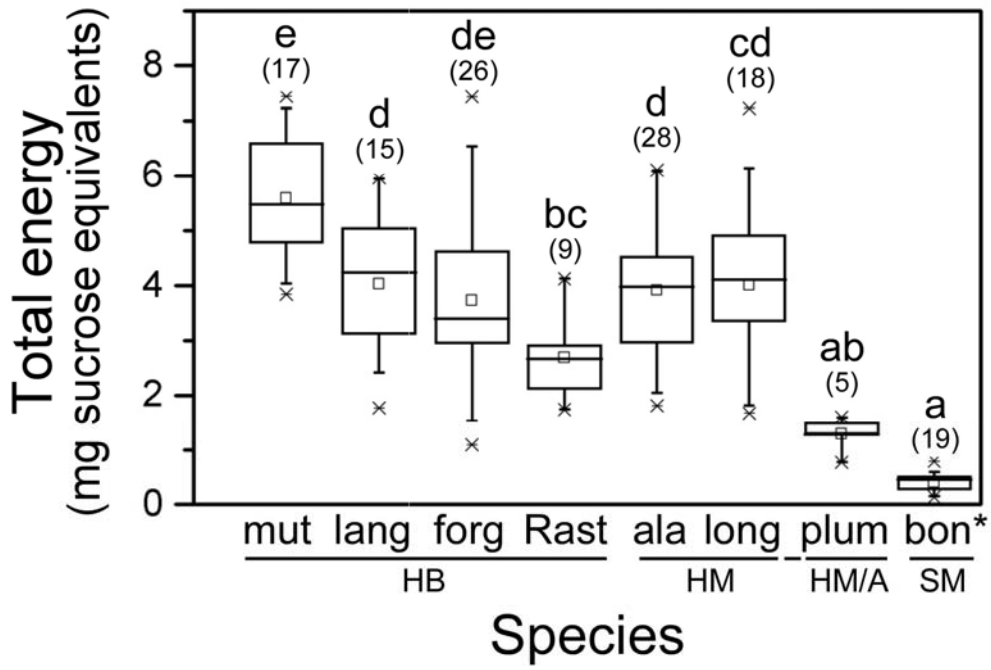

Total energy
(mg sucrose equivalents)
0
2
4
6
8
e
(17)
d
(15)
de
(26)
bc
(9)
d
(28)
cd
(18)
ab
(5)
a
(19)
mut
lang
forg
Rast
ala
long
plum
bon*
HB
HM
HM/A
SM
Species

Figure 6. Nectar volume, concentration, and energy as a function of corolla length. Species symbols correspond to those in Fig. 3, 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.

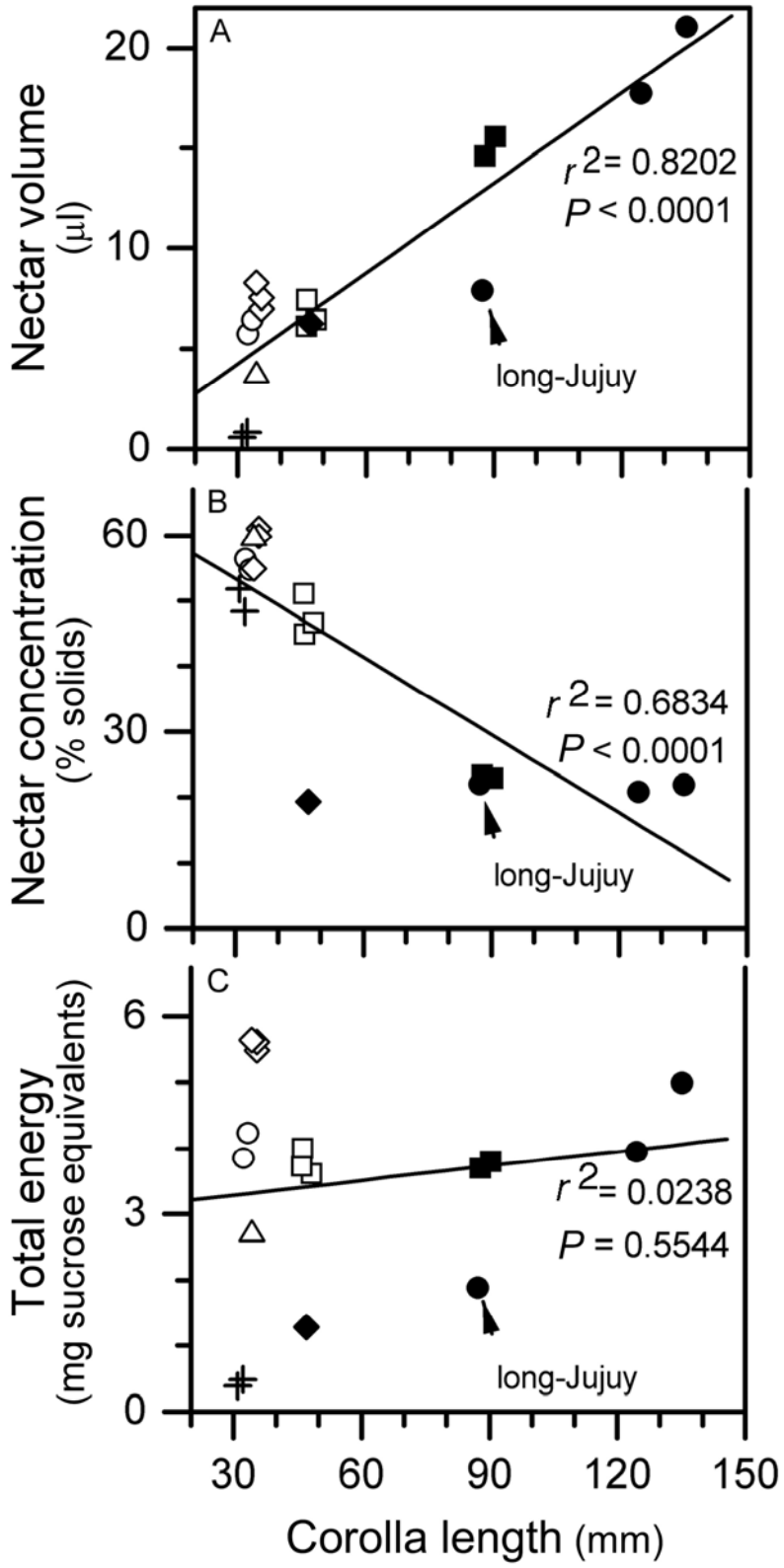

A
Nectar volume
(µl)
20
10
0
r^2= 0.8202
P < 0.0001
long-Jujuy
B
Nectar concentration
(% solids)
60
30
0
r^2= 0.6834
P < 0.0001
long-Jujuy
C
Total energy
(mg sucrose equivalents)
6
3
0
r^2= 0.0238
P = 0.5544
long-Jujuy
30
60
90
120
150
Corolla length (mm)

Figure 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 $\alpha = 0.01$). Six samples were collected from each species. (*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.)

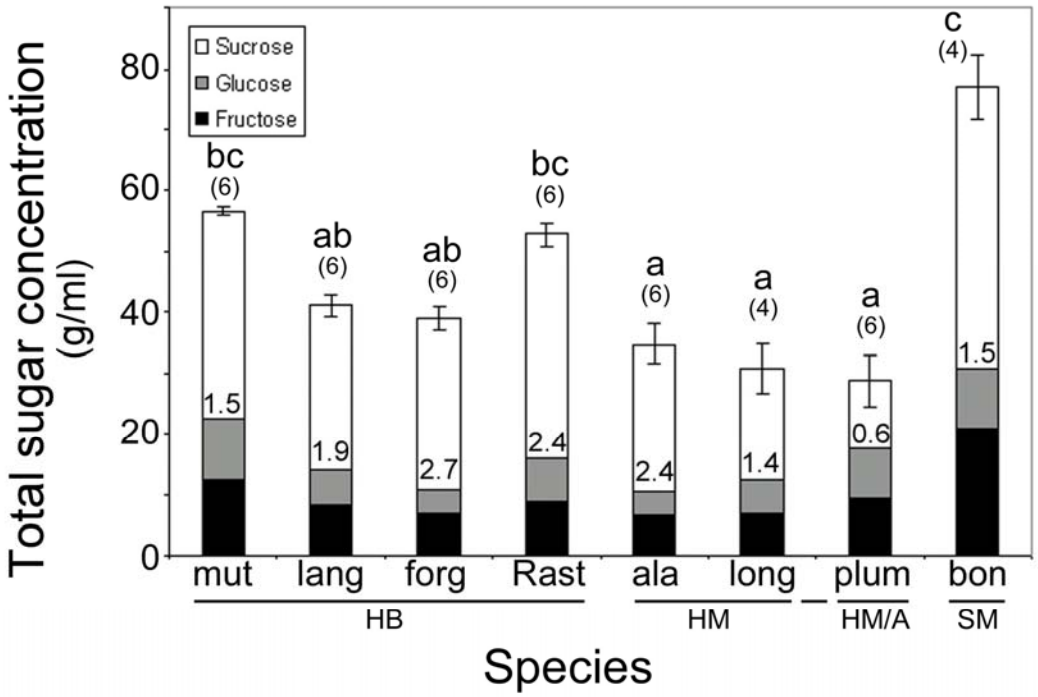
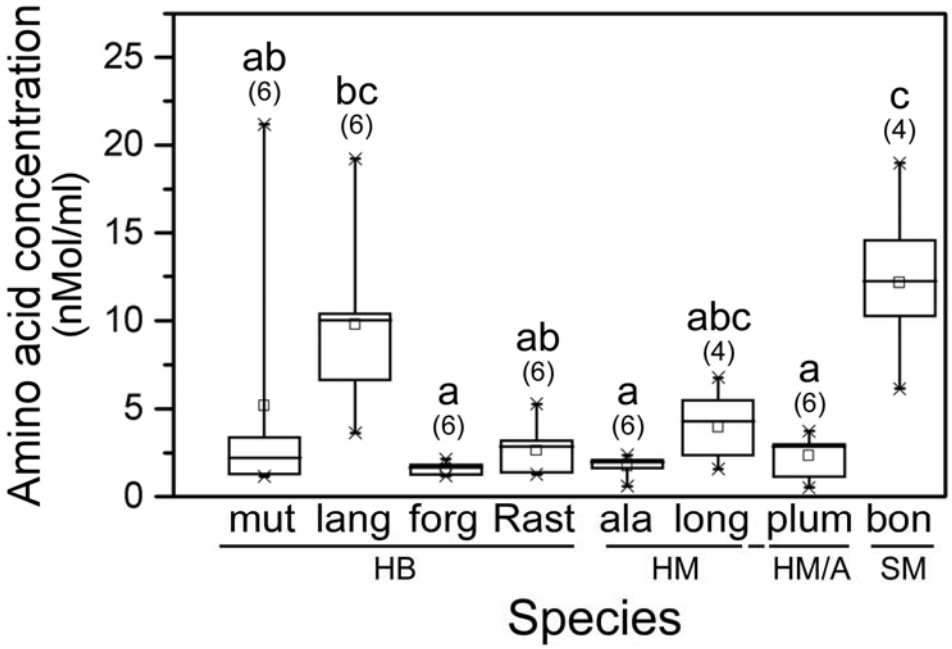

Total sugar concentration
(g/ml)
Sucrose
Glucose
Fructose
0
20
40
60
80
bc
(6)
ab
(6)
ab
(6)
bc
(6)
a
(6)
a
(4)
a
(6)
c
(4)
1.5
1.9
2.7
2.4
2.4
1.4
0.6
1.5
mut
lang
forg
Rast
ala
long
plum
bon
HB
HM
HM/A
SM
Species

Figure 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 α = 0.01) among species. Six samples were collected from each species. (*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.)


Amino acid concentration
(nMol/ml)
25
20
15
10
5
0
ab
(6)
bc
(6)
a
(6)
ab
(6)
a
(6)
abc
(4)
a
(6)
c
(4)
mut
lang
forg
Rast
ala
long
plum
bon
HB
HM
HM/A
SM
Species

CHAPTER 3

NECTAR TRAITS OF PLANTS FROM NATURAL POPULATIONS OF *NICOTIANA* SPECIES AND THE COMPARISON OF GREENHOUSE- AND FIELD-GROWN PLANTS

ABSTRACT

Nine populations of five different *Nicotiana* species in 2004 and one population of a putative new species in 2001 were studied to characterize nectar traits over the first day after anthesis. Nectar traits were measured at the onset of anthesis and 24 h later from flowers that were either bagged or exposed to floral visitors. Significant effects of time and bagging were detected in some populations. The difference between bagged and exposed flowers at 24 h after anthesis was used as an estimate of the amount of nectar removed by floral visitors. Most populations had significantly less nectar in flowers exposed to visitors compared to bagged flowers. Nectar traits of bagged flowers were also compared to previously documented greenhouse data (Kaczorowski et al., 2005). Naturally-growing plants had significantly lower nectar concentration when compared to greenhouse-grown plants from the same population. Nectar traits were also characterized for all flowers on two to three plants from most of the natural populations, where floral position and ecological variables (robbing, florivores, and wilted flowers) were also documented. There were some effects of position and ecological variables detected in nectar traits. Additionally, a flower color-changing species exhibited a significant change in nectar volume and energy content between color phases. This study demonstrates the

context dependence of nectar traits and, accordingly, the importance of field studies to understand what pollinators are likely to experience when visiting plants.

INTRODUCTION

Nectar is considered the most common and important floral reward for pollinators (Simpson and Neff, 1983), and therefore has the potential to greatly affect pollination. Variation in nectar traits can be considerable, even when plants are grown in the controlled environment of a greenhouse (Vickery and Sutherland, 1994; Kaczorowski et al., 2005). In natural populations, extensive variation in nectar traits is found both within and among plants (see Rathcke, 1992). A large part of the observed variation in nectar traits of naturally-growing plants is likely due to the high degree of environmental variation that is typically encountered in the field (Zimmerman and Pyke, 1988; Wyatt et al., 1992). However, part of the variation may also be a result of intrinsic plant factors (Pleasants and Chaplin, 1983; Hodges, 1993). Variation in nectar traits can affect pollinator behavior, as higher nectar volumes or higher energetic rewards tend to increase pollinator visitation frequency or duration to individual plants, inflorescences, or flowers (Zimmerman, 1983; Galen and Plowright, 1985; Thomson, 1988; Cresswell, 1990; Neff and Simpson, 1990; Mitchell, 1993). On the other hand, pollinators and other floral visitors themselves can also add to the variation in nectar traits in the field.

Pollinators and floral antagonists, such as nectar robbers or florivores, can modify nectar traits directly or through selective pressure, possibly in conflicting directions (Adler and Bronstein, 2004; Ordano and Ornelas, 2004). The composition of nectar components (i.e., amino acids) can be altered by floral visitors through direct contact with

nectar, by dislodging pollen into the nectar, or by physically damaging floral tissues, which could release cellular contents into the nectar (Willmer, 1980). Nectar concentration can be modified by nectar robbers; robbed flowers may have higher nectar concentrations, likely due to accelerated evaporation from the hole produced by the robber (Pleasants, 1983; Irwin and Brody, 1998), or lower concentration, likely due to additional secretion of nectar with less sugar (Arizmendi et al., 1996). But perhaps the most obvious effect of floral visitors on nectar is the depletion of nectar resources. Nectar volumes may be reduced legitimately by pollinators or illegitimately by nectar robbers; any reduction in nectar volume has the potential to stimulate additional nectar secretion in certain species (Ordano and Ornelas, 2004) and alter the nectar presentation to subsequent floral visitors.

The standing crop, or quantity of nectar or nectar sugar in a flower at a given time, varies as a result of nectar production rate differences and depletion of nectar by floral visitors (Zimmerman, 1988). Standing crop values frequently change while visitors continue to deplete nectar resources. Therefore, many pollinators may experience standing crops that are significantly lower than the total amount of nectar produced because other floral visitors deplete the available nectar (Itino et al., 1991; Mallick, 2000; McDade and Weeks, 2004b). It has been argued that standing crop may be more ecologically important to pollinators than the genetic potential for nectar production because it reflects nectar quantities they actually encounter during foraging (McDade and Weeks, 2004b); however, standing crops are likely to vary significantly across space and time. Standing crops are significantly correlated with nectar production rate soon after anthesis, but that correlation weakens as floral visitors continue to forage (Zimmerman,

1988; McDade and Weeks, 2004b). Standing crop has been shown to vary in relation to time of day, time of season, ambient temperature, nectar robbing, and flower position (see Zimmerman, 1988; Rathcke, 1992). Many studies of flower position effects on nectar traits have demonstrated nectar gradients on plants with simple inflorescences of spikes and racemes (Percival and Morgan, 1965; Pyke, 1978; Corbet et al., 1981; Waddington, 1981; Best and Bierzychudek, 1981; Pyke, 1982; Haddock and Chaplin, 1982; Galen and Plowright, 1985; Hodges, 1985; Devlin et al., 1987).

Many nectar traits are phenotypically correlated (Mitchell and Shaw, 1993; Campbell, 1996; Klinkhamer and van der Veen-van Wijk, 1999) and show additional correlations with morphological traits (Plowright, 1987; Duffield et al., 1993; Mitchell and Shaw, 1993; Campbell, 1996; Davis, 1997; Klinkhamer and van der Veen-van Wijk, 1999; Kaczorowski et al., 2005). Morphological traits may be important in the aid or restriction of foraging by certain pollinators (Campbell et al., 1996; Lange et al., 2000; Ree, 2005; Darrault and Schlindwein, 2005). Therefore, morphological traits could have more selection pressure placed on them than nectar traits. Phenotypic correlations can result from significant genetic or environmental correlations. Genetic correlations usually result from pleiotropy, where one gene affects multiple traits. Pleiotropic size effects are often exhibited in morphological traits (Conner, 1997; Juenger et al., 2000) and could have an effect on nectar traits, i.e. larger flowers may have larger nectaries that can produce more nectar or more sugar. Also, the indirect selection of nectar traits can be constrained or facilitated through negative or positive genetic correlations with morphological traits. Environmental correlations approximate the extent to which different traits respond to the same environmental conditions. Certain morphological

factors, such as floral size and shape, could influence the extent to which environmental conditions affect nectar traits by changing the microenvironment surrounding the nectar. Although only phenotypic correlations can be estimated in this study, I did investigate genetic correlations in conjunction with the heritability of morphological and nectar traits in a separate study (chapter 4).

I conducted a previous study in the greenhouse to investigate the variation of nectar traits within *Nicotiana* section *Alatae* in relation to pollinators and mating system. I found that most nectar traits were more similar between species that had the same predominant pollinator than between species that had different predominant pollinators (Kaczorowski et al., 2005). This pattern could have resulted from past pollinator selection such that nectar traits of the species within *Alatae* are more or less matched with pollinator preferences. But nectar traits could have evolved in association with other floral traits, with or without selection by pollinators. The greenhouse study also found that flower size was significantly correlated with nectar volume (positively) and nectar concentration (negatively), but not total energy content (Kaczorowski et al., 2005). These significant correlations could affect the indirect selection pressure on nectar traits if there is a significant genetic component. However, variable environmental conditions in the field could increase the variation observed in nectar traits and mask any underlying genetic correlations. Therefore, field measurements are critical for understanding how the environment and pollinator activity affect nectar and other floral traits in natural populations and to verify the accuracy of greenhouse data. However, I found only one other study that compared nectar traits from wild and greenhouse populations (Vickery and Sutherland, 1994).

This study describes natural nectar variation in 9 wild populations of 5 species within *Nicotiana* section *Alatae* in Southern Brazil and compares the nectar traits from plants growing in these natural populations to those grown in the greenhouse. The objectives of this study were to 1) determine distributions of nectar traits (volume, concentration, and energy content [total sugar]) for plants growing in natural populations and assess how these nectar traits change over the first day a flower is open, 2) compare nectar traits of flowers protected from floral visitors to those exposed to floral visitors to estimate the amount of nectar removed by pollinators, 3) determine whether there are any significant phenotypic correlations between nectar and morphological traits in plants growing in natural populations, 4) compare the nectar traits of plants growing in natural populations to greenhouse-grown plants from the same populations, 5) assess nectar traits of whole plants (i.e., standing crop) and determine whether positional and ecological factors affect those nectar traits, and 6) determine the correlation between color phase and nectar traits in a flower color-changing species, *N. mutabilis*.

MATERIALS AND METHODS

Study system

Nicotiana section *Alatae* is a monophyletic group (Ippolito, 2000; Chase et al., 2003; Knapp et al., 2004) composed of seven named species, from which five species were included in this study. *Nicotiana alata* Link and Otto, *N. forgetiana* hort. ex Hemsl., *N. langsdorffii* Schrank, *N. bonariensis* Lehm., and *N. mutabilis* Stehmann and Semir (Stehmann et al., 2002) are all self-incompatible and native to southeastern Brazil, Paraguay, Uruguay and eastern Argentina (Goodspeed, 1954), and are often found in

disturbed areas along roadsides, along the banks of rocky streams, or rocky outcrops and cliff crevices. Hawkmoths (Sphingidae) are the predominant pollinators of *Nicotiana alata*, small perching moths (Noctuidae) are the presumed primary pollinators of *N. bonariensis* (few such moths, nor any other pollinator, were observed), and hummingbirds (Trochilidae) are the predominant pollinators of the remaining three species (Kaczorowski et al., 2005). A putative new species (hummingbird-pollinated) within this section, "Rastroensis", was found in 2004, only in low abundance and at inaccessible heights in the canyon wall and so was not included in the analyses of the 2004 data. However, I have similar data on this population from 2001 and present it here separately from the other data. In 2004, nine populations (abbreviations of which can be found in Table 1 and population locations in Fig. 1) of five species from *Nicotiana* section *Alatae* were investigated in natural populations in southern Brazil (Table 1). All populations in this study were allopatric; even the populations of the two species (*N. langsdorffii* and *N. bonariensis*) at Morro da Igreja were separated by about 500 m in elevation. The five species, along with Rastroensis, comprise a monophyletic clade within section *Alatae* (Ippolito, 2000; Chase et al., 2003; Knapp et al., 2004).

Nicotiana mutabilis is a newly described species that has only been located in the Serra do Umbu in the northeast region of Rio Grande do Sul, Brazil, at altitudes of 700-900 m (Stehmann et al., 2002). The corollas of the flowers are white in bud and at anthesis, but gradually become pinker with age, until flowers are a deep magenta at abscission. In this study, I recognize three color variations (white, light pink, and dark pink). Flowers are white only for the first day after anthesis. By the second day after anthesis, flowers are light pink, and may remain so for another day. Flowers tend to be

darker pink by the third or fourth day after anthesis (R. Kaczorowski, pers. obs.). Preliminary observation suggests hummingbirds prefer new, white flowers over older, dark pink flowers (R. Kaczorowski and A. Ippolito, pers. obs.), but this preference remains to be experimentally tested.

Natural population data

The survey of nectar traits within natural populations for both 2004 and 2001 (Rastroensis only) included the sampling of flowers at approximately the time of anthesis (or bud opening, 0 h, based on observation at each population but similar to times determined from a greenhouse-based daily phenology study; Kaczorowski et al., 2005) and approximately 24 h later. In addition, for most plants sampling included flowers with and without bags to compare nectar from flowers protected from floral visitors to those exposed to floral visitors at both time periods.

In each population, 7-18 plants, from which 2-30 flowers per plant, were sampled in one of four treatments (0 h bagged, 0 h exposed, 24 h bagged, 24 h exposed), providing a total of 40-175 flowers per population. To prevent nectar removal by floral visitors, bagged flowers were covered with small plastic bags the day before anthesis (mesh bags used only in the 2001 Rastroensis sampling) or the same day as anthesis, but well before the flowers opened. Most flowers were bagged on the first day of sampling, although flowers in the *N. mutabilis* and *N. langsdorffii* populations were bagged the day prior. If bagged flowers did not open when expected, sampling was postponed until the day of their anthesis; therefore all populations likely had flowers bagged the day prior to anthesis. Flower sampling began within a few hours of anthesis (0 h) and approximately 24 hours later and sampling periods lasted between 1-5 hours. Upon sampling, exposed

flowers were matched by age and size to those that were bagged and subsequently sampled. In most cases, age-matching was performed by tagging exposed flowers that were close to anthesis while the other flowers were bagged, but in a few cases age-matching involved estimating the age at the time of sampling based on position and appearance relative to bagged flowers. Exposed flowers were only sampled at 24 h after anthesis in the 2001 Rastroensis population, on the assumption that there was little difference between bagged and exposed flowers at the time of anthesis. Based upon the results from other species sampled in 2004, this assumption was justified, but it restricted my ability to include interactions of bagging and time in the analysis.

As sample flowers were removed from the plant, the time (within a sampling period) was recorded and morphological measurements (corolla tube length, limb width, mouth diameter) were taken using calipers. Then each flower was destructively sampled for nectar volume and concentration measurements, making it necessary to use different flowers for the different time periods. 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; therefore, dissection of flowers was necessary to collect the trace amounts of nectar along the corolla tube. Nectar was collected and the volume estimated with glass micropipette tubes (50 µl [10 µl for *N. bonariensis*] Drummond "Microcaps"). Concentration measurements were estimated as the percentage of solids in solution (calibrated as sucrose equivalents [sucEq]) using a temperature-compensated refractometer. Dilutions were performed as necessary to keep the concentration readings within the range of the refractometer (0 – 50% solids). Total energy content was

calculated as the product of nectar volume and concentration, which was converted from weight/weight to weight/volume, as suggested by Bolton et al. (1979).

Statistical analysis

I used population, time after anthesis (0 and 24 h), and presence of bags as independent variables, in addition to their interactions, to analyze nectar volume, concentration, and energy content, and corolla tube length, limb width, and mouth diameter (PROC GLM in SAS 9.1 [SAS Institute Inc., Cary, NC, USA]). I also included time within sampling period and daily precipitation conditions (coded as 1 = dry, 2 = wet, 3 = rainy) as covariates in the model. *Nicotiana langsdorffii* was represented by only one population, so a population nested within species design could not be used; therefore species and population were concatenated into one independent variable. Rastroensis was analyzed similarly by itself, including only time and presence of bags as independent variables. Interactions of time and presence of bags could not be included in the model for Rastroensis because flowers were only bagged to be sampled at 24 h after anthesis. Because variances were unequal and did not equalize with various transformations, the data were ranked prior to analysis (PROC RANK in SAS 9.1). I 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. Contrast statements compared only data for bagged flowers within the groupings created. General differences between populations (all data included) were determined using Fisher's Least Significant Difference (LSD with means statement in SAS 9.1). Comparisons across different times and bagging treatments within populations were determined using least square means (lsmeans in SAS 9.1). Because multiple tests were

being performed, a more rigorous alpha of 0.01 was chosen to reduce type II errors, as opposed to performing Bonferroni corrections, which can illogically mask relevant results (Moran, 2003).

An estimation of the amount of nectar removed by floral visitors was calculated by subtracting the mean volume of nectar in exposed flowers from the mean volume of nectar in bagged flowers per population at 24 h after anthesis. The difference between these means was determined through least square means, as stated above (comparison among bagging treatments within 24 h after anthesis). A significant difference between bagged and exposed flowers at anthesis, most likely due to early bagging, required the *N. mutabilis* data to be adjusted before the estimation could be made. Data were adjusted by subtracting the mean difference in nectar volume between bagged and exposed flowers at anthesis from the volume of each flower sampled at 24 h after anthesis. The means and standard errors for the *N. mutabilis* populations were determined from the adjusted data. Significance for the amount of nectar removed in *N. mutabilis* was determined through two-sample t-tests (assuming unequal variances, in Microsoft Excel 2002). An alpha of 0.05 was retained for these a priori comparisons of special interest.

Comparison with greenhouse data

The nectar traits of plants in natural populations were compared to previously reported nectar traits from greenhouse-grown plants from the same populations (Kaczorowski et al., 2005). In natural populations, nectar volume and concentration were recorded for each flower while an average nectar volume was recorded for the multiple flowers needed to obtain a single concentration measurement per plant in the greenhouse. Therefore, the per-flower natural population data were averaged over each plant before

the data sets were combined. Only bagged flower measurements from the natural populations were included to match the greenhouse conditions, which effectively excludes pollinators.

Statistical analysis

I used environment (greenhouse or natural population), population, and time after anthesis (0 h and 24 h) as independent variables, in addition to their interactions, to analyze nectar volume, concentration, and energy content (PROC GLM in SAS 9.1). Rastroensis was analyzed similarly by itself, including only environment and time after anthesis, and their interaction, as independent variables. Comparisons were determined using post-hoc on ranked data (lsmeans and PROC RANK in SAS 9.1), and the more rigorous alpha of 0.01 was used to reduce the risk of Type II errors.

Trait correlations

Traits were averaged over plants by time and bagging treatment, within each population. Pearson correlation coefficients were determined for each trait combination at both time periods, using bagged flowers only (PROC CORR, SAS 9.1) to avoid the effects of nectar removal by floral visitors. An alpha of 0.01 was used to determine significance of the correlations.

Whole-plant standing crop

The standing crop for all flowers on a plant was estimated for two to three plants at peak bloom from eight of the nine populations, where all flowers from a plant were removed at one time and sampled for nectar volume and concentration, from which total energy content was calculated. The SM population of *N. forgetiana* was excluded because the population was mowed before it could be sampled again. Plants with

relatively few (as few as 7) and many (up to 130) flowers were selected to evaluate a range of plant sizes. The nectar volumes and concentrations were measured and recorded as described previously, along with the location of the flower on the plant (see Fig. 2: order of the branch and an approximation of the distance from the top of the plant by quartiles [determined by dividing each secondary branch by the total number of secondary branches and partitioning the plant into four sections along the primary branch]) and any other floral details deemed pertinent to nectar characteristics, including flower color. Of the floral details noted, I determined three variables to be present at a reasonable frequency and variable across populations, including the presence of holes or slits on the corolla tube (evidence of nectar robbing), larvae or bugs within the corolla tube (potential florivores), or signs of old age (wilted flowers retained on the plant). These ecological variables were analyzed to determine their correlation to each other (PROC CORR in SAS 9.1) and their variation among populations (PROC ANOVA with Fisher's LSD test used to determine post-hoc comparisons [$\alpha = 0.05$] in SAS 9.1).

Whole-plant standing crop analysis

Population and plant were used to analyze the total amount of nectar, the average nectar concentration, and the total energy content across the whole plant (PROC GLM in SAS 9.1). The number of open flowers on the plant was used as a covariate. Residuals were distributed normally, therefore the data were not ranked, and an alpha of 0.05 was retained for the whole-plant nectar analysis. The correlation between open flowers per plant and nectar traits was estimated by plotting nectar traits as a function of flower number and testing the linear fit of the regressions (in Origin 6.0, Microcal Software Inc., Northhampton, MA, USA)

Nectar trait per flower (positional) analyses

Because not all branch orders were present within each quartile, including both branch order and quartile as independent variables resulted in a model that was not full-rank; therefore two different models were used to analyze the effects of branch order and distance from the top of the plant by quartile. I used population, along with either branch order or quartile, as independent variables in two different models, in addition to the interactions, to analyze nectar volume, concentration, and energy content per flower, with plant defined as the experimental unit (PROC MIXED in SAS 9.1). I also included the ecological variables of holes or slits, larvae or bugs, or old age (coded as 0 or 1) as covariates in both models. Because few plants were large enough to have more than third order branches, only three branch order groups were defined– first order, second order, and third and higher. Because all plants from *N. bonariensis* MdI had only first and second order branches, that population was removed from the analysis of order effects to allow for comparisons among other species. Comparisons were determined using post-hoc tests on raw data (least square means [$\alpha = 0.05$, lsmeans in SAS 9.1]).

Color and nectar correlations in N. mutabilis

Within the *N. mutabilis* populations, flower color was used as an independent variable to analyze nectar volume, concentration, and energy content (PROC GLM in SAS 9.1) and contrasts were determined using post-hoc tests on raw data (least significant difference [$\alpha = 0.05$, lsd lines in SAS 9.1]).

RESULTS

Natural population data

Because there are many effects to consider in the general analysis of the natural population data, results are presented by main effect. Interactions will be presented within the main effect that makes the most sense.

Pollinator group effects

Nicotiana species that had the same primary pollinator were grouped together into what will be subsequently referred to as "pollinator groups" to compare the differences of nectar traits among *Nicotiana* species with different pollinators. Pollinator group contrasts found that all three pollinator groups were significantly different from each other ($P < 0.01$) in nectar volume and energy content, but not in nectar concentration (Table 2). The hummingbird-pollinated group (3 species, 2001 Rastroensis not included) had significantly more nectar volume and energy content than the single hawkmoth-pollinated species, which had significantly more nectar volume and energy content than the single small-moth pollinated species. The hawkmoth-pollinated group had significantly lower nectar concentrations than the hummingbird-pollinated group and the small moth-pollinated group, which were not significantly different from each other ($F_{1,812} = 0.13$, P = 0.7195). The pollination group contrast between hawkmoth- and small moth-pollinated groups is the same as the species contrast between *N. alata* and *N. bonariensis*, where they are significantly different from each other in all nectar traits. Interpretation of the results of post-hoc contrasts on pollinator groups is given with caution, due to the fact that these contrasts are unbalanced because only the hummingbird-pollinated group had more than one representative in this study.

Plant species effects

Most among-species contrasts were significantly different (at $\alpha = 0.01$) for all nectar traits, although a few species grouped together with similar nectar volume, concentration, and/or energy content (Table 3). When only bagged flowers were considered, *N. langsdorffii* was not significantly different from *N. alata* in any of the nectar traits, while it was not significantly different from *N. mutabilis* in nectar volume. *Nicotiana bonariensis* was not significantly different from *N. forgetiana* in nectar concentration.

Population effects

Even with only two populations within each of four species, there was a significant population effect for nectar volume, concentration and energy content ($P < 0.0001$; $F_{8,881} = 62.29$, $F_{8,812} = 40.81$, $F_{8,812} = 38.06$, respectively), when all data were included (Table 4), but this is confounded with interspecific differences. Post-hoc tests (LSD) showed only *N. mutabilis* populations were significantly different from each other (at $\alpha = 0.01$) across times and treatments for all nectar traits. However, *N. alata* populations were significantly different from each other in nectar concentration and *N. bonariensis* populations were significantly different from each other in nectar concentration and energy content. Some populations exhibited similar nectar traits to populations of other species (see Table 4).

When considering only bagged flowers, separated by time, population effects were different than those found when all data was considered. There were few significant differences (at $\alpha = 0.01$) in nectar traits among populations of the same species (Fig. 3). Nectar volume was not significantly different among populations within the same species, except for the *N. alata* populations at 0 h after anthesis (Fig. 3, A & D). Nectar concentration was not significantly different among populations within the same species,

except for the *N. alata* populations at 0 h after anthesis and the *N. bonariensis* populations at 0 and 24 h after anthesis (Fig. 3, B & E). Nectar energy content was not significantly different among any populations within a species at either 0 or 24 h after anthesis (Fig. 3, C & F). Only one population of *N. langsdorffii* was sampled, and it tended to be most similar in all nectar traits to one or both populations of *N. forgetiana* (a similarly pollinated species by hummingbirds) and/or *N. alata* (a differently pollinated species by hawkmoths).

Daily precipitation effects

All nectar traits of 2004 populations were significantly affected by the degree of precipitation the plants endured during each sampling day (see Table 1 for daily conditions; volume: $F_{1,879} = 8.20$, $P = 0.0043$; concentration: $F_{1,810} = 43.19$, $P < 0.0001$; energy: $F_{1,810} = 21.91$, $P < 0.0001$). Nectar traits were also significantly affected by precipitation within the 2001 Rastroensis population, although only for nectar volume unless the less rigorous α of 0.05 is retained (volume: $F_{1,33} = 8.78$, $P = 0.0056$; concentration: $F_{1,26} = 4.87$, $P = 0.0364$; energy: $F_{1,26} = 6.49$, $P = 0.0171$).

Time effects

Nectar traits of 2004 populations were not significantly affected by the time flowers were sampled within a sampling period (volume: $F_{1,879} = 2.44$, $P = 0.1188$; concentration: $F_{1,810} = 0.24$, $P = 0.6221$; energy: $F_{1,810} = 1.20$, $P = 0.2731$), nor were those of the 2001 Rastroensis population (volume: $F_{1,33} = 1.04$, $P = 0.3161$; concentration: $F_{1,26} = 1.22$, $P = 0.2794$; energy: $F_{1,26} = 0.25$, $P = 0.6194$). However, there were some significant effects of time after anthesis (or interactions involving time after anthesis) for

all nectar traits of 2004 populations. Therefore, all time effects presented and discussed hereafter will be in relation to time after anthesis.

Nectar volume was significantly affected by time after anthesis ($F_{1,879} = 18.38$, $P < 0.0001$), and there was also a significant population by time interaction ($F_{8,879} = 8.33$, $P < 0.0001$). All populations exhibited a general increase over time in nectar volume for bagged flowers, although post-hoc tests found significant ($\alpha = 0.01$) increases in only five (*N. bonariensis* ST, *N. mutabilis* BdO, *N. forgetiana* SM and both *N. alata* populations) of the nine populations (Fig. 4, Table 5: 24hB v 0hB). There was no apparent trend for changes in nectar volume over time in exposed flowers. The two populations that exhibited significant changes in nectar volume for exposed flowers had changes in opposite directions (*N. alata* SB had a significant increase, while *N. forgetiana* CdS had a significant decrease in nectar volume over time; Fig. 4, Table 5: 24hE v 0hE).

Nectar concentration was not significantly affected by time after anthesis ($F_{1,810} = 0.00$, $P = 0.9599$), although there was a significant population by time interaction ($F_{8,810} = 6.29$, $P < 0.0001$). This is due to only one (*N. forgetiana* SM) of the nine populations showing a significant decrease in the nectar concentration of bagged flowers, while bagged flowers in other populations did not significantly change in nectar concentration over time (Fig. 4, Table 5: 24hB v 0hB). Only two (*N. forgetiana* SM and *N. mutabilis* BdO) of the nine populations exhibited a significant decrease in the nectar concentration of exposed flowers over time (Fig. 4, Table 5: 24hE v 0hE).

Total energy content was significantly affected by time after anthesis ($F_{1,810} = 7.41$, $P = 0.0066$), and there was also a significant population by time interaction ($F_{8,810} = 4.49$, $P < 0.0001$). All populations tended to increase in total energy content in bagged

flowers, although only three (*N. bonariensis* ST, *N. forgetiana* SM and *N. alata* RP) of the nine populations had a significant increase in energy content over time (Table 5: 24hB v 0hB). There was no apparent trend for changes in nectar energy content over time in exposed flowers. The two populations that exhibited significant changes in nectar energy content for exposed flowers had changes in opposite directions (*N. alata* SB had a significant increase, while *N. forgetiana* CdS had a significant decrease in nectar energy content over time (Table 5: 24hE v 0hE).

Overall, time after anthesis did not significantly affect nectar traits within the 2001 Rastroensis population (volume: $F_{1,33} = 0.02$, $P = 0.8958$; concentration: $F_{1,26} = 1.42$, $P = 0.2438$; energy: $F_{1,26} = 6.01$, $P = 0.0212$) under the full model. However, if the covariates are left out of the model, all nectar traits were significantly affected by time after anthesis (volume: $F_{1,35} = 12.89$, $P = 0.0010$; concentration: $F_{1,28} = 25.62$, $P < 0.0001$; energy: $F_{1,28} = 55.02$, $P < 0.0001$). All nectar traits increased over time (Table 6; natural population means).

Bagging effects

Bagging flowers allows nectar to accumulate by restricting nectar removal by pollinators. There was a significant increase in nectar volume over time with the presence of bags ($F_{1,879} = 63.59$, $P < 0.0001$), but only in six (both of the *N. mutabilis* and *N. forgetiana* populations, the *N. langsdorffii* population, and *N. alata* RP) out of the nine populations ($\alpha = 0.05$; Table 7). This resulted in a significant population by bag effect ($F_{8,879} = 7.89$, $P < 0.0001$), a significant time by bag effect ($F_{1,879} = 8.48$, $P = 0.0037$), and a significant population by time by bag effect for nectar volume ($F_{1,879} = 2.82$, $P = 0.0043$). Nectar volume was not significantly different between bagged and exposed

flowers at approximately the time of anthesis (0 h), except that bagged flowers had significantly more nectar than exposed flowers in both *N. mutabilis* populations (Fig 2, Table 5: 0hB v 0hE). Some flowers in these populations were bagged the day prior to sampling, and an approximate anthesis time to begin sampling the following day was determined. However, this species exhibited the most variation in anthesis time in the greenhouse (Kaczorowski et al., 2005) and environmental conditions also contribute to variation in anthesis time. Therefore, all flowers were already open by the time they were sampled and the accumulated nectar that was present in exposed flowers at the time of anthesis (Table 6) was likely to have been depleted from some flowers by floral visitors before I had the opportunity to sample them. The *N. bonariensis* populations and the *N. alata* SB population had no significant differences in nectar volume (at $\alpha = 0.05$) between bagged and exposed flowers at 24 h after anthesis (Fig. 4, Table 7).

Bagging flowers did not affect nectar concentration overall ($F_{1,810} = 1.36$, $P = 0.2431$). However, bagged flowers had significantly higher nectar concentrations than exposed flowers at 24 h after anthesis in the *N. forgetiana* SM population (Fig. 4, Table 5: 24hB v 24hE).

There was a significant increase in total energy content with the presence of bags ($F_{1,810} = 39.94$, $P < 0.0001$), but only in six (the same populations as in nectar volume, from Table 7) out of the nine populations (Table 5: 24hB v 24hE), resulting in a significant population by bag effect ($F_{8,810} = 5.69$, $P < 0.0001$) for nectar energy content. Nectar energy content was not significantly different between bagged and exposed flowers at approximately the time of anthesis (0 h), except that bagged flowers had more energy content in their nectar than exposed flowers in both *N. mutabilis* populations

because of the difference in nectar volumes for reasons explained previously (Table 5: 0hB v 0hE).

Bagging significantly affected both nectar volume and energy content ($P < 0.0001$; $F_{1,33} = 32.27$, $F_{1,26} = 44.08$, respectively), but not nectar concentration ($F_{1,26} = 4.03$, $P = 0.0551$) in the 2001 Rastroensis population at 24 h after anthesis. Nectar volume and energy content were significantly higher at 24 h after anthesis in bagged flowers than in exposed flowers (Table 6 for bagged flowers [natural population data]; exposed flowers, mean ± SE; volume: 0.72 ± 0.18 µl; energy: 0.42 ± 0.15 mg sucEq). The interaction of bagging and time could not be investigated because exposed flowers were only sampled at 24 h after anthesis in the 2001 Rastroensis population.

Comparison with greenhouse data

A cautionary result with respect to studying interspecific variation in the greenhouse is demonstrated by the significant difference in nectar traits between plants grown in natural populations and greenhouse-grown plants from the same populations (Fig. 5). There was a significant effect of environment (greenhouse or natural population) for nectar volume, concentration, and energy content ($P < 0.0001$; $F_{1,232} = 22.87$, $F_{1,225} = 807.22$, $F_{1,225} = 38.33$, respectively). Greenhouse-grown plants tended to have lower average nectar volumes than naturally-grown plants, although only *N. mutabilis* [QC] and *N. forgetiana* SM had significantly less nectar in the greenhouse-grown plants than in the naturally-grown plants overall. Furthermore, *N. alata* [RP] had significantly more nectar in the greenhouse-grown plants than in the naturally-grown plants (Fig. 5, Table 6). All populations had significantly higher nectar concentrations in the greenhouse-grown plants than in the naturally-grown plants, both overall and for each time comparison (Fig.

5, Table 6). Greenhouse-grown plants also tended to have more total energy content than naturally-grown plants, although only *N. langsdorffii* [MdI], *N. alata* [RP], and *N. forgetiana* CdS had significantly more energy content in greenhouse-grown plants than the naturally-grown plants overall (Fig. 5, Table 6). There was also a significant environment by population effect for all of the nectar traits measured ($P < 0.0001$; volume: $F_{5,232} = 15.58$, concentration: $F_{5,225} = 15.89$, energy: $F_{5,225} = 9.91$) and environment by time effect for concentration and energy content ($F_{1,225} = 26.63$, $P < 0.0001$; $F_{1,225} = 7.20$, $P = 0.0078$, respectively).

For the 2001 Rastroensis population, nectar concentration was the only nectar trait that was significantly different between natural populations and the greenhouse ($F_{1,40} = 86.56$, $P < 0.0001$), while nectar volume and energy content were not significantly different ($F_{1,47} = 1.94$, $P = 0.1700$; $F_{1,40} = 2.65$, $P = 0.1111$, respectively). Nectar concentration was significantly higher in flowers from the greenhouse than it was in flowers from the natural population at the time of anthesis and 24 h later (Table 6).

Trait correlations

There were no significant correlations ($\alpha = 0.01$) between nectar traits and corolla tube length or limb width at either 0 or 24 h after anthesis (Table 8). However, corolla mouth diameter was significantly correlated with nectar volume at 0 and 24 h after anthesis and with total nectar energy content at 24 h after anthesis (Table 8). Additionally, all morphological traits were significantly correlated with each other and total nectar energy content was significantly correlated with nectar volume and concentration. Despite the significant negative correlation between nectar volume and

concentration in the previous greenhouse study (Kaczorowski et al., 2005), they were not significantly correlated with each other at either time period in this study (Fig. 6).

Whole-plant standing crop

Nectar traits across the whole plant

The average nectar concentration across the whole plant was significantly affected by population ($F_{7,9} = 7.13$, $P = 0.0044$), but the total amount of nectar and energy (sugar) presented across the plant was not ($F_{7,10} = 1.90$, $P = 0.1729$; $F_{7,9} = 2.00$, $P = 0.1639$, respectively). However, I sampled non-randomly with respect to flower number, which could have affected the within population error. The total amount of nectar and energy (sugar) across the whole plant was significantly affected by the total number of flowers on the plant ($F_{7,10} = 10.88$, $P = 0.0080$; $F_{7,9} = 12.13$, $P = 0.0069$, respectively), while the average nectar concentration across the plant was not ($F_{7,9} = 2.10$, $P = 0.1810$). Both nectar volume and energy content available across a plant were positively correlated with flower number per plant (Fig. 7, nectar energy content only; $P < 0.0001$; volume: $n = 21$, $r^2 = 0.670$; energy: $n = 20$, $r^2 = 0.684$).

Nectar traits per flower

When branch order and quartile effects were analyzed in different models, both including the ecological variables as covariates, population effects were significant in both the branch order and quartile model for nectar concentration per flower across the whole plant ($F_{6,11} = 5.22$, $P = 0.0090$ and $F_{7,13} = 4.32$, $P = 0.0131$, respectively), and in the quartile model for nectar energy content per flower ($F_{7,12} = 4.32$, $P = 0.0035$). There were no significant population effects in either the branch order or quartile models for nectar volume per flower across the whole plant ($F_{6,11} = 0.53$, $P = 0.7716$ and $F_{7,13} = 2.24$,

$P = 0.0988$, respectively), or in the branch order model of nectar energy content per flower ($F_{6,11} = 0.99$, $P = 0.4762$).

Ecological variables

There was a significant difference among populations in the percentage of flowers with holes or slits ($F_{7,19} = 21.36$, $P < 0.0001$), with larvae or bugs ($F_{7,19} = 5.00$, $P = 0.0075$), or with old, wilted flowers ($F_{7,19} = 14.15$, $P < 0.0001$). Most populations were significantly different from others in the average frequency of one or more ecological variable (Fig. 8). There were also some significant correlations among the ecological variables. The presence of larvae and bugs was significantly negatively correlated with the presence of holes or slits ($r^2 = -0.1225$, $P = 0.0018$) and old, wilted flowers ($r^2 = -0.1036$, $P = 0.0084$), while there was no significant correlation between the presence of holes or slits and old, wilted flowers ($r^2 = -0.0468$, $P = 0.2349$). This suggests that larvae and bugs may avoid old, wilted flowers and robbed flowers, or perhaps robbers avoid flowers with larvae or bugs in them.

All nectar traits were significantly lower overall in the presence of one or more of the recorded ecological variables. Nectar volume per flower was significantly reduced by all ecological variables in both the branch order (holes/slits: $F_{1,561} = 12.97$, $P = 0.0003$; larvae/bugs: $F_{1,561} = 14.84$, $P = 0.0001$; old age: $F_{1,561} = 12.71$, $P = 0.0004$) and quartile model (holes/slits: $F_{1,564} = 12.12$, $P = 0.0005$; larvae/bugs: $F_{1,564} = 10.03$, $P = 0.0016$; old age: $F_{1,564} = 10.81$, $P = 0.0011$). Nectar concentration was significantly lower in the presence of bugs or larvae in both the branch order and quartile model ($P < 0.0001$; $F_{1,375} = 34.35$, $F_{1,362} = 30.83$, respectively). Nectar energy content was significantly reduced overall in the presence of holes or slits in both the branch order and quartile model ($F_{1,375}$

= 11.39, $P = 0.0008$; $F_{1,362} = 8.64$, $P = 0.0035$, respectively) and the presence of bugs or larvae only in the branch order model ($F_{1,375} = 4.78$, $P = 0.0294$).

Nectar traits were differentially expressed among populations in the presence of the ecological variables (Table 9). In the presence of nectar robbing (holes/slits), flowers in the *N. forgetiana* and *N. mutabilis* QC populations had both significantly reduced nectar volume and energy content. Additionally, robbed flowers had significantly lower nectar volume in the *N. bonariensis* MdI population and energy content in the *N. langsdorffii* population. However, nectar energy content was significantly higher in flowers with holes or slits in the *N. alata* RP population due to the significantly higher nectar concentration in flowers with holes or slits compared to those that lacked them. Although nectar concentration was not significantly affected overall by the presence of holes or slits, nectar concentration was significantly higher in flowers with holes or slits in the *N. forgetiana* and *N. alata* RP populations and significantly lower in flowers with holes or slits in the *N. langsdorffii* and *N. mutabilis* QC populations. In the presence of larvae or bugs in the corolla, flowers had significantly lower nectar volumes and energy content in the *N. alata* SB population and lower nectar concentration in the *N. mutabilis* BdO and both *N. alata* populations. Significantly lower nectar volumes were found in the older, retained flowers of the *N. forgetiana*, *N. mutabilis* BdO and *N. alata* RP populations. Despite the fact that nectar energy content was significantly affected by the presence of old flowers overall, there were no significant effects detected within any of the populations.

Positional effects

There were no significant differences (based on lsmeans, $\alpha = 0.05$) among populations, branch orders, or their interactions for nectar volume and energy content in the branch order model, but the quartile model detected some significant differences for these nectar traits among populations and among some quartiles within populations. The quartile model found that both *N. bonariensis* populations had significantly less nectar volume per flower than *N. alata* SB and *N. mutabilis* BdO, while *N. bonariensis* MdI had significantly less nectar per flower than *N. langsdorffii* as well. The quartile model also found less nectar energy content per flower in both *N. bonariensis* populations compared to *N. mutabilis* BdO and in *N. bonariensis* ST compared to *N. alata* RP and *N. forgetiana* CdS, while *N. alata* SB had significantly more nectar energy content than all other populations. Among the few significant differences in nectar volume and energy content per flower among quartiles within a population, no positional trends were apparent (data not shown).

There were significant differences among populations in both the branch order and quartile model for nectar concentration, and there were also some significant interactions in the quartile model. In both models, *N. alata* SB had significantly higher nectar concentration per flower than in *N. bonariensis* ST and *N. langsdorffii*, while *N. mutabilis* QC had significantly higher nectar concentration per flower than *N. mutabilis* BdO, *N. forgetiana* CdS, and *N. bonariensis* ST. Additionally, *N. mutabilis* QC had significantly higher nectar concentration per flower than *N. alata* RP and *N. langsdorffii* in the branch order model and significantly higher nectar concentration per flower than *N. bonariensis* MdI in the quartile model. No positional trends were apparent among the few significant quartile differences within a population for nectar concentration.

Flower color effects in N. mutabilis

Within the *N. mutabilis* populations, a significant flower color effect was detected for nectar volume and energy content per flower across a plant ($F_{2,413} = 123.30$, $P < 0.0001$; $F_{2,249} = 18.90$, $P < 0.0001$, respectively), but not nectar concentration ($F_{2,249} = 0.85$, $P = 0.4306$). Newer, white flowers had significantly more nectar than the light pink flowers, which had significantly more nectar than the dark pink flowers (Fig. 9). Nectar energy content was significantly greater in the newer, white flowers than the light pink or dark pink flowers, but there was no significant difference between the light pink and dark pink flowers in total energy content (data not shown).

DISCUSSION

Pollinator group effects

Nicotiana species with different pollinators were found to be significantly different from each other in all nectar traits, except the hummingbird-pollinated group was not significantly different from the small moth-pollinated group in nectar concentration (Table 2). Previous greenhouse experiments found similar results, except nectar energy content was not significantly different between hummingbird- and hawkmoth-pollinated groups, as they were in the field. In the greenhouse, the hawkmoth-pollinated group tended to have a greater amount of less concentrated nectar than the hummingbird- and small moth-pollinated groups, and the small moth-pollinated group had less nectar than hummingbird-pollinated group, but of similar concentration (Kaczorowski et al., 2005). Trends for nectar traits among pollinator groups were similar

in the field to those in the greenhouse, except the hummingbird-pollinated group had more nectar energy content than the only hawkmoth-pollinated species in the field study, *N. alata*. The similarity of nectar energy content between hummingbird- and hawkmoth-pollinated groups in the greenhouse suggested that there may be constraints on the amount of energy a plant could allocate towards nectar rewards. However, the significant difference in nectar energy content in the field does not support this prediction. The environmental variation in the field could have altered nectar traits enough to mask any possible trade-offs, suggesting that if plants have constraints on the potential for nectar rewards, they may not be realized under the environmental variation present in the field. Other factors could also contribute to this difference in energy content between hummingbird- and hawkmoth-pollinated species in the field, such as the inclusion of exposed flowers, in which flowers had been exposed to nectar depletion by floral visitors. Hummingbirds and hawkmoths may have affected nectar traits differently in the exposed flowers. These differences could not be accounted for in these pollinator group contrasts.

The exclusion of *N. longiflora* and *N. plumbaginifolia* from this study reduced the number of species within the hawkmoth-pollinated group to only one representative (*N. alata*), while only one small moth-pollinated species (*N. bonariensis*) is represented in *Nicotiana* section *Alatae*. With only the hummingbird-pollinated group represented by multiple species, post-hoc contrasts on pollinator group could be misleading. Additionally, species within or among pollinator groups are not necessarily independent of each other due to shared phylogenetic history. Conclusions drawn from these results would be strengthened by mapping nectar traits onto a phylogeny (Maddison, 1991; Cunningham et al., 1998; Martins, 1999; Pagel, 1999) to determine whether nectar trait

changes were associated with pollinator shifts. However, the phylogeny of these species is not yet resolved (Ippolito, 2000; Chase et al., 2003; Knapp et al, 2004).

Species effects

For all nectar traits, most species in this study were significantly different from each other overall (Table 3). More species were significantly different from each other in the greenhouse for each of the nectar traits (Kaczorowski et al., 2005). *Nicotiana bonariensis* had a similar nectar concentration to *N. mutabilis* and *N. langsdorffii* in the greenhouse, but their concentrations differed significantly in the field. This was likely due to one of the *N. bonariensis* populations exhibiting significantly higher nectar concentrations than any other species in the field. Also in the field, *N. forgetiana* was significantly different from *N. mutabilis* and *N. langsdorffii* in nectar volume and energy content and different from *N. alata* in energy content, while these differences were not exhibited in the greenhouse. The only two species that had significantly different nectar traits in the greenhouse, but not in the field, was *N. alata* and *N. langsdorffii*. These two species have the greatest size disparity of any of the species, in addition to having different pollinators, so it was interesting that these were the only species to have similarity in all nectar traits in the field. Species within a pollinator group were more similar to each other in the greenhouse than in the field, whereas there were fewer significant differences among species of different pollinator groups in the field. Even though these comparisons were made using bagged flowers, environmental variation may have an effect on plants that contributes to the greater variation among species in the field, although there are likely to be other factors contributing to differences of nectar traits between the field and greenhouse environments.

Population effects

Most populations within a species were significantly different from each other in all nectar traits when all data was considered. However, when only bagged flowers were considered, more populations within species were found to be similar (compare Table 4 and Fig. 3). Bagged flowers should better reflect the nectar traits provided by the plant before floral visitors have an opportunity to alter the nectar, therefore more similarities would be expected among populations within a species. Even though there were fewer population effects within species when only bagged flowers were considered, there were still a few significant population effects for certain nectar traits (Fig. 3). The *N. forgetiana* populations were significantly different only in nectar volume at anthesis. It was rainy in the Caxias do Sul population on the first day of sampling, while it was dry both sampling days at the São Marcos population; therefore the significant difference at anthesis was likely due to the different weather conditions (see Table 1). Additionally, this difference was not detected when the precipitation covariate was not included in the model. For similar reasons, weather was likely the main factor contributing to the significant difference of nectar concentration at anthesis between the *N. alata* populations. Both sampling days at the Rio Pelotas population were rainy compared to the dry sampling days at the São Bernardo population. The *N. bonariensis* populations were significantly different in nectar concentration at both 0 and 24 h after anthesis, which was likely due more to the general climate differences between the populations than the weather conditions. The Santa Tereza population was found along a very dry dirt roadside with very little vegetation and relatively high temperatures, although it did rain on one of the sampling days. In contrast, the Morro da Igreja population was at the top of

a mountain in the cloud forest, which is often moist and cool and sampling on all three days occurred during extensive rains. The significant effect of environmental conditions on nectar traits emphasizes the importance of sampling over a long time scale or standardizing for weather conditions. Although weather and general climate conditions are the most apparent factors likely affecting these populations, other factors are also likely to contribute to these differences. Population effects were rare in the greenhouse experiments, suggesting that the differences in the field are likely due to environmental variation.

Time effects

Nectar volume was the only nectar trait that was significantly affected by time overall, although all nectar traits had a significant time by population interaction. All populations exhibited a general increase over time in nectar volume and energy content of bagged flowers, though not all significantly (Table 5 & 6). Continuous nectar production for a period of time after anthesis is typical for many species (Cruden et al., 1983). Furthermore, nectar production in *Nicotiana* continues until at least 48 h after anthesis (R. W. Thornburg, pers. comm.); therefore the increase in nectar volume is likely due to continuous nectar production. The nectar concentration of bagged flowers both increased and decreased over time, though only significantly in *N. forgetiana* SM (Table 5 & 6). Nectar concentrations can be differentially affected by environmental conditions (Park, 1929; Wyatt et al., 1992), evaporation (Plowright, 1987), robbing (Pleasants, 1983; Irwin and Brody; 1998; Arizmendi et al., 1996), and likely other factors. It is likely that multiple factors contributed to the differences in nectar concentration over time. Nectar concentration in the greenhouse experiments significantly increased in some species

(hummingbird-pollinated), but not in others (hawkmoth-pollinated). However, nectar concentration never decreased over time (Kaczorowski et al., 2005). Time had different effects on bagged and exposed flowers, discussed in the following section.

Bagging effects

This study used plastic bags to restrict pollinators from removing or otherwise altering nectar of flowers. This treatment was assumed to be analogous to conditions in the greenhouse. However, temperature and relative humidity can be higher within plastic bags, which could significantly increase nectar volume and reduce nectar concentration (Wyatt et al., 1992). It is possible that the use of plastic bags altered the nectar traits, but comparisons between bagged and exposed flowers suggest that microenvironment effects are minimal (i.e., no overall bagging effects on nectar concentration). Most concentration differences between bagged and exposed flowers within populations were non-significant, but *N. forgetiana* SM did exhibit a significantly higher nectar concentration in bagged compared to exposed flowers at 24 h after anthesis (Table 5). This difference could be due to some effects induced by bagging (i.e., evaporation increasing concentration), but nectar concentration significantly decreased over time among bagged and exposed flowers in the *N. forgetiana* SM population. Therefore, there must have been a greater reduction in the nectar concentration of exposed flowers compared to bagged flowers. It is unclear why nectar concentrations decreased in this population since there was no precipitation while sampling this population, but perhaps bagged flowers were more protected from the unknown effects. Alternatively, robbing slits on the bagged flowers could have increased nectar evaporation from flowers in the bags and reduced the concentration decline in those flowers.

Greenhouse environments are not conducive for the investigation of floral visitor effects on nectar traits because most floral visitors are restricted from entering these environments. Plants growing in their native habitat are likely to be visited frequently by floral visitors, including pollinators, at certain times in the season. The use of bags restricted floral visitors from removing nectar. Therefore, nectar volume in bagged flowers over time should represent nectar accumulation, while the nectar volume of exposed flowers over time tends to deplete as pollinators remove nectar during foraging (Zimmerman, 1988). Significant nectar accumulation in bagged flowers has been demonstrated previously (McDade and Weeks, 2004a), and is supported by this study, as the nectar volume of bagged flowers increased over time in all populations investigated, although it significantly accumulated in only five populations (Table 5).

The standing crop of nectar, estimated in exposed flowers, is likely to exhibit a considerable amount of variation because it is dependent upon pollinator foraging activity, as well as other factors (Zimmerman, 1988). Standing crop is expected to be low when pollinator activity is high, but when pollinator activity is low standing crop is more representative of nectar production (Zimmerman, 1988; McDade and Weeks, 2004b). Two populations in this study exhibited a significant change in nectar volume in exposed flowers over time, albeit in opposite directions (Table 5). The *N. alata* SB population exhibited a significant volume increase in the exposed flowers, likely due to a lack of nectar removal by pollinators. This population was situated along a roadside ravine, where most plants were scattered along the slope.Icould only sample the plants at the top, along the roadside, where pollinators may have been less likely to visit, although at least one foraging hawkmoth was observed during sampling. The *N. forgetiana* CdS

population exhibited a significant decrease in volume over time among the exposed flowers. Many plants were sampled in this population over approximately 4 h around the time of anthesis. There was sufficient time for the 0 h flowers to accumulate some nectar. However, many hummingbirds (pollinators) and bumblebees (robbers) were observed in this population and they most likely significantly reduced the amount of nectar present in the exposed flowers. The comparison of bagged and exposed flowers at 24 h after anthesis should estimate nectar removal by floral visitors.

If nectar volumes are not significantly different between bagged and exposed flowers at the time of anthesis (0 h), than nectar removal can be estimated by subtracting the mean volume of flowers exposed to floral visitors (exposed) from the mean volume of flowers restricted from floral visitors (bagged) for each population at 24 h after anthesis (see Table 7). Because *N. mutabilis* nectar volumes were significantly higher in bagged compared to exposed flowers at 0 h, the bagged nectar volumes had to be adjusted to compare differences for estimations of nectar removal. Nevertheless, there was still a significant ($\alpha = 0.05$) amount of nectar removed from *N. mutabilis* flowers, although the adjustment changed the significance of the difference in the Quebra Cabo population (from $P = 0.0006$ to $P = 0.0217$; see Table 5; 24hB v 24hE). All but three populations had more nectar in bagged flowers than exposed flowers at 24 h after anthesis. The comparison of bagged and exposed flowers at 24 h after anthesis is only a rough estimation of nectar removal because other factors may contribute to these differences. Nectar removal has also been shown to stimulate additional nectar production in many species (Navarro, 1999; Ordano and Ornelas, 2004; McDade and Weeks, 2004b), while accumulated nectar can be reabsorbed into surrounding tissues in some species (Burquez

and Corbet, 1991; Jakobsen and Kristjansson, 1994; Nicolson, 1995; Luyt and Johnson, 2002; Nepi and Stpiczynska, 2007). If nectar removal stimulates nectar replacement in *Nicotiana* species, the nectar volumes of exposed flowers may be an underestimation of the amount of nectar removed by pollinators. If *Nicotiana* species tend to reabsorb nectar into surrounding tissues, the nectar volumes of bagged flowers are more likely to be overestimated than exposed flowers because they are more likely to have accumulated nectar. Regardless, other studies comparing nectar volumes of bagged and exposed flowers found that exposed flowers had significantly less nectar than bagged flowers, and attribute these differences to removal by floral visitors (Itino et al., 1991; Mallick, 2000; McDade and Weeks, 2004b), while our results support these findings.

Comparison with greenhouse data

Plants growing in natural populations had significantly different nectar traits from those grown in the greenhouse. The differences between greenhouse-grown and naturally-grown plants were likely due, in part, to the different environmental conditions each group of plants was exposed to. Floral nectar traits can be considerably affected by environmental conditions. Environmental factors that are known to affect nectar traits include temperature (Freeman and Head, 1990; Jakobsen and Kristjansson, 1994), humidity (Park, 1929; Corbet, 1978; Corbet et al., 1979; Bertsch, 1988; Wyatt et al., 1992), carbon dioxide levels (Lake and Hughes, 1999; Davis, 2003), mineral content (Shuel, 1957; Gardener and Gillman, 2001), and soil moisture (Pleasants, 1983; Zimmerman, 1988; Wyatt et al., 1992; Carroll et al., 2001). Although none of these factors were effectively measured in the field, they all have the potential to differ greatly

from what would be found in the greenhouse. These factors are also likely to vary more readily over time in natural populations, as well as across different natural populations.

The difference in environmental conditions inside and outside the greenhouse was not the only important environmental aspect; the conditions each natural population was exposed to prior to and during the time of sampling could have significantly affected the nectar traits observed. Environmental conditions varied greatly across populations during the time of sampling (Table 1), but the overall climate was also noticeably different in certain populations. The greatest difference noted was between the 2 populations of *N. bonariensis* (discussed in the species effects section). The distinct environmental difference between the two *N. bonariensis* populations is likely a major factor contributing to the large difference in nectar concentration (Fig 3). Although the conditions among the other populations were not as noticeably different as they were for the *N. bonariensis* populations, they may have been different enough to differentially affect the traits of plants within the populations.

Another factor that could have differentially affected nectar traits between the two environments would be the floral visitors. Nectar removal can alter nectar traits within the flowers from which nectar was removed, as well as in the other flowers on the plant. Additionally, plants deprived of pollinators may invest more into nectar resources to increase the chances of enticing pollinators. This could potentially explain why greenhouse-grown plants produced nectar with much higher concentrations. However, there is likely to be a suite of factors that contribute to differences in nectar traits between greenhouse and natural population environments.

Trait correlations

Previous greenhouse experiments investigated the correlation of nectar traits to flower size (corolla tube length). There was a significant positive correlation between nectar volume and corolla length, while nectar concentration had a significant negative correlation with corolla length, resulting in a non-significant correlation between total energy content and corolla length (Kaczorowski et al., 2005). These correlations were determined from regressions based on population means of two different sets of plants (one set for nectar traits, the other for floral morphology traits). In this field study, nectar and morphological data were taken on each flower sampled in the natural populations and therefore more information was available for natural population correlations than for greenhouse correlations. However, natural population measurements were averaged per plant, although more information was used for plant averages in natural populations. No significant correlations were detected between nectar traits and corolla tube length. However, nectar volume (at 0 h and 24 h after anthesis) and nectar energy content (at 24 h after anthesis) were significantly correlated with corolla mouth diameter (Table 8). Although this was not the morphological trait I hypothesized would have the greatest effect on nectar, it has the potential for restricting pollinator foraging (Lange et al., 2000) and therefore could fall under selective pressure. Mouth diameter may also be important for controlling air flow into the corolla tube, thereby affecting evaporation. However, if this were the case, I would expect nectar concentration to be more affected than nectar volume. Nectar concentration is not significantly correlated with any other measured trait in this study, not even nectar volume, to which it was strongly negatively correlated in the previous greenhouse study (Kaczorowski et al., 2005). The differences in correlations detected between the two studies could be a result of different methodologies in obtaining

the estimates (regressions versus PROC CORR in SAS 9.1) or could be due to environmental variation significantly affecting traits of plants growing in natural populations.

Whole-plant standing crop

Whole-plant sampling quantifies the amount of nectar reward available to a pollinator across the whole plant. It is not surprising that the total amount of nectar and nectar sugar (energy content) is significantly positively correlated to the number of flowers on the plant (Fig. 6). Zimmerman and Pyke (1988) found relatively similar rates of nectar production across different treatments of defoliation and bud removal, suggesting a somewhat constant demand for energy content by flowers to produce nectar. Therefore, the more flowers produced by a plant, the more nectar reward should be available. However, this correlation could be disrupted as a result of significant environmental variation (see Rathcke, 1992). Nectar concentration tends to be even more stabile than nectar volumes, with coefficients of variation (CVs) ranging between 5 and less than 25 %, compared to CVs between 40 and over 100 % for nectar volume (Rathcke, 1992).

It is interesting that nectar volume was not significantly affected by either of the positional variables, but nectar concentration and energy content were significantly affected by some aspect of flower position. Nectar energy content was affected by the most variables (or interactions). This may be important to pollinator behavior because nectar energy content integrates both volume and concentration and so has the potential to impact pollinators more than nectar volume. The position effects in nectar energy content present potential for pollinators to alter foraging behaviors based on flower

position. Many of the previous studies of positional effects on nectar have demonstrated that nectar volume tends to decrease with increasing height on the inflorescence (Pyke, 1978; Waddington, 1981; Pyke, 1982; Haddock and Chaplin, 1982; Galen and Plowright, 1985; Hodges, 1985; but vice versa in Corbet et al., 1981). Additionally, total sugar (energy content) was found to follow a similar trend (Best and Bierzychudek, 1982; Devlin et al., 1987), but sugar concentration was shown to increase with increasing floral height on the inflorescence of a few *Digitalis* species (Percival and Morgan, 1965). In this study, there was no clear trend for nectar to vary in relation to the height on the inflorescence. However, due to the complexity of the panicle-like inflorescence and methodology used for estimating position, trends may have gone undetected.

Nectar traits were significantly affected by some ecological variables, such as robbing, florivores or flower retention. These ecological variables were differentially distributed among some populations (Fig. 8). For example, *N. mutabilis* QC was heavily robbed, but completely lacked larvae in the corolla or retained flowers, while *N. mutabilis* BdO showed evidence of robbing only on one flower, but had a higher occurrence of larvae and retained flowers. These populations were very different in their structure; the Barra do Ouro population consisted of only a few (~20) individuals spread out over about 500 m along the roadside, on the edge of a tree line, while the Quebra Cabo population was immense with thousands of plants covering a large open hillside. Pollinator activity was also very different, likely as a result of the population size differences, with sparse pollinator activity in the small population and heavy pollinator (hummingbird) and robber (bumblebee) activity in the large population (R. Kaczorowski, pers.obs.).

A number of studies have investigated the effects of nectar robbing on plants. The effects of nectar robbing are complicated, as they depend upon the type of robbers, the type of legitimate pollinators, the quantity of nectar removed during robbing, and the amount of resources available to the plants in a population (Maloof and Inouye, 2000). A review of studies investigating the effects of nectar robbing on seed set found an equal number of negative, positive, and neutral effects (Maloof and Inouye, 2000). Therefore, the hypothesis put forth by Darwin (1872), and adopted by many since, that nectar robbing must have a negative impact on plants, may not always hold true. Nectar robbing decreases the standing crop of nectar (McDade and Kinsman, 1980; Pleasants, 1983; Irwin and Brody, 1998), demonstrated also in this study. This may impart a negative effect on plant fitness through resource costs of replacing removed nectar (Pyke, 1991; Navarro, 1999), or by decreasing the attractiveness of robbed flowers to legitimate pollinators (Irwin and Brody, 1998). Negative effects of nectar robbing may also result from damage to reproductive tissues (Galen, 1983; Traveset et al., 1998) or direct deterrence of pollinators by robbers (Roubik, 1982). However, nectar robbing could have positive effects on plant fitness if robbers effectively pollinate while manipulating the flowers (Maloof and Inouye, 2000), or when lower nectar volumes cause pollinators to visit more flowers (Cushman and Beatie, 1991) or increase distances flown between inflorescences (Zimmerman and Cook, 1985; Maloof, 2001).

When a nectar robbing effect was detected within a population, there was significantly more nectar in flowers that were not robbed (Table 9). This is likely due to the robbers removing a significant amount of nectar. Nectar robbers are expected to exert selective pressure on nectar traits. Rates of nectar production are expected to be higher in

the presence of nectar robbing (Pyke, 1991). The difference in robbing frequency between the two populations of *N. mutabilis* offers a comparison in the average amount of nectar produced between a population likely under pressure from robbers and one that is not. Whole-plant standing crop was significantly higher in the Barra do Ouro population that lacked robbers ($t = 1.97$, $P = 0.0001$); however I find no significant difference between the two *N. mutabilis* populations in the nectar volume of bagged flowers (Fig. 3), which more nearly represents nectar production rate for the populations. Therefore, this study does not offer evidence of increased nectar production in the presence of robbing.

Nectar robbing can increase nectar concentration, likely a result of accelerated evaporation from the holes produced by the robber (Pleasants, 1983; Irwin and Brody, 1998), and it can also decrease nectar concentration, perhaps because the removal of nectar stimulates additional nectar secretion without additional sugar production (Arizmendi et al., 1996). Both of these patterns can be seen in different populations of this study. Nectar concentration was significantly higher in the *N. forgetiana* and *N. alata* RP populations, while it was significantly lower in the *N. langsdorffii* and *N. mutabilis* QC populations (Table 9). The lack of a significant nectar robbing effect on nectar concentration overall may be a consequence of these contrasting patterns in robbing effects among populations.

Florivores and other herbivores are expected to have negative effects on plants, although they may also benefit plants by stimulating growth and allocation towards reproduction (Agrawal, 2000). Nectar rewards have been associated with the paradigm of attracting both pollinators and floral antagonists, and therefore may be under conflicting

selective pressure from these two types of floral visitors (Adler and Bronstein, 2004; Ordano and Ornelas, 2004). Although I do not have evidence to support or refute this hypothesis, I did find that all florivore effects on nectar traits were negative (Table 9), which could have indirect reproductive consequences for the plants.

Many of the populations in this study had some evidence of old flower retention (Fig. 8). A possible function of flower retention is to increase floral display, which may be an important factor in attracting pollinators (Klinkhamer and de Jong, 1993; Harder and Barrett, 1995; Robertson and Macnair, 1995). However, larger floral displays can cause increased geitonogamy, pollen transfer among flowers within a plant (de Jong et al., 1992; Klinkhamer et al., 1994; Harder and Barrett, 1995), which can reduce plant fitness (de Jong et al., 1993). Although, the *Nicotiana* species included in this study are self-incompatible (McClure et al., 1990); therefore there is no risk of geitonogamy reducing fitness within these plants. The greatest occurrence of flower retention was observed in the *N. mutabilis* BdO population.

Pollinator frequency was vastly different between the two populations of *N. mutabilis*. In the Quebra Cabo population, where pollinator frequency was great, there was no evidence of retained flowers. In contrast, old and wilted flowers constituted approximately 35% of flowers sampled in the Barra do Ouro population, where pollinator frequency was low. This lends support to the possibility that flowers are retained to increase floral display because it seems to be occurring when plants are in greater need of additional attractiveness to entice pollinators (e.g., flower longevity reflects pollination status), although flowers may simply be retained until they are either pollinated or senesced. Additionally, the dark pink color of the old retained flowers, in contrast with

the new white flowers, may aid in the apparency of the plant to pollinators at long distances, while offering a cue for pollinators to discriminate upon at short distances (Gori, 1989; Weiss, 1991; Oberrath and Böhning-Gaese, 1999).

Hummingbirds, the predominant pollinators of *N. mutabilis*, are more often associated with red flowers than white, although evidence suggests they develop preferences for traits associated with rewarding species (Bené, 1941; Grant, 1966; Grant and Grant, 1968; Raven, 1972; Meléndez-Ackerman et al., 1997). Despite an initial preference for red over white flowers of equal reward, hummingbirds will abandon that preference when white flowers are more rewarding (Meléndez-Ackerman et al., 1997). Many other studies also suggest that hummingbird color preference can be learned in conjunction with differential reward (Bené, 1941; Collias and Collias, 1968; Miller and Miller, 1971; Stiles, 1976). In the *N. mutabilis* populations, the amount of nectar and sugar in flowers could be predicted by flower color. Therefore, it is not surprising that preliminary observations suggest hummingbirds preferentially visit white flowers over pink ones. Selection driving floral color changes should occur when plants somehow benefit. Consequently, the flower color change in *N. mutabilis* is likely to increase plant fitness, although the fitness consequences were not investigated in this study.

Implications

Natural populations are subject to biotic and abiotic factors that cannot be experienced by greenhouse-grown plants. This study has demonstrated many effects on nectar traits from factors that plants may be subjected to in the field. Environmental variation and floral visitors can have significant effects on nectar traits. Pollinators and floral antagonists may affect nectar traits in different ways and may place selective

pressure on nectar traits in opposite directions (Adler and Bronstein, 2004; Ordano and Ornelas, 2004). All *Nicotiana* populations investigated in this field study exhibited significant differences in at least one measured nectar trait (concentration was always different) when compared to greenhouse-grown plants from the same populations. Species within a pollinator group were more similar to species from other pollinator groups in the field than they were in the greenhouse, likely due to higher environmental variation. However, comparisons of nectar traits between pollinator groups were generally similar across the different environments of the field and greenhouse. Nectar traits can affect plant fitness through effects on pollinator behavior (Zimmerman, 1983; Real and Rathcke, 1991; Mitchell and Waser, 1992; Mitchell, 1993; Hodges, 1995). Therefore, it is important to understand the nectar profile that pollinators are likely to encounter, which requires characterization of nectar traits in the field. There are many potential sources, often conflicting, for selective pressure on nectar traits. A more thorough understanding of the factors that can affect nectar traits in the field can allow for a better interpretation of how different selective pressures may interact to shape current nectar profiles. Although this study identifies many factors that affect nectar traits, more studies are necessary to determine how these factors may affect plant fitness.

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Table 1. Population information for *Nicotiana* species studied in natural populations.

Species	Population (abbr.)	State [a]	Latitude	Longitude	Altitude (m)	Accession [b]	Date sampled [c]	Conditions [d]
N. mutabilis	Barra do Ouro (BdO)	RS	S 29° 34.920'	W 50° 18.708'	628	92220	Oct 23-24, 2004	1,2: rain
"	Quebra Cabo (QC)	RS	S 29° 32.582'	W 50° 21.426'	892	92221	Oct 26-28, 2004	1,3: dry; 2: wet
N. forgetiana	Caxias do Sul (CdS)	RS	S 29° 5.377'	W 51° 4.905'	746	92224	Nov 3-4, 2004	1: rain; 2: dry
"	Sao Marcos (SM)	RS	S 28° 59.928'	W 51° 4.555'	689	92225	Nov 8-9, 2004	1,2: dry
N. alata	Rio Pelotas (RP)	RS/SC	S 28° 13.234'	W 50° 45.474'	742	92218	Dec 4-5, 2004	1,2: rain
"	Sao Bernardo (SB)	RS	S 28° 51.587'	W 51° 7.312'	619	92219	Nov 6-7, 2004	1,2: dry
N. bonariensis	Santa Tereza (ST)	RS	S 29° 23.788'	W 50° 22.007'	889	92222	Nov 24-25, 2004	1: dry; 2: rain
"	Morro da Igreja (MdI)	SC	S 28° 7.071'	W 49° 28.942'	1744	92226	Dec 21-23, 2004	1-3: rain
N. langsdorffii	Morro da Igreja (MdI)	SC	S 28° 4.570'	W 49° 30.882'	1347	92227	Dec 22-24, 2004	1,3: dry; 2: wet
"Rastroensis" [e]	Bom Jardim da Serra	SC	S 28° 23.38'	W 49° 31.42'	765	—	Nov 21-23, 2001	1,3: dry; 2: wet

[a] State of Brazil: RS: Rio Grande do Sul, SC: Santa Catarina

[b] Accessions were collected by Rainee Kaczorowski and deposited at the Federal University of Minas Gerais Herbarium in Belo Horizonte, Minas Gerais, Brazil (BHCB).

[c] Dates represent when treatments were applied to the plants in each population. Whole-plant standing crop data was obtained during this period or shortly thereafter.

[d] Numbers represent day of sampling (first, second or third)

[e] Sampled on a different year than all other species and therefore analyzed in separate analyses.

Table 2. Pollinator group contrasts for each nectar trait in *Nicotiana* species. Table entries denote significance using contrast statements applied to ranked data (bagged flowers only) following PROC GLM (SAS 9.1): ***: $P \leq 0.0001$, **: $P < 0.01$, ns: not significant.

		Pollinator group	
Pollinator group	Nectar trait	Hawkmoth	Small moth
Hummingbird	Volume	***	***
"	Concentration	**	ns
"	Energy content	**	***
Hawkmoth	Volume		***
"	Concentration		***
"	Energy content		***

Table 3. Species contrasts for each nectar trait in *Nicotiana* species. Table entries denote significance using contrast statements applied to ranked data (bagged flowers only) following PROC GLM (SAS 9.1): ***: $P \leq 0.0001$, **: $P < 0.01$, ns: not significant.

		Nicotiana species			
Nicotiana species	Nectar Trait	*N. forgetiana*	*N. alata*	*N. bonariensis*	*N. langsdorffii*
N. mutabilis	Volume	***	***	***	ns
"	Concentration	***	***	**	***
"	Energy content	***	***	***	***
N. forgetiana	Volume		***	***	***
"	Concentration		***	ns	***
"	Energy content		**	***	**
N. alata	Volume			***	ns
"	Concentration			***	ns
"	Energy content			***	ns
N. bonariensis	Volume				***
"	Concentration				***
"	Energy content				***

Table 4. General population effects (all data included) in *Nicotiana* species for each nectar trait. Table entries denote significance by LSD ($\alpha = 0.01$) applied to ranked data following PROC GLM (SAS 9.1): **: $P < 0.01$, ns: not significant.

			Nicotiana species / population								
			N. mutabilis		*N. forgetiana*		*N. alata*		*N. bonariensis*		*N. langsdorffii*
Nicotiana species	Population	Nectar trait	BdO	QC	CdS	SM	RP	SB	ST	MdI	MdI
N. mutabilis	BdO	Volume		**	ns	ns	**	**	**	**	**
"	"	Concentration		**	ns	ns	**	ns	**	**	**
"	"	Energy content		**	**	ns	**	**	**	**	**
"	QC	Volume			**	**	**	**	**	**	**
"	"	Concentration			**	**	**	**	ns	**	**
"	"	Energy content			**	**	**	**	**	**	**
N. forgetiana	CdS	Volume				ns	**	**	**	**	ns
"	"	Concentration				ns	**	ns	**	**	**
"	"	Energy content				ns	**	ns	**	**	ns
"	SM	Volume					**	**	**	**	ns
"	"	Concentration					ns	**	**	**	**
"	"	Energy content					ns	ns	**	**	**
N. alata	RP	Volume						ns	**	**	ns
"	"	Concentration						**	**	ns	ns
"	"	Energy content						ns	**	**	ns
"	SB	Volume							**	**	**
"	"	Concentration							**	**	**
"	"	Energy content							**	**	ns

Table 4. Continued.

			Nicotiana species / population								
			N. mutabilis		*N. forgetiana*		*N. alata*		*N. bonariensis*		*N. langsdorffii*
Nicotiana species	Population	Nectar trait	BdO	QC	CdS	SM	RP	SB	ST	MdI	MdI
N. bonariensis	ST	Volume								ns	**
″	"	Concentration								**	**
″	"	Energy content								**	**
″	MdI	Volume									**
″	"	Concentration									ns
″	"	Energy content									**

Table 5. Effects of time after anthesis (0 and 24 h) and presence of bags (B, E) in nectar traits among *Nicotiana* species. Table entries denote significance in the direction of the difference, determined by lsmeans following PROC GLM (SAS 9.1): +++/- - -: $P < 0.0001$, increase/decrease; ++/- -: $P < 0.01$, increase/decrease; ns: not significant. See Table 1 for population abbreviations.

		Nicotiana species / populations								
		N. alata		*N. forgetiana*		*N. mutabilis*		*N. bonariensis*		*N. langsdorffii*
Nectar traits	Comparisons [a]	RP	SB	CdS	SM	QC	BdO	ST	MdI	MdI
Nectar volume	0hB v 0hE	ns	ns	ns	ns	++	+++	ns	ns	ns
"	24hB v 24hE	ns	ns	+++	+++	++	+++	ns	ns	++
"	24hB v 0hB	+++	+++	ns	+++	ns	++	++	ns	ns
"	24hE v 0hE	ns	+++	- - -	ns	ns	ns	ns	ns	ns
Nectar concentration	0hB v 0hE	ns	ns	ns	ns	ns	ns	ns	ns	ns
"	24hB v 24hE	ns	ns	ns	++	ns	ns	ns	ns	ns
"	24hB v 0hB	ns	ns	ns	- -	ns	ns	ns	ns	ns
"	24hE v 0hE	ns	ns	ns	- -	ns	- - -	ns	ns	ns
Total nectar energy	0hB v 0hE	ns	ns	ns	ns	++	+++	ns	ns	ns
"	24hB v 24hE	++	ns	+++	+++	++	+++	ns	ns	++
"	24hB v 0hB	++	ns	ns	++	ns	ns	++	ns	ns
"	24hE v 0hE	ns	++	- - -	ns	ns	ns	ns	ns	ns

[a] 0hB: 0 h, bagged; 0hE: 0 h, exposed; 24hB: 24 h, bagged; 24hE: 24 h, exposed

Table 6. Means and standard errors of nectar volume, concentration, and energy content for all *Nicotiana* section *Alatae* species studied in both the greenhouse and in natural populations. Only bagged flowers were included in the natural population data. Asterisks denote significantly higher values than those found in the other environment, determined from lsmeans (SAS 9.1): ***: $P < 0.0001$, **: $P < 0.01$.

			Greenhouse Means ± SE		
Species	Population	Time	Volume (µl)	Concentration (%)	Energy (mg sucEq)
N. mutabilis	Quebra Cabo	0 h	3.74 ± 0.67	46.47 ± 2.19 ***	2.04 ± 0.28
"	"	24 h	7.62 ± 0.44	58.68 ± 1.73 ***	5.59 ± 0.26
N. forgetiana	Caxias do Sul	0 h	4.53 ± 0.88	36.75 ± 2.00 ***	2.01 ± 0.46
"	"	24 h	7.43 ± 0.92	44.94 ± 2.46 ***	4.00 ± 0.45 **
"	Sao Marcos	0 h	4.10 ± 1.12	36.58 ± 3.17 ***	2.34 ± 0.42
"	"	24 h	6.12 ± 0.98	51.06 ± 3.07 ***	3.73 ± 0.61
N. alata	Rio Pelotas	0 h	7.59 ± 0.53 **	21.86 ± 0.94 ***	1.79 ± 0.14 **
"	"	24 h	15.54 ± 1.76 **	22.86 ± 0.71 ***	3.79 ± 0.37 ***
N. bonariensis	Santa Tereza	0 h	0.24 ± 0.04	40.39 ± 2.14 ***	0.12 ± 0.02
"	"	24 h	0.68 ± 0.07	48.93 ± 2.21 ***	0.41 ± 0.05
N. langsdorffii	Morro da Igreja	0 h	3.32 ± 1.00	42.58 ± 2.33 ***	2.02 ± 0.44
"	"	24 h	6.46 ± 1.04	54.71 ± 3.89 ***	4.23 ± 0.60 ***
"Rastroensis" [a]	Bom Jardim	0 h	1.83 ± 0.78	48.79 ± 2.73 ***	1.31 ± 0.58
"	"	24 h	3.66 ± 0.48	59.50 ± 2.60 ***	2.68 ± 0.28

[a] Rastroensis natural population data was collected in 2001, not in 2004 with the other populations and therefore analyzed separately.

Table 6. Continued.

Species	Population	Time	Natural Population Means ± SE											
			Volume (µl)				Concentration (%)			Energy (mg sucEq)				
N. mutabilis	Quebra Cabo	0 h	15.49	±	1.95	***	23.99	±	0.90	4.12	±	0.45	***
"	"	24 h	18.63	±	2.47	**	20.79	±	1.23	4.48	±	0.66	
N. forgetiana	Caxias do Sul	0 h	6.67	±	0.88		16.43	±	1.32	1.32	±	0.23	
"	"	24 h	13.05	±	2.49		17.96	±	1.15	2.41	±	0.42	
"	Sao Marcos	0 h	4.79	±	0.93		23.34	±	1.04	1.28	±	0.26	
"	"	24 h	11.72	±	1.24	**	19.65	±	0.85	2.52	±	0.32	
N. alata	Rio Pelotas	0 h	3.20	±	0.48		16.08	±	0.78	0.55	±	0.08	
"	"	24 h	10.10	±	2.76		14.98	±	0.92	1.69	±	0.43	
N. bonariensis	Santa Tereza	0 h	0.92	±	0.11		27.18	±	1.61	0.31	±	0.04	
"	"	24 h	1.59	±	0.35		24.13	±	2.03	0.44	±	0.08	
N. langsdorffii	Morro da Igreja	0 h	6.46	±	2.12		13.00	±	1.29	1.06	±	0.44	
"	"	24 h	9.82	±	1.84		14.01	±	2.43	1.78	±	0.47	
"Rastroensis" [a]	Bom Jardim	0 h	2.07	±	0.44		25.93	±	1.81	0.69	±	0.12	
"	"	24 h	4.48	±	0.49		42.23	±	1.41	2.58	±	0.26	

[a] Rastroensis natural population data was collected in 2001, not in 2004 with the other populations and therefore analyzed separately.

Table 7. Nectar removal estimates from flowers in natural populations of *Nicotiana*. Estimated volume removed was determined by subtracting the mean volume of exposed flowers from the mean volume of bagged flowers at 24 h. Percentage removed was determined by dividing the mean volume of exposed flowers by the mean volume of bagged flowers at 24 h. Significance of estimated removal determined by lsmeans applied to ranked data following PROC GLM (SAS 9.1): ***: $P < 0.0001$, **: $P < 0.01$, *: $P < 0.05$, ns: not significant.

			Nectar volume (mean ± SE, µl)		Estimated volume removed [b]	
Species	Population	N_B, N_E [a]	24 h Bagged	24 h Exposed	(µl)	(%)
N. mutabilis	Barra do Ouro	37, 30	13.74 ± 1.87 [c]	4.08 ± 0.96	9.66 *** [d]	30%
"	Quebra Cabo	37, 7	15.62 ± 1.63 [c]	7.43 ± 1.66	8.19 * [d]	48%
N. forgetiana	Caxias do Sul	47, 26	11.68 ± 2.10	3.35 ± 1.09	8.33 ***	29%
"	Sao Marcos	26, 9	12.25 ± 1.12	4.47 ± 0.46	7.78 ***	36%
N. alata	Rio Pelotas	23, 10	10.20 ± 1.67	4.23 ± 0.52	5.97 *	41%
"	Sao Bernardo	18, 7	7.60 ± 0.82	10.46 ± 0.90	-2.86 ns	
N. bonariensis	Santa Tereza	34, 12	1.58 ± 0.27	0.70 ± 0.12	0.88 ns	
"	Morro da Igreja	10, 2	0.70 ± 0.14	0.46 ± 0.12	0.24 ns	
N. langsdorffii	Morro da Igreja	13, 10	9.62 ± 1.35	2.35 ± 0.40	7.27 **	24%

[a] N_B = sample size of bagged flowers, N_E = sample size of exposed flowers, both at 24 h.

[b] Significance is similar to that found in Table 5: 24hB v 24hE, although an alpha of 0.05 was used here.

[c] Mean and SE determined from adjusted data to account for the significant volume differences between bagged and exposed flowers at 0 h

[d] Significance determined through t-test of adjusted 24 h bagged data and normal 24 h exposed data

Table 8. Pearson correlation coefficients determined for pairs of traits measured in natural populations of *Nicotiana*. Traits were averaged over plants (N = 81-88, bagged flowers only) and analyzed using PROC CORR (SAS 9.1). ***: $P \leq 0.0001$, **: $P < 0.01$, ns: not significant.

Traits	Time (h)	Limb width	Mouth diameter	Nectar volume	Nectar concentration	Total nectar energy
Tube length	0	0.9096 ***	0.5423 ***	-0.1834 ns	-0.2249 ns	-0.2174 ns
"	24	0.9138 ***	0.5349 ***	0.0638 ns	-0.0928 ns	-0.0425 ns
Limb width	0		0.4028 ***	-0.2418 ns	-0.1697 ns	-0.2608 ns
"	24		0.4237 ***	0.0043 ns	-0.0614 ns	-0.0820 ns
Mouth diameter	0			0.3161 **	-0.2053 ns	0.2483 ns
"	24			0.4461 ***	-0.0883 ns	0.3535 **
Nectar volume	0				0.1830 ns	0.9773 ***
"	24				0.2195 ns	0.9581 ***
Nectar concentration	0					0.3063 **
"	24					0.3714 **

Table 9. Effects of ecological variables within *Nicotiana* populations. Significance was determined using PROC TTEST (in SAS 9.1), Satterthwaite method when variances were unequal, pooled variance estimator method when variances were equal (variance equality tested with Folded *F*' statistic). Positive signs denote a significant increase of the trait in the presence of the ecological variable and negative signs denote a significant decrease of the trait in the presence of the ecological variable. +++/- - -: $P < 0.0001$, ++/- -: $P < 0.01$, +/-: $P < 0.05$, ns: not significant, —: could not be tested, insufficient number of flowers with ecological variable.

Ecological variable	Species / Population [a]							
Nectar trait	ala RP	ala SB	bon MdI	bon ST	forg CdS	lang MdI	mut BdO	mut QC
Holes or slits (robbing)								
Nectar volume	ns	ns	- -	—	- - -	ns	—	-
Nectar concentration	++	ns	—	—	++	-	—	-
Total nectar energy	+++	ns	—	—	- -	-	—	-
Larvae or bugs (florivores)								
Nectar volume	ns	- - -	ns	ns	ns	—	ns	—
Nectar concentration	- -	-	ns	ns	ns	—	- - -	—
Total nectar energy	ns	- -	ns	ns	ns	—	ns	—
Old age (retained flowers)								
Nectar volume	- - -	ns	—	—	- - -	—	- -	—
Nectar concentration	—	ns	—	—	ns	—	ns	—
Total nectar energy	—	ns	—	—	ns	—	ns	—

[a] Species abbreviations: ala = *N. alata*, bon = *N. bonariensis*, forg = *N. forgetiana*, lang = *N. langsdorffii*, mut = *N. mutabilis*; population abbreviations in Table 1

Figure 1. Map of the *Nicotiana* populations sampled in the two most southern states of Brazil, Rio Grande do Sul and Santa Catarina. See also Table 1.

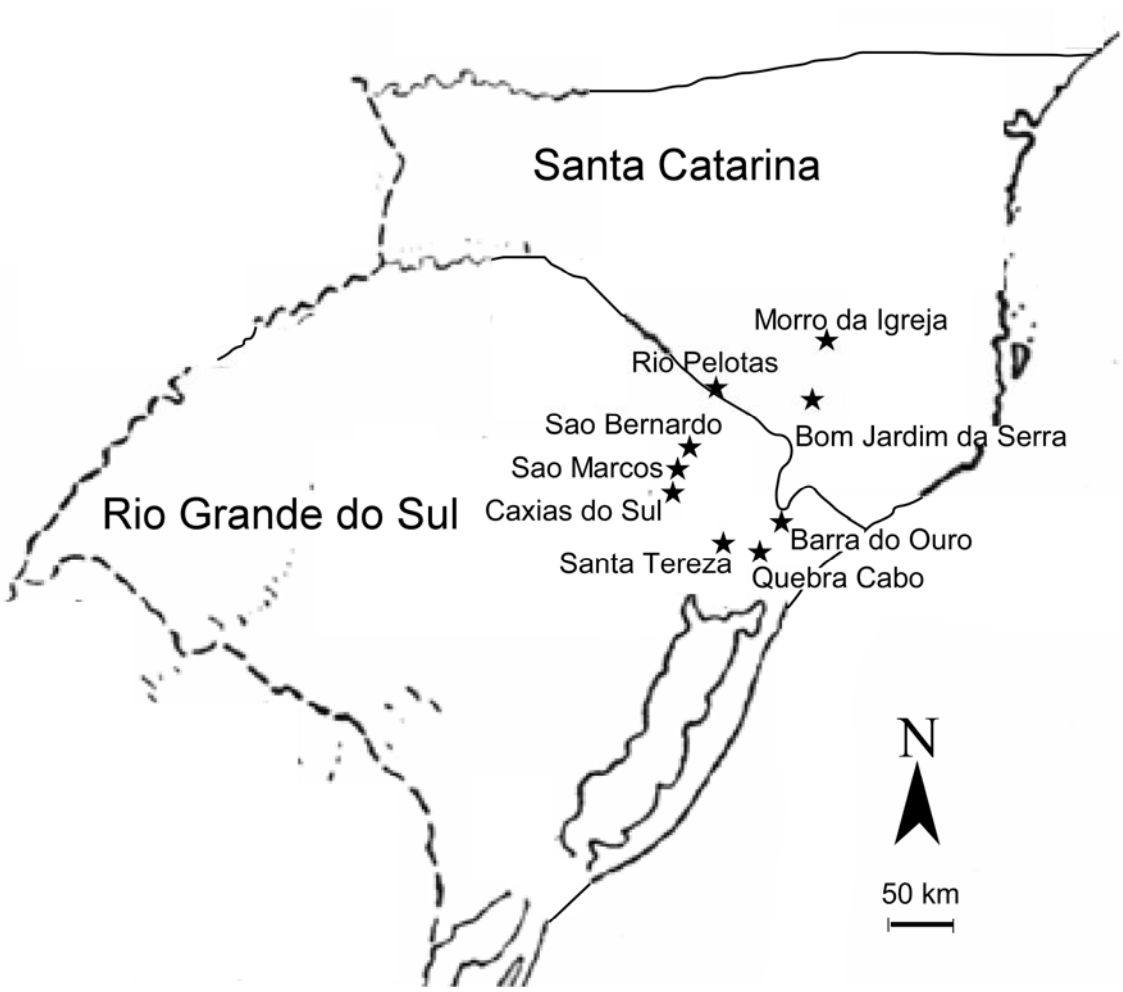
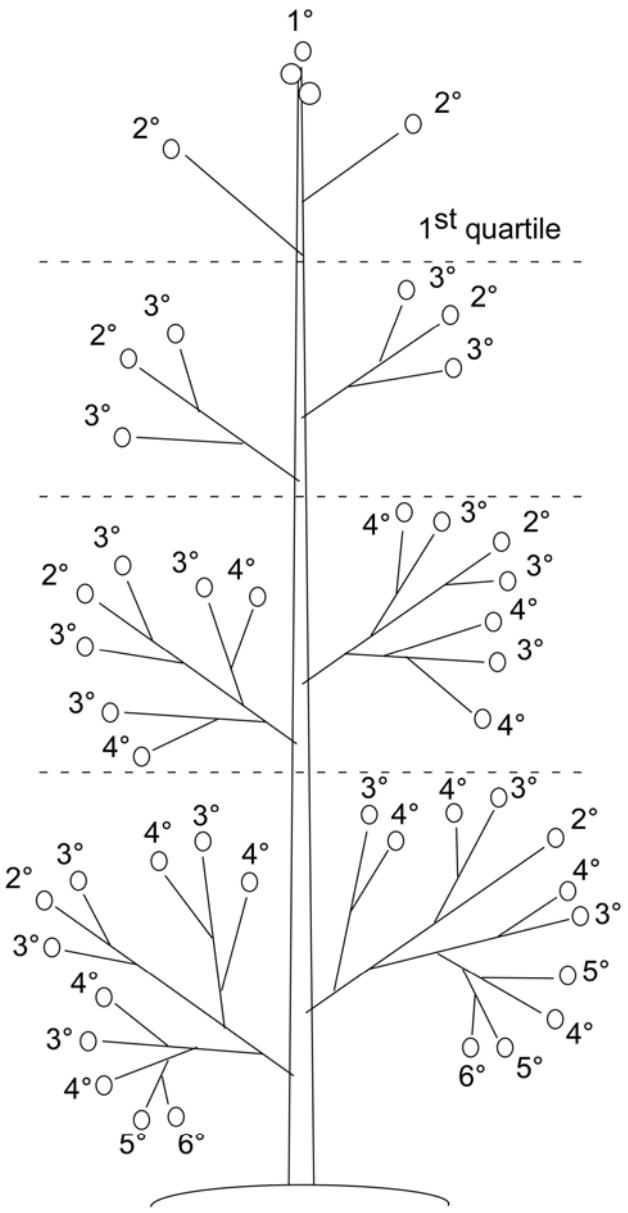

Santa Catarina
Rio Grande do Sul
Morro da Igreja
Rio Pelotas
Sao Bernardo
Bom Jardim da Serra
Sao Marcos
Caxias do Sul
Barra do Ouro
Santa Tereza
Quebra Cabo
N
50 km

Figure 2. A stylized depiction of plant architecture and flower positions in *Nicotiana*. Circles represent open flowers, which may number up to 3 on the higher order branches, but only one open flower per branch was typically found. Branch order begins with the shoots arising from the basal rosette (not shown) at the ground, which were denoted as primary branches (1°). Secondary branches (2°) emerge from a primary branch, and order progresses into lower orders (up to sixth order [6°]) as branches emerge from other branches. To approximate the distance from the top of the plant, each secondary branch was numbered consecutively beginning at the apex of the primary branch, then this number was divided by the total number of secondary branches on the plant and that percentage was broken up into quartiles (note dashed lines). The first quartile represents the apex of the plant, while the fourth quartile represents the base. Lower order branches fall into the same quartile as the secondary branch with which they are associated.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6°
5°

Figure 3. Distribution of nectar volume, concentration, and energy at approximately the time of anthesis (0 h) and 24 hours after anthesis (24 h) in multiple populations of *Nicotiana* species. Box plots represent raw data from bagged flowers only. 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 below each box plot. Letters above box plots denote species groups (a–f) identified using least squares (adjusted) means (lsmeans, SAS 9.1) of ranked data, where species sharing a letter are not significantly different (at $\alpha = 0.01$). A-C) Nectar volume, concentration, and energy at 0 h (time of anthesis). D-E) Nectar volume, concentration, and energy at 24 h after anthesis. Species abbreviations: ala = *N. alata*, forg = *N. forgetiana*, mut = *N. mutabilis*, lang = *N. langsdorffii*, bon = *N. bonariensis*. See Table 1 for population abbreviations.

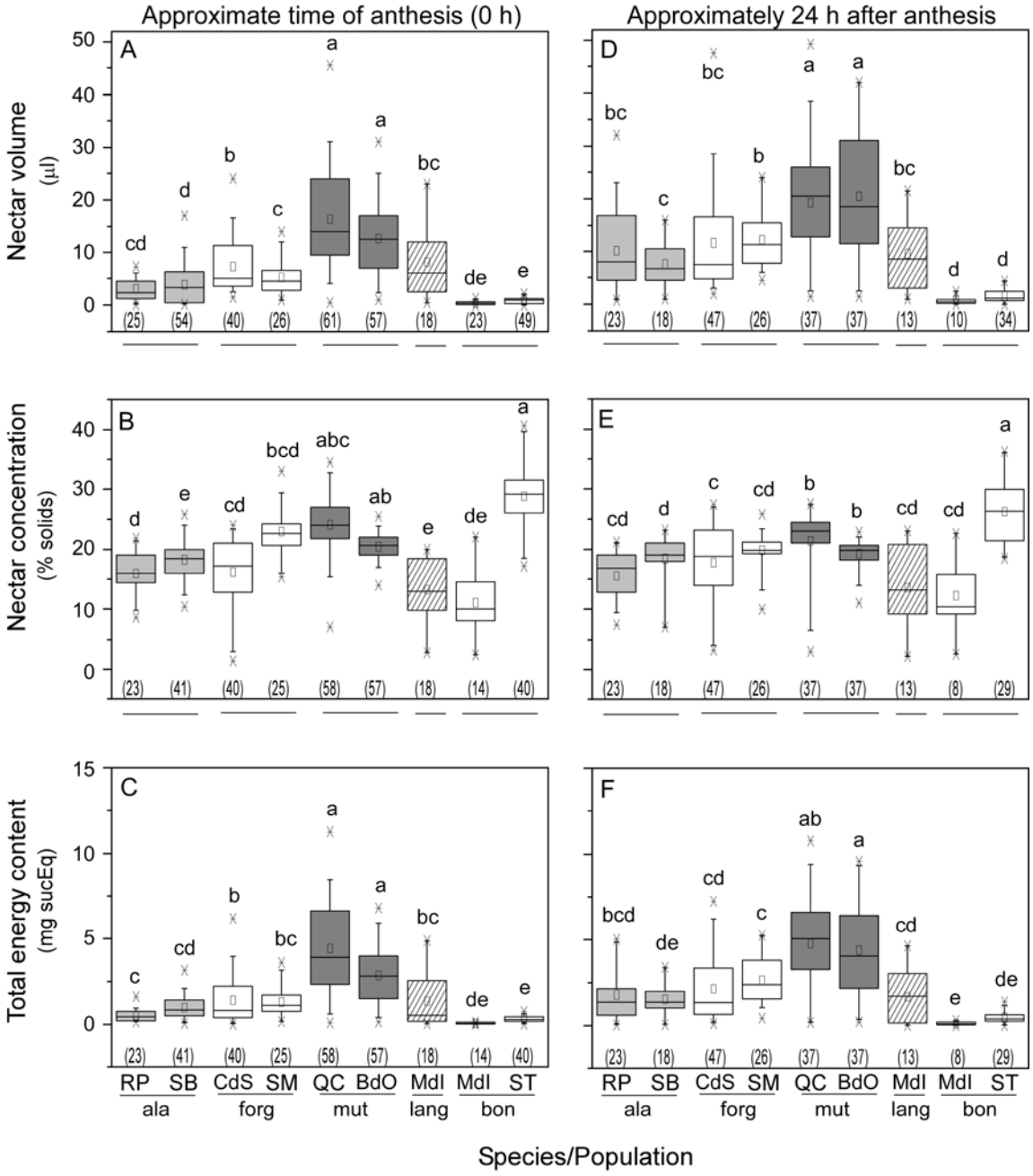
Approximate time of anthesis (0 h)
Approximately 24 h after anthesis
A
B
C
D
E
F
Nectar volume (µl)
Nectar concentration (% solids)
Total energy content (mg sucEq)
RP SB CdS SM QC BdO MdI MdI ST
ala forg mut lang bon
Species/Population

Figure 4. Mean nectar volume and concentration at approximately 0 and 24 h after anthesis in multiple populations of *Nicotiana* species. The mean and SE for both bagged (solid symbols) and exposed (open symbols) flowers were included. Nectar volume is represented by squares and is plotted on the left y-axis, while concentration is represented by triangles and is plotted on the right y-axis. The line connecting the 0 and 24 h data points is given to show the general direction of the change in both nectar traits over 24 h, although this change may not necessarily be linear. See Table 2 for significance of comparisons. Species abbreviations: ala = *N. alata*, forg = *N. forgetiana*, mut = *N. mutabilis*, lang = *N. langsdorffii*, bon = *N. bonariensis*. See Table 1 for population abbreviations.

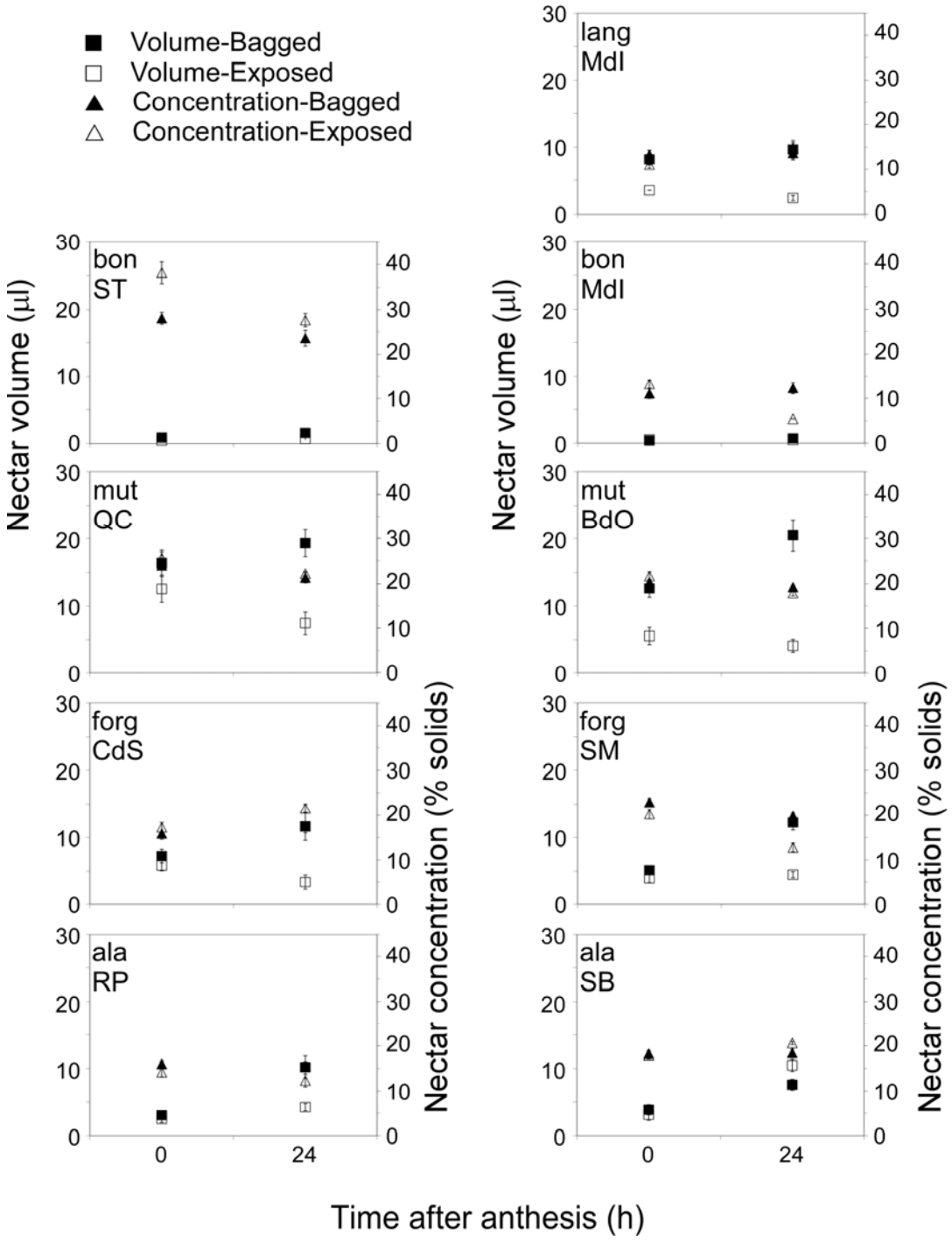
Volume-Bagged
Volume-Exposed
Concentration-Bagged
Concentration-Exposed
bon ST
mut QC
forg CdS
ala RP
lang MdI
bon MdI
mut BdO
forg SM
ala SB
Nectar volume (µl)
Nectar concentration (% solids)
Time after anthesis (h)
0
24
0
10
20
30
40

Figure 5. Greenhouse versus natural population reaction norms for nectar volume, concentration, and energy content at 0 and 24 h after anthesis. Only means included for natural populations of *Nicotiana* sampled in 2004 that were also investigated in the greenhouse. See Table 6 for standard errors and significance.

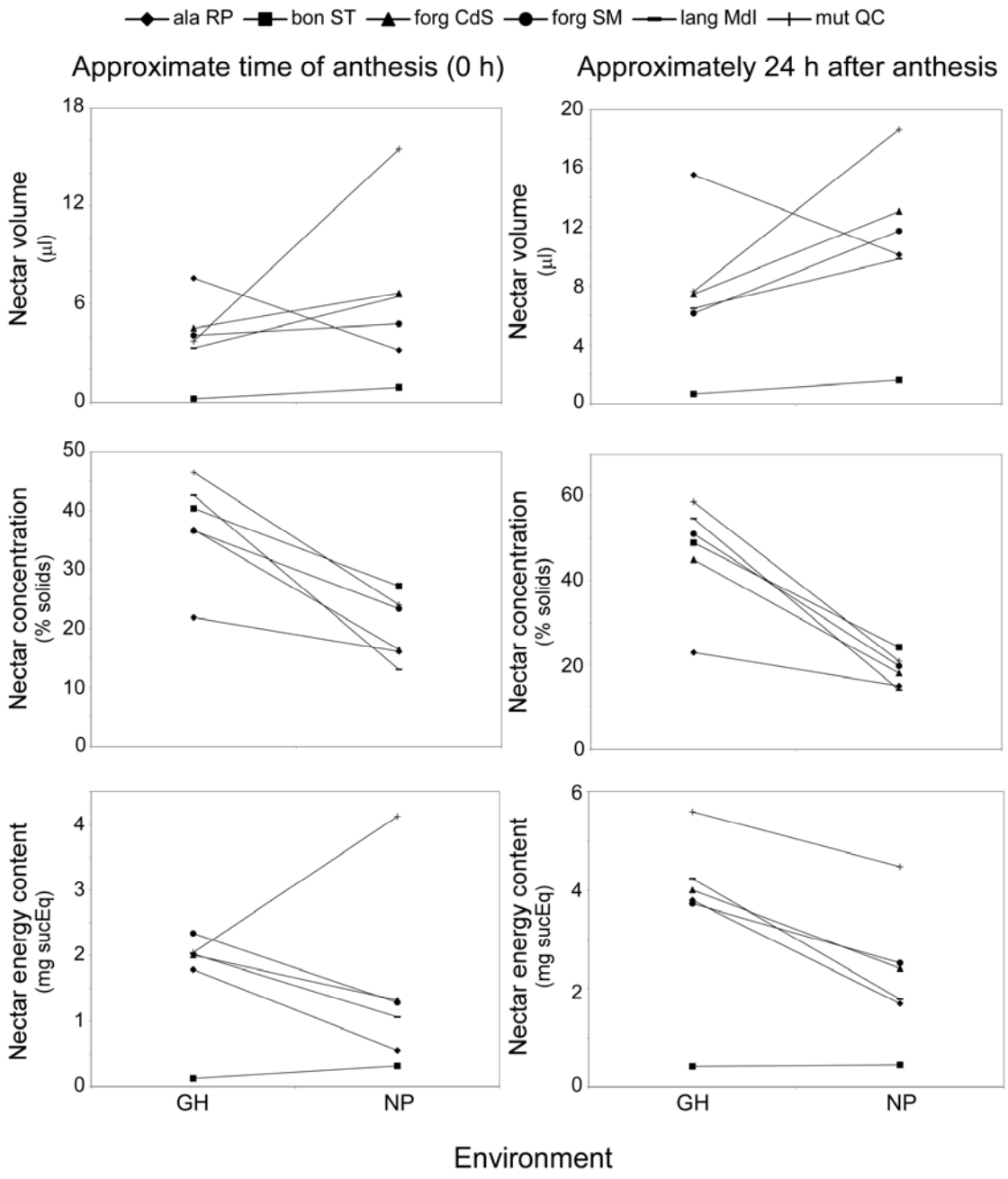
ala RP
bon ST
forg CdS
forg SM
lang MdI
mut QC
Approximate time of anthesis (0 h)
Approximately 24 h after anthesis
Nectar volume (µl)
Nectar concentration (% solids)
Nectar energy content (mg sucEq)
GH
NP
Environment

Figure 5. Nectar volume as a function of nectar concentration at 0 and 24 h after anthesis. Each data point denotes the mean nectar volume and concentration for each plant sampled in all sampled *Nicotiana* populations at 0 and 24 h after anthesis. Diamond symbols (◆) represent correlations at 0 h, while square symbols (■) represent correlations at 24 h after anthesis. The lines represent the best linear fit (0 h: dashed line, 24 h: solid lined). Correlations were not significant (see Table 8 for Pearson correlation coefficients).

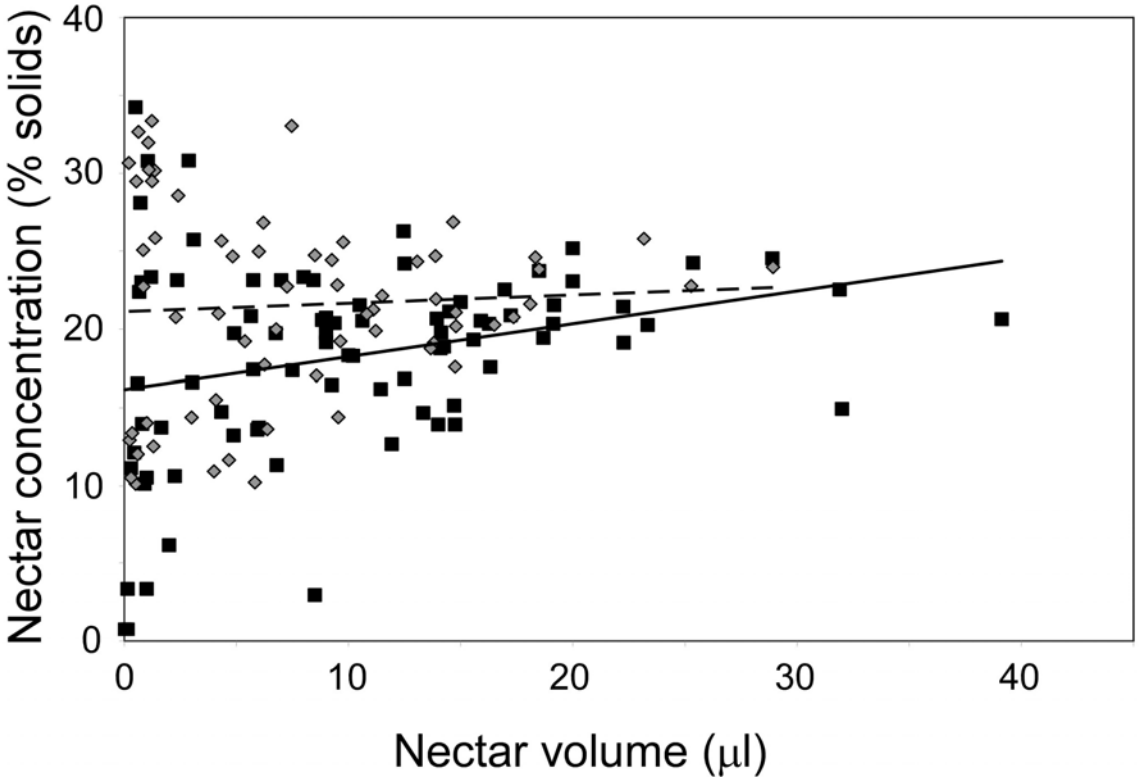
Nectar concentration (% solids)
40
30
20
10
0
0
10
20
30
40
Nectar volume (μl)

Figure 6. Whole-plant total energy as a function of the total number of flowers on *Nicotiana* plants. Each symbol represents an individual plant, indicating the nectar energy content and total number of flowers for each plant; therefore no error bars could be included. Different populations of the same species are represented by solid or open symbols of the same shape: *N. alata* (RP:▲, SB:△), *N. bonariensis* (MdI:▼, ST:▽), *N. mutabilis* (BdO:◆, QC:◇), *N. forgetiana* (CdS:■, SM:□), *N. langsdorffii* (MdI:●). The line represents the best linear fit ($r^2 = 0.6863$, $P < 0.0001$).

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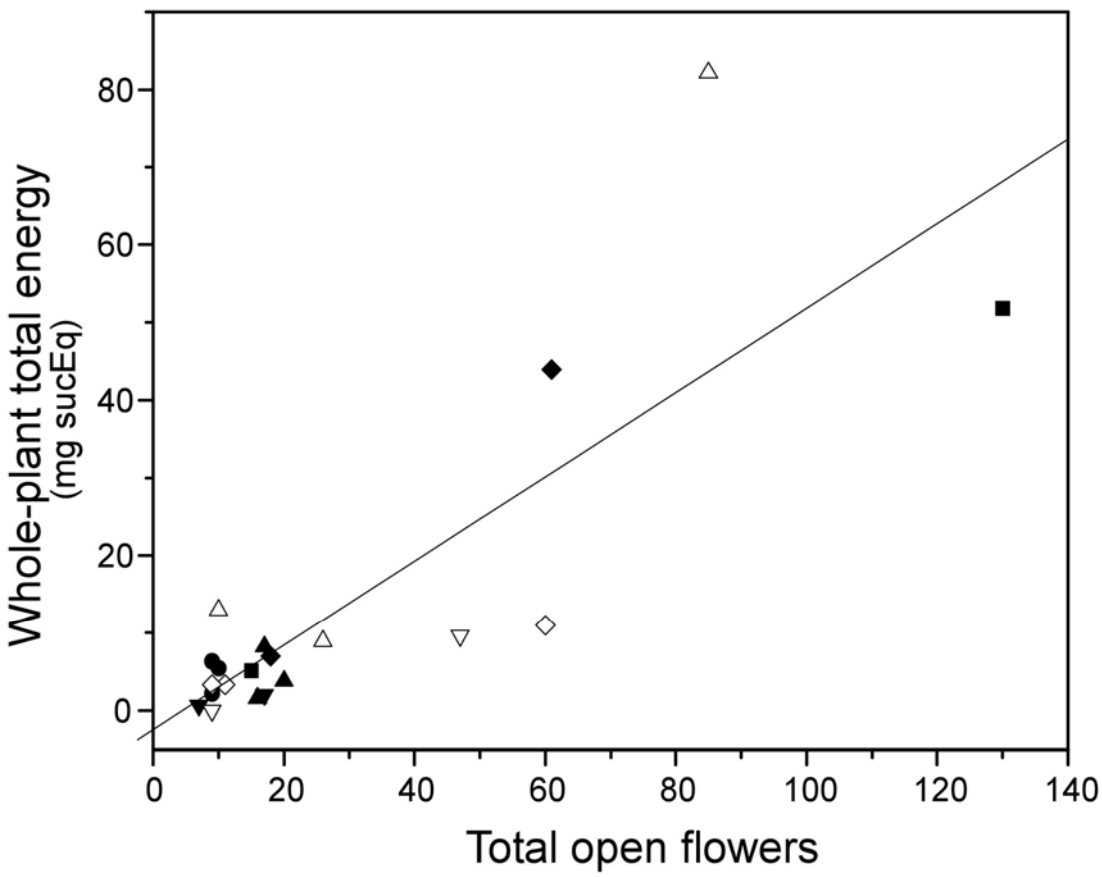

Whole-plant total energy
(mg sucEq)
0
20
40
60
80
0
20
40
60
80
100
120
140
Total open flowers

Figure 7. Average frequency of ecological factors among populations of *Nicotiana*. Bars show the percentage (mean and SE) of flowers exhibiting holes or slits, bugs or larvae, or signs of old age, averaged across all sampled plants within a population (sample size for each population shown in parentheses). Letters above bars denote population groups (a-d) within each ecological factor, identified using post-hoc tests (LSD, SAS 9.1) on raw data, where populations that share a letter are not significantly different (at $\alpha = 0.05$). Species abbreviations: ala = *N. alata*, forg = *N. forgetiana*, mut = *N. mutabilis*, lang = *N. langsdorffii*, bon = *N. bonariensis*. See Table 1 for population abbreviations.

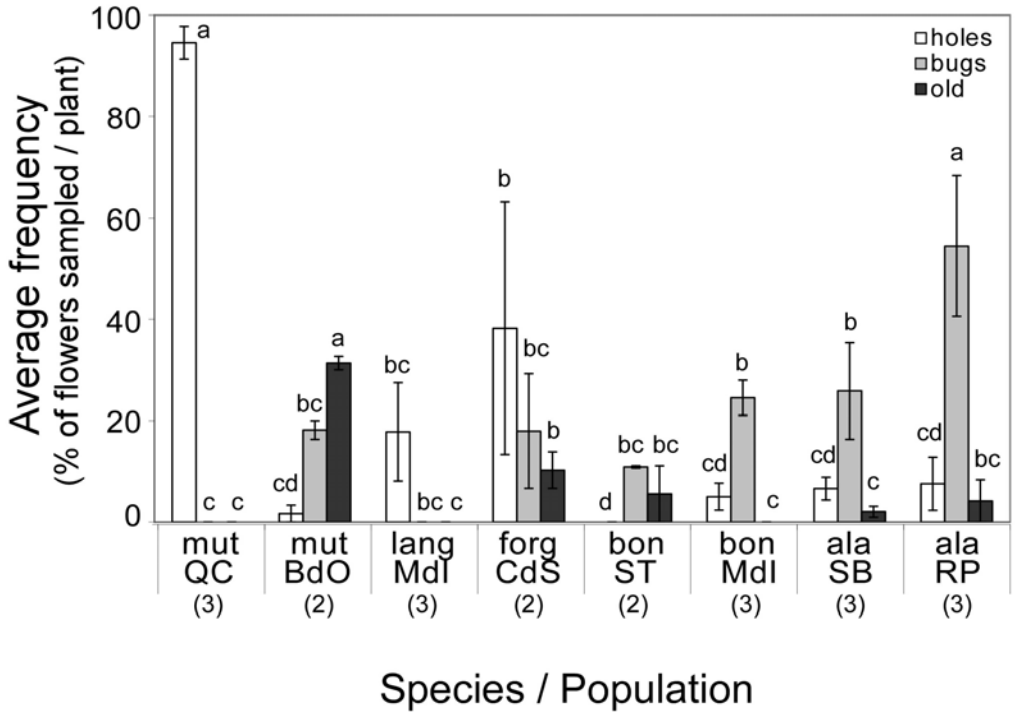
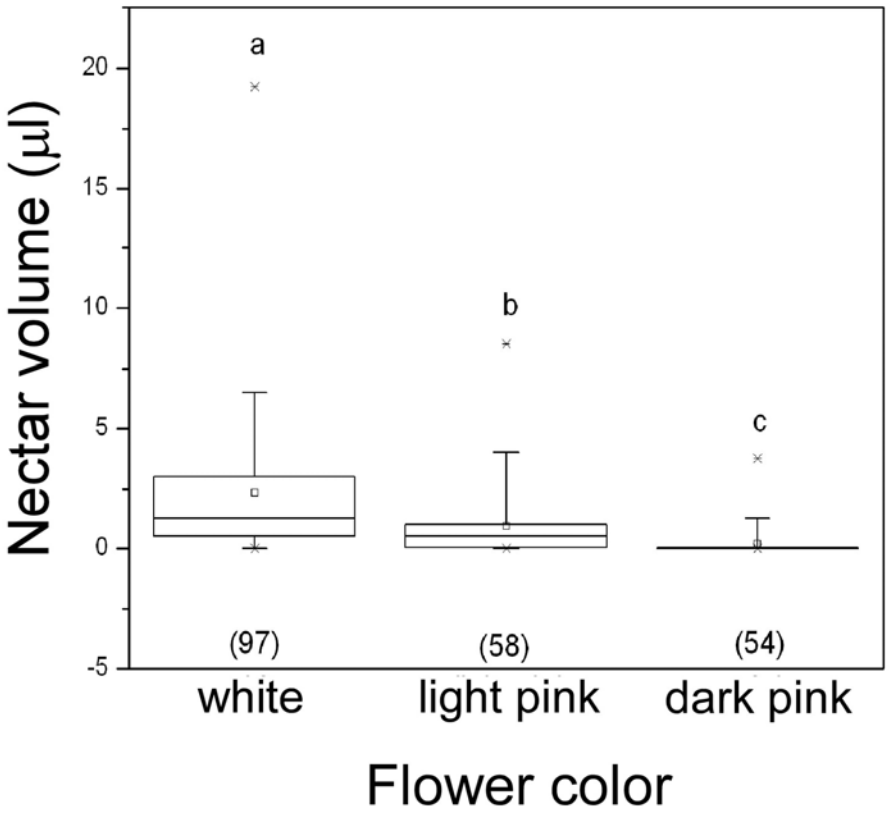

Average frequency
(% of flowers sampled / plant)
100
80
60
40
20
0
holes
bugs
old
a
c c
cd
bc
a
bc
bc c
b
bc
b
d
bc bc
cd
b
c
cd
b
c
cd
a
bc
mut QC (3)
mut BdO (2)
lang MdI (3)
forg CdS (2)
bon ST (2)
bon MdI (3)
ala SB (3)
ala RP (3)
Species / Population

Figure 8. Nectar volume according to flower color in *Nicotiana mutabilis*. Flower color in *N. mutabilis* is also a function of flower age, given that young, new flowers are white and gradually turn pinker over time. Different letters (a-c) represent significant differences (at $\alpha = 0.05$) determined by post-hoc tests (lsd lines, SAS 9.1) on raw data. Plant sample sizes are denoted in parentheses.


a
b
c
20
15
10
5
0
-5
Nectar volume (µl)
(97)
(58)
(54)
white
light pink
dark pink
Flower color

CHAPTER 4

HERITABILITY AND CORRELATION OF NECTAR AND FLORAL MORPHOLOGY TRAITS IN *NICOTIANA ALATA*

ABSTRACT

The heritability and genetic basis of nectar traits have been rarely studied in the field, where plants are exposed to environmental factors that could mask underlying genetic components. Heritabilities and variance components were estimated for nectar and morphological traits of the wild species, *Nicotiana alata*, using a partial diallel design under field conditions. I found significant heritability for nectar volume and energy content, as well as for corolla tube length. Phenotypic correlations were significant for all traits and correlations between nectar volume and energy content, and between corolla limb width and mouth diameter had a genetic basis. The genetic structure of morphological and nectar correlations in *N. alata* was significantly different from the interspecific correlations among *N. alata* and its seven closest relatives (Kaczorowski et al., 2005). There were no significant genotype by density interactions detected in one environment (Missouri), though corolla limb width and nectar volume and energy did exhibit significant genotype by environment interactions across novel (Missouri) vs. native (Brazil) habitats. This study is an important contribution to the relatively few studies of evolutionary potential and constraint on nectar traits.

INTRODUCTION

Nectar has an important role in attracting various animals to flowers to aid in pollination. Some nectar traits exhibit significant phenotypic variation (see Zimmerman, 1988; Rathcke, 1992), which has been linked to pollinator behavior. Larger nectar rewards tend to increase the frequency or duration of pollinator visitation to individual plants, inflorescences, or flowers (Zimmerman, 1983; Galen and Plowright, 1985; Thomson, 1988; Cresswell, 1990; Neff and Simpson, 1990; Mitchell, 1993). Nectar's effect on pollinator behavior is likely to increase the chances for pollen import and export, which could ultimately increase components of male and female fitness (Zimmerman, 1983; Real and Rathcke, 1991; Mitchell and Waser, 1992; Mitchell, 1993; Hodges, 1995). The link between plant fitness and variation in nectar traits suggests that there is the potential for natural selection to act upon nectar traits, but the trait must also possess significant heritability for evolutionary change to occur (e.g., Falconer, 1989).

Relatively few studies to date have investigated the heritability of nectar traits (Mitchell, 2004). Several agricultural studies on crop species detected significant genetic variation in nectar production (Pedersen, 1953; Bond and Fyfe, 1968; Hawkins, 1971; Teuber and Barnes, 1979; Teuber et al., 1990), although only two such studies estimated the heritability for this trait (0.92 for *Lotus corniculatus*, Murrell et al., 1982; 0.12 for *Capsicum annuum*, Rabinowitch et al., 1992). When investigated in a controlled environment, studies on wild species have found significant heritabilities for nectar volume (h^2 = 0.13 – 0.64; Mitchell and Shaw, 1993; Boose, 1997; Vogler et al., 1999; Klinkhamer and van der Veen-van Wijk, 1999; Worley and Barrett, 2000; Leiss et al., 2004) and total sugar (h^2 = 0.37 – 0.62; Mitchell and Shaw, 1993; Klinkhamer and van der Veen-van Wijk, 1999). Heritability for nectar concentration was significant in one of

two controlled environment studies (0.62, Klinkhamer and van der Veen-van Wijk, 1999; not significant in Mitchell and Shaw, 1993). Only two studies investigating nectar trait heritability in wild species were conducted in semi-natural field conditions. One field study estimated significant heritability for nectar production (0.26, Leiss et al., 2004); while the other field study estimated heritabilities for nectar volume and concentration that were not significantly different from zero (Campbell, 1996).

Mitchell (2004) claims that the current deficiencies in nectar research include genotype by environment (G×E) interactions, within-plant phenotypic variability, and environmental variability in the field. Field measurements are likely to include more sources of environmental variance than in the controlled environment of a greenhouse or growth chamber (Coyne and Beecham, 1987; Montalvo and Shaw, 1994; Schoen et al., 1994; Simons and Roff, 1994; Thiede, 1998, Conner et al., 2003). Because many nectar traits are sensitive to environmental conditions (see Zimmerman, 1988; Rathcke, 1992), the reduction in environmental variance in controlled environments may inflate estimates of heritability from what would be observed under natural conditions (Falconer, 1989). However, the only study to quantitatively investigate the heritability of nectar production in both controlled and field conditions calculated a higher realized heritability in the field than in the growth chamber (Leiss et al., 2004). They attribute this unexpected result to genotype by environment interactions. G×E interactions occur when the phenotypic expression of genotypes differs according to environmental conditions (Falconer, 1989). G×E effects can affect the rate and direction of evolution of a trait (Via and Lande, 1985), so it is important to estimate these when using common garden experiments to tell you about heritability in natural environments.

Phenotypic correlations between floral morphology traits and nectar traits are common (Plowright, 1987; Duffield et al., 1993; Mitchell and Shaw, 1993; Campbell, 1996; Davis, 1997; Klinkhamer and van der Veen-van Wijk, 1999). These correlations are often assumed to be adaptive, as floral traits may offer an honest cue to the nectar status within the flower. Morphological traits can often restrict access to nectar by pollinators (Campbell et al., 1996; Lange et al, 2000; Ree, 2005; Darrault and Schlindwein, 2005) and, therefore, may be more important to successful pollinator visitation than nectar traits themselves. The adaptive nature of phenotypic correlations between nectar and floral morphology traits depends upon underlying genetics. Genetic correlations between nectar traits and morphological traits could allow pollinators to select on a floral morphology trait with benefits in increased reward. Significant genetic correlations have been demonstrated between nectar traits and morphological traits (Mitchell and Shaw, 1993; Campbell, 1996; Klinkhamer and van der Veen-van Wijk, 1999; Vogler et al., 1999). Significant phenotypic correlations between nectar and morphological traits were found across species in previous studies (Kaczorowski et al., 2005 [chapter 2] and chapter 3). If those correlations were due to some degree of constraint, I might also expect to find significant genetic correlations for the same trait combinations within a species.

Heritability can be estimated through a variety of means. Many of the studies investigating the heritability of nectar traits used clonal replication techniques to estimate broad-sense heritability (Mitchell and Shaw, 1993; Boose, 1997; Klinkhamer and van der Veen-van Wijk, 1999; Vogler et al., 1999), which is expected to inflate narrow sense heritability estimates because they include non-additive sources of genetic variation and

provide more of an upper-bound on narrow-sense heritability (Falconer, 1989). Leiss et al. (2004) estimated realized heritability based on response to one generation of selection on nectar production, while Worley and Barrett (2000) inferred significant heritability in nectar production through a correlated response to selection on flower number and size. Only two studies on wild species used breeding designs and sibling analysis to estimate narrow-sense heritability, both of which used a nested half-sib design (Mitchell and Shaw, 1993; Campbell, 1996). Campbell (1996) also used a parent-offspring regression to compare with the nested half-sib design.

I used a partial diallel crossing design to estimate narrow-sense heritability and genetic correlations. This method is good when there are a limited number of parental plants. A complete diallel, where all possible crosses between a set of parents is made, can be quite impractical when many parents are included in the design. A partial diallel generates the same sib relationships of the complete diallel, but with only a fraction of the possible crosses (Lynch and Walsh, 1998). Although the diallel methods require relatively larger sample sizes and more complicated analyses compared to most alternatives, the additional information gained from such methods generally outweigh these disadvantages. More variance components (e.g., maternal, parental interaction and dominance variance components) can be estimated more precisely when using diallel designs compared to other sibling analyses because more sib relationships are produced (Lynch and Walsh, 1998). Diallel designs can often accommodate more sires than possible in nested half-sib designs and therefore could generate better estimates for genetic correlations. Diallel designs can also have the advantage over realized heritability and selection responses in that heritability can be accurately estimated for multiple traits

on a given set of plants simultaneously, while only one trait can be selected for in a given set of plants in selection-based experiments (Conner and Hartl, 2004).

I previously established that there was significant phenotypic variation in nectar volume, concentration, and energy content within *Nicotiana alata* and other *Nicotiana* species that tended to correspond to the predominant pollinator of the species (Kaczorowski et al., 2005). In this study, I performed a large field experiment in Missouri to estimate heritability and genetic correlations. I also performed a smaller field experiment in Brazil to compare the environmental variance of traits in Missouri versus Brazil. This comparison sheds light on whether heritability estimates in Missouri are a good approximation of heritability in Brazilian populations. The objectives of this study were 1) to determine the heritability of nectar traits and morphological traits in a large common garden, 2) to determine whether there were any significant genotypic or phenotypic correlations among and between nectar and morphological traits, 3) to determine whether there were any significant density effects or family by density interactions within the Missouri experimental plot, and 4) to determine whether there were any significant Missouri vs. Brazil G×E interactions in nectar and morphological traits within *Nicotiana alata*.

MATERIALS AND METHODS

Study system

Nicotiana alata Link and Otto is a member of the monophyletic clade of section *Alatae* (Ippolito et al. 2000; Chase et al., 2003). It has several features of a hawkmoth pollination syndrome including long, tubular, white flowers that emit a strong fragrance

at night (Raguso et al., 2003). Plants are self-incompatible (McClure et al., 1990) and native to southern Brazil and adjacent areas in Paraguay, Uruguay and eastern Argentina (Goodspeed, 1954). Typical habitats of *N. alata* are rocky slopes and stream sides, but it commonly spreads along roadsides. The source population of the seeds used in this study is Rio Pelotas, which can be found where the Pelotas River intersects highway BR-116 at the state lines of Santa Catarina and Rio Grande do Sul, Brazil. Plants of this population can be found emerging from the rocky slopes, along the river, and along the road.

Quantitative genetic experiment (Missouri)

Experimental design

Seeds collected from 44 different wild plants from the source population (Rio Pelotas) were germinated in a greenhouse (14-h days at c. 24°C and 10-h nights at c. 13°C at the University of Missouri–Columbia), where pots were watered as necessary. One progeny from each of the 44 different plants was chosen to serve in a partial diallel cross design, where each plant served as a dam in four crosses and as a sire in four different crosses (Fig 1). Twelve progeny (sometimes less due to lack of germination) from each of the 176 full-sib families were grown in the greenhouse (and watered as necessary) until they were large enough to be transplanted outdoors. In May 2004, plants were transplanted into the experimental plot (approximately 30 × 22 m) at the University of Missouri-Columbia Genetics Farm. While plants were young and small, the plot was occasionally weeded and additional water was given as needed, but no fertilizer was ever used. The experimental plot consisted of two blocks with two replicates each, and replicates containing three different density treatments (high [approximately 15 cm. apart within rows], medium [30 cm.], or low [45 cm.]). Each treatment contained one plant

from each of the 176 different full-sib families. The entire plot was surrounded by border plants to reduce edge effects.

Some plants died before they could be placed into the experimental plot and mortality occurred throughout the experiment. To evaluate the status of each plant, a survey was taken on 24 June, 2004 (early survey), as well as immediately before or after the sampling period for each replicate (late survey). Since mortality disrupted our three density categories, I estimated a continuous metric of "crowding" as the average distance to the next plant in the row on either side of the sampled plant at the early and late survey.

Sampling

Each of the four replicates was sampled completely on separate nights, so that the block term included night to night variation. On each plant within a replicate, at least 2 flowers that appeared ready to open on the following day were enclosed individually with hand-perforated plastic bags (beaten with hand-made nail stick). On the following day, sampling began around 1800 CDT, when most flowers were beginning to open, and continued for five to seven hours. An open, bagged flower from each plant was chosen as the plant sample and removed from the plant. The time of flower removal was recorded because nectar traits can change over time (Rathcke, 1992; Kaczorowski et al., 2005). Morphological measurements (corolla tube length, limb width, and mouth diameter) were taken immediately with calipers and then the flower was destructively sampled to collect the nectar. To access the nectar, 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 with 50 µl calibrated glass micropipette tubes (Drummond "Microcaps") and the volume was

recorded. Concentration measurements were determined using a temperature-compensated refractometer, as the percentage solids in solution (sucrose equivalents [sucEq]). Total energy was calculated as the product of nectar volume and concentration, which was converted from wt/wt to wt/vol, as suggested by Bolton et al. (1979).

Quantitative genetic analysis

Phenotypic variation in *Nicotiana* nectar and floral traits was partitioned into genetic and environmental components of variation using a mixed model approach under a full biological model (Cockerham and Weir, 1977; Shaw 1987; McLean et al., 1991; Searle et al., 1992; Lynch and Walsh, 1998). This model partitions the total phenotypic variance into the following six casual components:

$$V_P = V_N + V_T + V_{mat} + V_{pat} + V_K + V_E;$$

where V_P represents the total phenotypic variance. Under standard interpretation of the bio-model, V_N is additive nuclear genetic variance (variance caused by the simple additive action of nuclear alleles), V_T is non-additive nuclear interaction variance (variance caused by the interaction of alleles at a nuclear locus, including both dominance and epistasis), V_{mat} is maternal variance caused by contributions other than those from nuclear genes (maternal environmental or cytoplasmic effects), V_{pat} is paternal variance caused by contributions other than those from nuclear genes (paternal environmental effects), V_K is variance caused by parental interactions other than among nuclear genes (nuclear-extranuclear and extranuclear-extranuclear interactions), and V_E is the environmental variance. In addition, spatial blocking, time of sampling, and the

crowding metric were included as fixed factors in the model used to estimate heritability, although separate analyses also explored density as a fixed factor to compare with the analyses using the crowding metric. Interactions between fixed factors and other factors in the full biological model were not explored because of low power given the large number of parameters under a full model, although density by genotype interactions were investigated in a simplified model (discussed below). The total phenotypic variance (V_P) for each trait was obtained by summing all of the variance components for the trait. The true additive genetic variance (V_A) was calculated as $4 \times V_N$ (V_N = covariance of half-sibs = $V_A / 4$; Lynch and Walsh, 1998). Heritability (h^2) was calculated as V_A / V_P (Falconer, 1989, Lynch and Walsh, 1998). The additive genetic coefficient of variation ($CV_A = 100 \times \sqrt{V_A}$ / mean), which standardizes V_A by the mean was also calculated.

Because the design was unbalanced, an iterative restricted maximum likelihood method (REML) was utilized for significance testing and the estimation of variance components using PROC MIXED in SAS (Shaw, 1987; Searle et al., 1992; Littell et al., 1996). Nectar volume and energy values were log (1+y) transformed before analysis to improve homoscedasticity. Likelihood tests were used to evaluate the statistical significance of individual components of variance. Programming code for the REML partitioning of a partial diallel using PROC MIXED follows that of Juenger et al. 2005.

I investigated phenotypic correlations among this set of plant traits using a standard Pearson product-moment correlation between the paired raw data from the large quantitative genetics experiment. I evaluated genetic correlations as the Pearson product-moment correlation between additive genetic breeding values as estimated by Best Linear Unbiased Prediction (BLUPs) under the full bio-model analysis. The significance of

phenotypic and genetic correlations was determined by *t*-tests after *z*-transformation of the correlation coefficient. Levels of significance for phenotypic and genetic correlations were not adjusted for multiple tests.

Density interactions

Because density main effects were significant for many variables (data not shown), a separate analysis was performed to explore the interactions between density and families (half- and full-sib). I used block, dam, sire, and density, including interactions, to analyze corolla tube length, limb width, mouth diameter, nectar concentration, volume, and total energy content (the latter two variables were log [1 + y] transformed before analysis; PROC GLM in SAS 9.1). A numeric time variable was also used as a covariate.

Interspecific phenotypic correlations

In addition to the intraspecific phenotypic correlations found in this study, phenotypic correlation coefficients from a previous *inter*specific study (Kaczorowski et al., 2005) were also estimated to compare with the intraspecific correlation coefficients found. Interspecific correlations were estimated through linear regressions of population means of nectar traits and morphological traits from different sets of plants produced from the same populations (see Volskay, 2002 for methods on morphological measurements).

Validation experiment (Brazil)

Experimental design

In order to determine whether heritability estimates in Missouri would adequately represent trends in the native habitat of Brazil, broad G × E interactions between

Missouri and Brazil were explored. Ten different full-sib families used in the Missouri field experiment were grown at the Experimental Station of Campestre in the state of Rio Grande do Sul, Brazil, within 25 km of natural *N. alata* populations. Seeds were germinated in a hoop greenhouse and seedlings grown until they were large enough to be transplanted outdoors, although transplants in the Missouri experiment were grown in the greenhouse longer and therefore were larger when transplanted. In December 2004, the small plants were transplanted into a plowed plot (approximately 14 × 18 m) and watered as necessary while the plants were small. No weeding or fertilizing occurred in this experimental plot. Although some biotic and abiotic factors can potentially differ between the farm plot and natural roadside populations, the environmental conditions were likely to be very similar between the two areas. The experimental plot consisted of two replicates, each with two density treatments (0.5 m and 1.0 m). Each of the four treatments contained two progeny of each of the ten families and was surrounded by border plants. Mortality was almost completely limited to border plants; only two of the 80 experimental plants died during the experiment, and therefore a crowding metric was not calculated.

Sampling

Sampling of the entire Brazil plot occurred on 25 January, 2005. The plants were much smaller, with fewer flowers, than the plants in the Missouri plot at the time of sampling, which could have affected nectar traits. Sampling occurred in generally the same fashion as the Missouri plot was sampled, though flowers were bagged just before sampling began as opposed to the day prior and up to three flowers per plant were

sampled as opposed to only one flower per plant, which also may have affected nectar traits. Sampling began around 1830 GMT-2 and lasted for approximately four hours.

Analysis

I used environment (Missouri and Brazil), full-sib family, and their interaction as independent variables to analyze nectar volume, concentration, and energy, plus corolla tube length, limb width, and mouth diameter (PROC GLM, SAS 9.1).

RESULTS

Quantitative genetic experiment (Missouri)

In the Missouri field plot, significant additive nuclear genetic variance (V_N) was detected for tube length, nectar volume and nectar energy (Table 1; $\chi^2 = 9.5$, $P < 0.0001$; $\chi^2 = 4.5$, $P < 0.05$; $\chi^2 = 8.0$, $P < 0.0001$; respectively), but not for limb width, mouth diameter, or nectar concentration. Other variance components (V_T, V_{mat}, V_{pat}, and V_K) were not significantly different from zero, suggesting that the genetic variation in these traits is relatively simple. However, our power to detect significant interactions (dominance and epistasis) was low. The highest CV_A values were estimated for the traits that had significant heritability, though the relationship between h^2 and CV_A was not very linear (corolla tube length had the highest h^2, but with a lower CV_A than nectar volume and energy; Table 2).

The highest degree of heritability was estimated for corolla tube length at 0.160, followed by nectar energy at 0.107 and nectar volume at 0.069 (Table 1). Block effects were highly significant for all traits, and included both a positional (north to south) and time component (four sampling nights from July to September), which were confounded.

Time effects within a sampling night were also significant for all traits because nectar volume increases throughout the night (Kaczorowski et al., 2005 [chapter 2]), although time effects were not as strong as block effects. Morphological traits were more affected by plant density than nectar traits ($P < 0.0001$; Fig 2, Table 1). Nectar concentration and energy lacked a significant density effect and nectar volume had only a mildly significant density effect ($P = 0.02$; Table 1). Similar results, with only a few differences in significance, were found when the crowding covariate, as opposed to the density factor, was used in the analyses (data not shown).

Density interactions

In the separate GLM model, no significant interactions between density and either half-sib or full-sib families were detected.

Correlations

Although significant phenotypic correlations were found for all trait combinations (positive; $P < 0.0001$, except $P = 0.01$ between corolla tube length and nectar concentration; Table 3, Fig. 3, above the diagonal), significant genotypic correlations were only detected between corolla limb width and mouth diameter, as well as between nectar volume and total energy (Table 3, Fig. 3, below the diagonal), which are computationally confounded. Full-sib family correlations detected more significant positive correlations (9 out of 15) than the BLUP-determined genotypic correlations, but fewer than the phenotypic correlations (Tables 3 and 4). Among full-sib families, tube length was not significantly correlated with any nectar trait and nectar concentration was not significantly correlated with any trait except nectar energy, with which it is

computationally confounded. All other full-sib family correlations were positive and significant ($\alpha = 0.01$).

Interspecific phenotypic correlations

Considerably fewer phenotypic correlations were found to be significant interspecifically (6 out of 15) in a previous study (Kaczorowski et al., 2005; Table 4), than those found intraspecifically in this study. Interspecifically, all phenotypic correlations between corolla tube length, limb width, nectar volume and nectar concentration were significant. However, those correlations involving nectar concentrations had negative correlations (Table 5) whereas no negative correlations were detected intraspecifically.

Missouri vs. Brazil

There was a significant environment effect between Missouri and Brazil for all traits except mouth diameter (Table 6). However, significant Missouri vs. Brazil G×E interactions were only detected in limb width, nectar volume and energy content (Table 6, Fig. 4). Therefore, these were the only traits for which the ranking of full-sib families was affected by which environment the plants were grown. Corolla tube lengths tended to be longer in Brazil than in Missouri, while nectar concentrations and energy content tended to be higher in Missouri than in Brazil. Mouth diameters were not significantly different between the two environments.

DISCUSSION

Heritability, though low, was significant for nectar volume and total energy (or total sugar production) (Table 1). To my knowledge, this is the second field study on a

wild plant species that has demonstrated significant heritability for nectar production (Leiss et al., 2004), though methods for estimating heritability were different. However, perhaps because it is not often investigated, this is the first field study to my knowledge that has demonstrated significant heritability in total sugar production. Total energy is simply the product of nectar volume and concentration, but has been argued to be the most appropriate nectar variable to consider for plant-pollinator interactions because it is more informative than just nectar volume or concentration alone (Rathcke, 1992). The significant genetic correlation between total energy and nectar volume (Table 3, Fig. 3) supports evidence that nectar volume is more responsible for the majority of the variation within total energy than nectar concentration (Rathcke, 1992). Despite the fact that total energy is dependent upon nectar volume, the heritability for total energy was almost double that of nectar volume in this study (Table 1). It is possible that total energy hay have greater homeostasis in relation to environmental effects because changes in volume could compensate for changes in concentration.

Heritabilities estimated for nectar volume and total energy in this study were the lowest significant values of any of the studies investigating nectar heritability. Because this study was performed in the field, not the greenhouse, there was probably a large amount of environmental variance that diminished the amount of additive variance detected for any trait. Campbell (1996) suggested that the low, non-significant heritability determined for nectar traits was a result of the high environmental variance present in the field because both nectar volume and concentration had higher additive variance components than most of the other traits measured. The other field study investigating nectar trait heritability used realized heritability estimates through artificial selection

(Leiss et al., 2004). This method is a more direct approach that often has greater statistical power than sibling analyses (Conner and Hartl, 2004) and therefore is more likely to detect significant heritability despite environmental variance.

Nectar concentration did not exhibit significant heritability in this study (Table 1). Only one study has estimated significant heritability for nectar concentration, using clonal repeatability (Klinkhamer and van der Veen-van Wijk, 1999), and therefore is likely to be an overestimation of the actual heritability in natural environments. However, with a broad-sense heritability of 0.62 in that study, there may still be a significant degree of heritability even after non-additive variance components are taken into account. The other two studies that investigated nectar concentration found that it lacked significant heritability, both in the field (Campbell, 1996) and in clones and crosses studied in the controlled environment of a lathhouse (Mitchell and Shaw, 1993).

Nectar concentration also lacked any significant genetic correlations with other traits (Table 3), despite the fact that this trait is used in calculating the total energy (or amount of sugar) in nectar. Campbell (1996) found a significant positive genetic correlation between nectar concentration and volume, but did not include total energy as a variable. The only other study to include nectar concentration as a variable in genetic correlation calculations was unable to estimate these values for this trait because of low sample sizes and/or negative estimates of V_A (Mitchell and Shaw, 1993).

The only morphological trait to exhibit significant heritability was corolla tube length, which exhibited the highest heritability value estimated in this study (Table 1). Corolla tube length can be an important character for the access to nectar by pollinators (Campbell et al., 1996; Ree, 2005; Darrault and Schlindwein, 2005). In fact, while typical

hawkmoth pollinators observed for *N. alata* (e.g., *Agrius cingulata* and *Eumorpha labruscae*) strongly preferred *N. alata* over *N. forgetiana* (a shorter-tubed hummingbird-pollinated species) in experimental sympatric plots; a smaller hawkmoth (likely *Callionima nomius*) preferred the shorter-tubed *N. forgetiana* over *N. alata* (Ippolito et al., 2004). This unexpected visitation was presumably because of the small hawkmoths' restricted access to the nectar in *N. alata*. However, nectar traits were not significantly genetically correlated with corolla tube length or any other floral morphology trait (Fig. 3). Therefore, the assumption that correlations between nectar and floral morphology traits may be adaptive by offering a cue of nectar status to pollinators is not supported in this study.

No significant heritability was found in the other morphological traits, corolla limb width and mouth diameter, though there was a significant genetic correlation between them (Table 3, Fig. 3). A recent analysis of many heritability studies determined that mean heritability for corolla traits was among the highest of the traits investigated and that genetic correlations between corolla size traits were usually high and positive (Ashman and Majetic, 2006), despite the fact that they were not detected here. Though significant genetic correlations between morphological traits and nectar traits have been detected in previous studies (Mitchell and Shaw, 1993; Campbell, 1996; Klinkhamer and van der Veen-van Wijk, 1999; Vogler et al., 1999), no such correlations were found to be significant in this study.

I chose plant density to be a known environmental effect in the study to quantify how these traits might respond to this environmental variance. Although the density treatments were disrupted by significant mortality, it apparently did not change the effect

of the initial planting density since the crowding metric gave very similar resuts. All morphological traits were strongly affected by plant density, while nectar volume was the only nectar trait affected, and only mildly so (Fig. 2, Table 1). However, the significant density effect suggests that plants in the higher density were competing for resources because flowers were smaller with less nectar under high density. Therefore, nectar volume and especially floral morphology traits appear to be more dependent upon resources than nectar concentration. Additionally, heritability estimates and genetic correlations are likely to be affected differentially between these traits because these traits are affected to different degrees by plant density (and potentially other environmental effects).

It can sometimes be misleading to discuss heritability measured between different traits and/or studies, in part because heritability is dependent upon the mean values of the trait under investigation, which are often unreported. Therefore the coefficient of additive genetic variation (CV_A) may be a more appropriate statistic for comparing data sets than heritability estimates because it standardizes V_A by the mean, while it can often be more informative about relative variabilities and evolvabilities than heritability values themselves (Houle, 1992). The high CV_A values for nectar volume and energy demonstrate that even though h^2 was low for these traits, there was still a large degree of additive variation present, possibly more than that in tube length which had a higher heritability estimate (Table 2).

All phenotypic correlations were determined to be significant (Table 3), though the strength of the phenotypic correlations ranged from very strong (nectar volume and energy, $R^2 = 0.88$, $P < 0.0001$) to very weak (nectar concentration and corolla tube

length, $R^2 = 0.01$, $P = 0.01$) (Fig. 3). The strongest phenotypic correlation between nectar volume and energy was also the stronger of the two significant genetic correlations. Detection of significance in the weak correlations is likely due to the large sample size (plant $N = 1100$). Full-sib family means were better at estimating genetic correlations than phenotypic correlations (Table 4). However, these correlations are likely to include dominance variance, which can mask genetic correlations, though the dominance / epistatic variance component (V_T) was not significant for any trait in this study (Table 1).

Results from the interspecific study showed that floral size (corolla tube length) was a good predictor for nectar volume or concentration across species within *Nicotiana* section *Alatae* (Kaczorowski et al., 2005). This result seems to hold within *N. alata* as well, though correlations between nectar concentration and other traits were in the opposite direction from that found interspecifically (compare Tables 3 and 5). However, there was no significant genetic basis for these correlations (Fig. 3). The structure of genetic variation may have changed as the species of *Nicotiana* section *Alatae* evolved.

Despite significant density effects for morphological traits and nectar volume (Fig.), there were no significant family (half- or full-sib) by density (G×E) interactions detected within the Missouri experimental plot. However, there were some significant full-sib family by environment interactions detected between Missouri and Brazil. Significant G×E effects between Missouri and Brazil were detected for corolla limb width, nectar volume and total energy (Fig. 4). However, because full-sib families were used in this comparison, it is possible that dominance or maternal effects are included in these estimates, although V_T and V_{mat} were not significantly different from zero in the

Missouri experiment (Table 1). Using half-sib families would have strengthened the comparison between Missouri and Brazil environments.

Significant family by density interactions have been detected previously for corolla traits (Mazer and Schick, 1991), but density has not been investigated in relation to nectar traits to our knowledge. However, significant G×E interactions for nectar traits have been detected when compared across different water treatments (Boose, 1997; Leiss and Klinkhamer, 2005), light availability (Boose, 1997), blooming years (Campbell, 1996), and between hot, cool, and potbound conditions (Vogler et al., 1999).

This study has detected significant heritability in the nectar volume and energy of *N. alata*, despite being estimated in the field, where environmental variance is typically high. This suggests that nectar traits could respond to selective pressure. Although genetic correlations were only significant among nectar traits (volume and energy) and morphological traits (limb width and mouth diameter), and not between them, significant phenotypic correlations did link morphological and nectar traits together. However, without a significant genetic correlation, indirect selection of nectar traits via morphological traits does not seem likely within *N. alata*.

Mitchell (2004) stated that 'G×E interaction is potentially the most important under-explored area of nectar research.' Our study contributes to the four that have previously investigated G×E interactions in nectar traits (Campbell, 1996; Boose, 1997; Vogler et al., 1999; Leiss and Klinkhamer, 2005). This study is the first to examine G×E effects in nectar traits across different plant densities. Even though the genotypic expression of nectar traits was not affected by density in the Missouri experiment, the differences in environment between Missouri and Brazil did effect the genotypic

expression of nectar volume and energy. However, I cannot determine which factors of the different environments contributed most to these effects. These significant G×E interactions would likely alter the results for the heritability and genetic correlations if the experiment was conducted in another environment. This demonstrates that using results from single experiments to generalize in a broader sense can often be misleading. Regardless, my results offer important evidence to support the other study (Leiss and Klinkhamer, 2005) showing that nectar traits can be heritable in the presence of the large degree of environmental variation encountered in the field. And that heritable component may allow for nectar traits to evolve under selective pressure by pollinators.

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Table 1. Heritability, variance components and significance of the various effects for each trait studied within *Nicotiana alata*. Significance for the variance components determined from likelihood tests, while the significance for the fixed effects determined in the bio-model analysis.

	Tube		Limb		Mouth		Concentration		Log(volume)		Log(energy)	
Narrow-sense heritability (h^2) [a]	0.170	*	0.034	ns	0.044	ns	0.016	ns	0.069	*	0.107	*
Additive variance (V_A) [b]	6.004		1.192		0.056		0.160		0.032		0.016	
Random Effects	Variance components											
Additive nuclear (V_N)	1.501	*	0.298	ns	0.014	ns	0.040	ns	0.008	*	0.004	*
Non-additive nuclear interactions (V_T)	0.676		0.970		0.039		0.343		0.000		0.000	
Paternal environmental (V_{pat})	0.000		0.000		0.000		0.000		0.000		0.000	
Maternal environmental (V_{mat})	0.000		0.000		0.011		0.076		0.000		0.000	
Extranuclear parental interactions (V_K)	0.000		0.000		0.000		0.000		0.000		0.000	
Environmental (V_E)	35.314	***	33.798	***	1.202	***	9.694	***	0.453	***	0.146	***
Total (V_P)	37.491		35.066		1.266		10.153		0.461		0.150	
Fixed Effects	Significance											
Block ($F_{1,1093}$)	***		***		***		***		***		***	
Time Covariate ($F_{1,1093}$)	*		***		**		***		***		***	
Density ($F_{2,1093}$)	***		***		***		ns		*		ns	

***: $P < 0.0001$, **: $P < 0.01$, *: $P < 0.05$, ns: not significant

[a] Calculated as $h^2 = V_A/V_P$ for each trait; significance based on that of V_N

[b] Calculated as $V_A = 4 \times V_N$

Table 2. Descriptive statistics for both Missouri and Brazil data and mean standardized statistic (CV_A) for the Missouri data determined from raw data for all traits measured in *Nicotiana alata*.

	Missouri statistics					Brazil statistics			
Character [a]	N_{MO} [b]	Mean	±	SE	V_A [c]	CV_A [d]	N_{BR} [b]	Mean	±	SE
Corolla tube length (mm)	1098	60.17	±	0.21	6.5996	4.27	313	63.76	±	0.36
Corolla limb width (mm)	1097	44.34	±	0.19	1.0444	2.30	312	46.64	±	0.34
Corolla mouth diameter (mm)	1096	7.79	±	0.03	0.0714	3.43	312	7.70	±	0.06
Nectar concentration (% solids)	1040	18.10	±	0.10	0.1818	2.36	287	5.79	±	0.28
Nectar volume (µl) [e]	1089	6.68	±	0.16	2.1032	21.72	307	16.10	±	0.21
Nectar energy (mg sucEq) [e]	1040	1.36	±	0.03	0.1046	23.74	287	1.10	±	0.06

[a] Units apply to mean statistics only

[b] Number of observations that include a measurement for the character

[c] $V_A = 4 \times V_N$ (additive nuclear variance component)

[d] Additive genetic coefficient of variation, $CV_A = 100 \times \sqrt{V_A} / \text{mean}$

[e] V_A differs from that reported in Table 1 using transformed data

Table 3. Genotypic and phenotypic correlation coefficients for all traits measured in *Nicotiana alata*. Genetic correlations (below the diagonal) were determined from additive genetic breeding values (Best Linear Unbiased Predictions [BLUPs]) for each parent (N = 44). Phenotypic correlations (above the diagonal) were determined from paired raw data (N = 1040-1098, see Table 2).

	Tube length	Limb width	Mouth diameter	Nectar concentration	(Log) Nectar volume [a]	(Log) Total energy [a]
Tube length	—	0.4437 ***	0.2814 ***	0.0794 **	0.1784 ***	0.1952 ***
Limb width	0.2641 ns	—	0.3925 ***	0.1249 ***	0.3472 ***	0.3486 ***
Mouth diameter	0.0066 ns	0.5912 ***	—	0.1879 ***	0.2542 ***	0.2854 ***
Nectar concentration	-0.2719 ns	0.0204 ns	0.0782 ns	—	0.1975 ***	0.4433 ***
(Log) Nectar volume [a]	0.0715 ns	0.1080 ns	0.1777 ns	0.1483 ns	—	0.9405 ***
(Log) Total energy [a]	-0.0160 ns	0.0861 ns	0.2878 ns	0.1933 ns	0.8926 ***	—

***: $P < 0.0001$, **: $P \leq 0.01$, ns: not significant

[a] Values were log(1+y) transformed prior to analysis.

Table 4. Pearson correlation coefficients for all traits measured in *Nicotiana alata* using full-sib family means (*N* = 173; PROC CORR in SAS 9.1).

	Tube length	Limb width	Mouth diameter	(Log) Nectar volume [a]	Nectar concentration	(Log) Total energy [a]
Tube length	—	0.4302 ***	0.2746 **	0.1001 ns	-0.0927 ns	0.0444 ns
Limb width		—	0.5035 ***	0.2629 **	0.0533 ns	0.2177 **
Mouth diameter			—	0.2156 **	0.0157 ns	0.2539 **
(Log) Nectar volume [a]				—	0.0949 ns	0.8628 ***
Nectar concentration					—	0.3286 ***
(Log) Total energy [a]						—

***: $P < 0.0001$, **: $P < 0.01$, ns: not significant

[a] All values were log(1+y) transformed prior to analysis.

Table 5. Interspecific (*Nicotiana* section *Alatae*) phenotypic correlation coefficients for all trait combinations. Correlations were determined from population means for all *Nicotiana* section *Alatae* species ($N = 17$).

	Tube length	Limb width	Mouth diameter	Nectar concentration	Nectar volume [a]
Tube length	—				
Limb width	0.9385 ***	—			
Mouth diameter	0.1554 ns	0.2714 ns	—		
Nectar concentration	-0.8358 ***	-0.7967 ***	0.0890 ns	—	
Nectar volume	0.8772 ***	0.8521 ***	0.3407 ns	-0.6771 **	—
Total energy	0.1405 ns	0.1157 ns	0.5507 ns	0.2127 ns	0.5023 ns

***: $P < 0.0001$, **: $P < 0.01$, ns: not significant

[a] Nectar volume and total energy were log (1+y) transformed before intraspecific correlations were made.

Table 6. ANOVA results for the independent variables that were included in the model comparing full-sib families (N = 10) between Missouri and Brazil environments.

		Tube length		Limb width		Mouth diameter		Concentration		Log(Volume)		Log(Energy)	
Source	df	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*
Environment	1	59.9	< 0.0001	19.28	< 0.0001	3.18	0.0754	84.36	< 0.0001	9.17	0.0027	20.03	< 0.0001
FS family	9	6.67	< 0.0001	6.13	< 0.0001	4.13	< 0.0001	1.33	0.2196	4.65	< 0.0001	3.13	0.0013
Env * FS fam	9	1.87	0.0560	2.25	0.0190	1.39	0.1921	1.54	0.1348	2.76	0.0041	1.93	0.0483

Figure 1. Partial diallel matrix used to produce crosses. Shaded cells represent crosses made, four crosses for each plant maternally and four crosses for each plant paternally. Note that more parents were used than what is shown (N = 44).

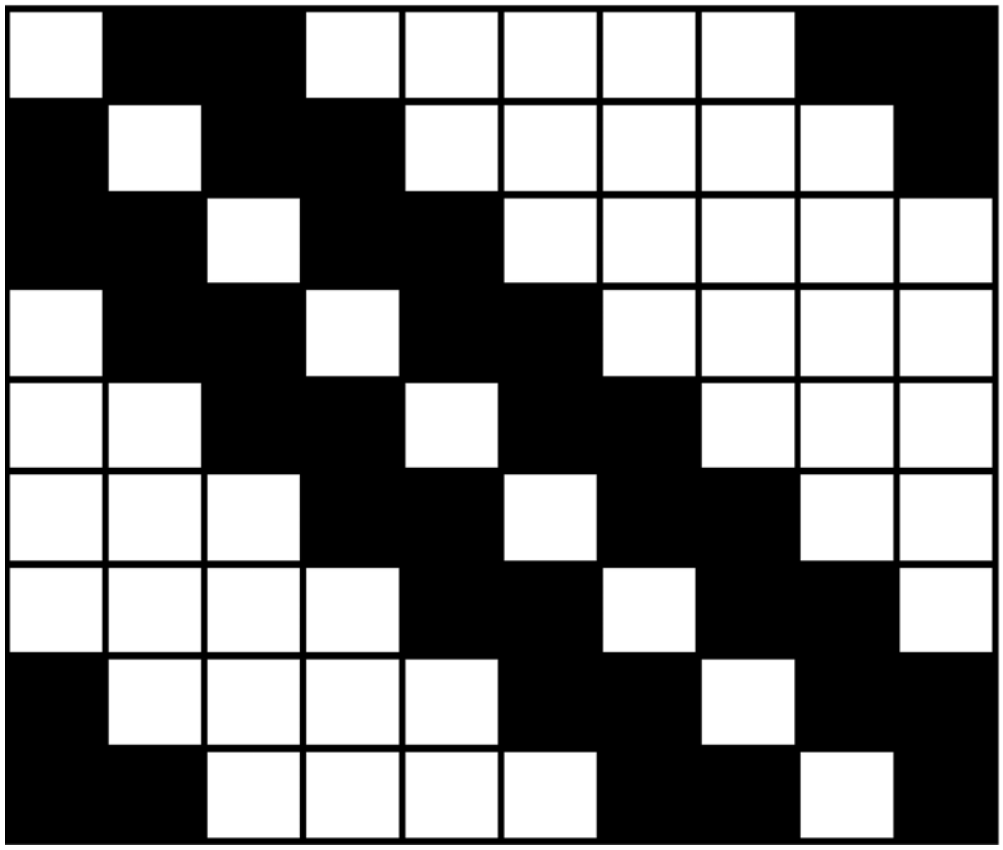


Figure 2. Density effects for all measured traits in *N. alata*. Plots show mean ± 95% confidence interval for each density treatment. Sample sizes are included above the plots. See Table 1 for significance.

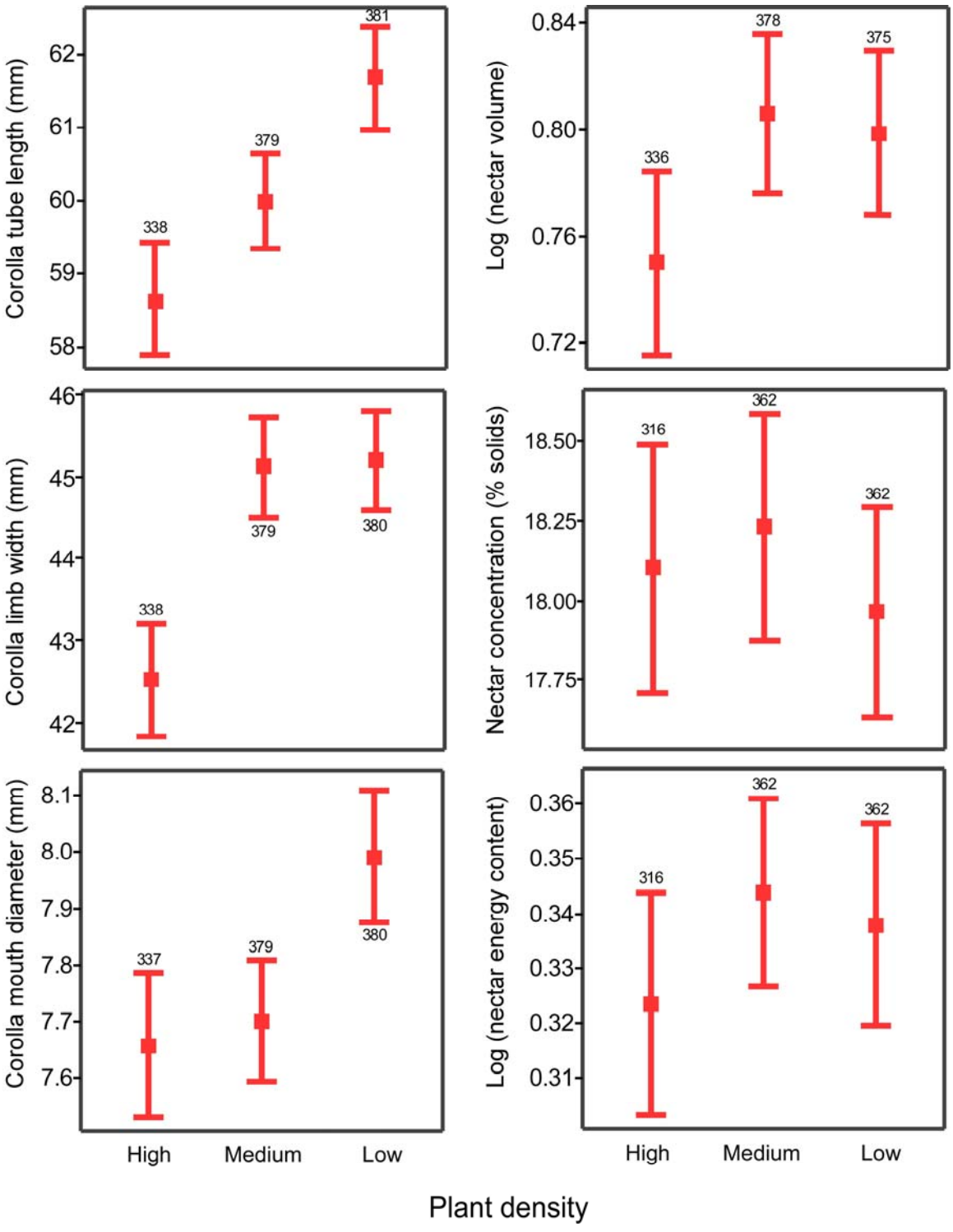

Corolla tube length (mm)
62
61
60
59
58
381
379
338
Log (nectar volume)
0.84
0.80
0.76
0.72
378
375
336
Corolla limb width (mm)
46
45
44
43
42
379
380
338
Nectar concentration (% solids)
18.50
18.25
18.00
17.75
362
316
362
Corolla mouth diameter (mm)
8.1
8.0
7.9
7.8
7.7
7.6
380
337
379
Log (nectar energy content)
0.36
0.35
0.34
0.33
0.32
0.31
362
316
362
High
Medium
Low
High
Medium
Low
Plant density

Figure 3. Scatterplot matrix of genetic and phenotypic correlations. Genetic correlation scatterplots produced with the additive genetic breeding values (Best Linear Unbiased Predictions [BLUPs]) estimated in the mixed model (below the diagonal, $N = 44$). Phenotypic correlations were determined using paired raw data (above the diagonal, $N =$ 1040-1098).

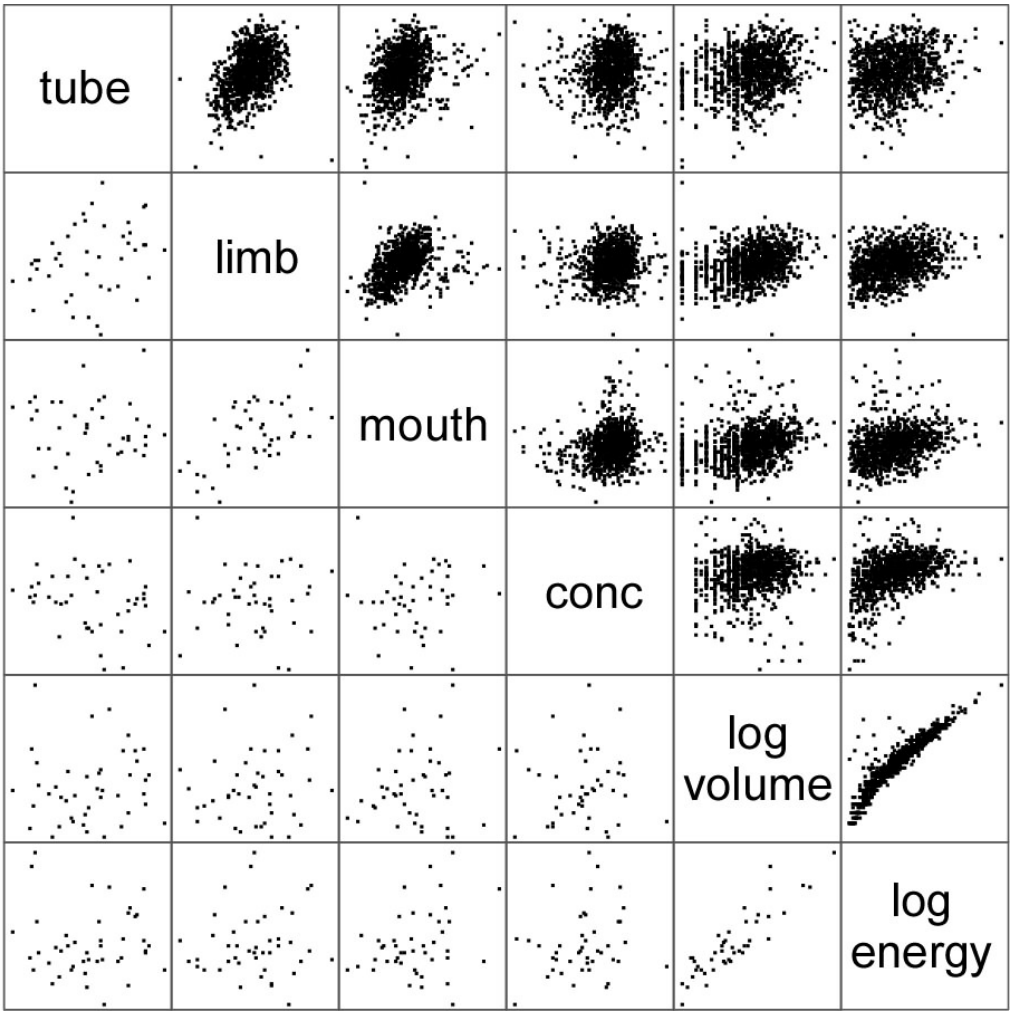
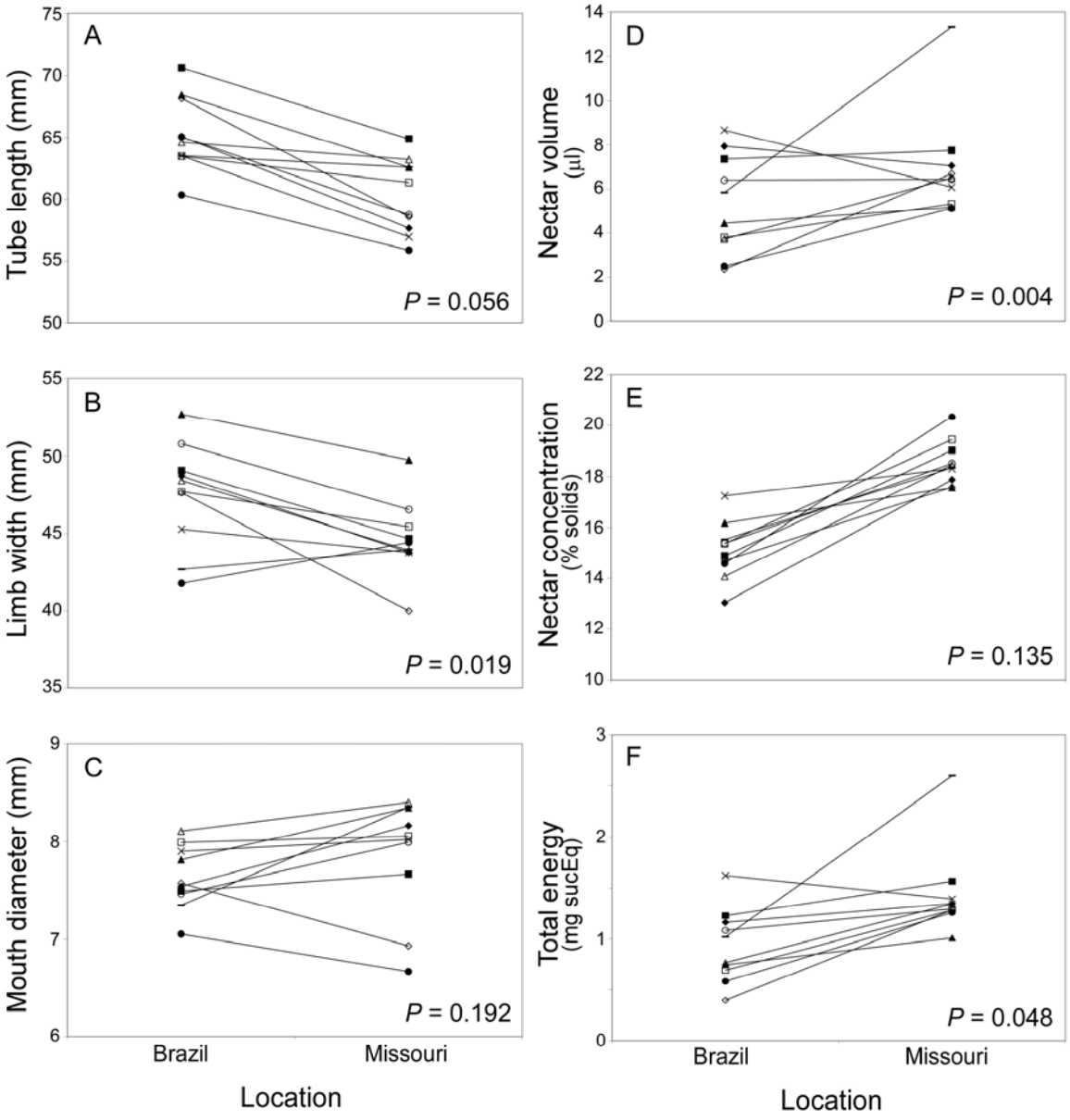

tube
limb
mouth
conc
log volume
log energy

Figure 4. Norms of reaction for all traits studied in the heritability experiments. Ten full-sib families from the Missouri-based heritability experiment were grown in a Brazilian experimental plot. Each line represents a different family. Points are means of all individuals from the family, averaged across different density treatments. Significance for the full-sib family by environment (G×E) interaction is given in the bottom right corner of each panel. A-C) Genotype by environment reaction norms for morphological traits: tube length, limb width, and mouth diameter. D-F) Genotype by environment reaction norms for nectar traits: nectar volume, concentration, and energy.


A
Tube length (mm)
75
70
65
60
55
50
P = 0.056
B
Limb width (mm)
55
50
45
40
35
P = 0.019
C
Mouth diameter (mm)
9
8
7
6
P = 0.192
Brazil
Missouri
Location
D
Nectar volume (µl)
14
12
10
8
6
4
2
0
P = 0.004
E
Nectar concentration (% solids)
22
20
18
16
14
12
10
P = 0.135
F
Total energy (mg sucEq)
3
2
1
0
P = 0.048
Brazil
Missouri
Location

CHAPTER 5

EFFECTS OF NECTAR AUGMENTATION ON SEED PRODUCTION IN *NICOTIANA ALATA*

ABSTRACT

Increased nectar quantities are expected to increase the reproductive success of plants via pollinator behavior. I tested the effects of nectar quantity on seed production per fruit by augmenting nectar of all flowers on individual *Nicotiana alata* plants differentially across consecutive nights. Seed production per fruit was not significantly affected by nectar augmentation. I also determined that seed production per fruit was not pollen limited by natural pollination in this population because there was no significant difference in seed production between hand-pollinated and naturally-pollinated control flowers. I explain some of the weaknesses of this study, but tentatively conclude that nectar production would not be affected by selection through female fitness.

INTRODUCTION

Nectar production is probably the most frequently studied nectar trait as it can have important implications for the pollination success of many plants. It is almost ubiquitously variable across plant species (see Rathcke, 1992) and has even been found to have a heritable component (see Mitchell, 2004; Leiss et al., 2004; Kaczorowski, Ch. 3 for *N. alata*). Nectar production can also have important implications for plant fitness.

Nectar rewards have been repeatedly linked to pollinator behavior. High nectar production can increase the frequency of pollinator visitation (Pedersen, 1953; Real and Rathcke, 1991; Burd, 1995; Nassar et al., 1997), the number of flowers visited within a plant or inflorescence (Zimmerman, 1983; Galen and Plowright, 1985; Mitchell, 1993; Hodges, 1995), or the duration of an individual flower probe (Thomson and Plowright, 1980; Zimmerman, 1983; Galen and Plowright, 1985; Mitchell and Waser, 1992; Cresswell, 1999; Manetas and Petropoulou, 2000; Kudo, 2003). Pollinator behaviors such as these have often been associated with increases in plant fitness, through increased pollen removal and/or deposition (Thomson and Plowright, 1980; Galen and Plowright, 1985; Thomson, 1986; Mitchell, 1993; Pleasants and Chaplin, 1983; Harder, 1990; Galen, 1992; Hodges, 1995), decreased fruit abortion (Manetas and Petropoulou, 2000), or increased seed set (Pedersen, 1953; Zimmerman, 1983; Real and Rathcke, 1991; Manetas and Petropoulou, 2000). However, a number of studies have failed to find a correlation between nectar and plant fitness, despite a correlation between nectar and pollinator behavior (Mitchell and Waser, 1992; Cresswell, 1999; Kudo, 2003). Additionally, even if certain components of plant fitness are affected by pollinator behavior (i.e., male fitness via pollen removal), other components may not be affected (i.e., seed set; Pleasants and Chaplin, 1983; Mitchell, 1993; Hodges, 1995).

In this study, I augmented nectar volumes on the same plants differentially over consecutive nights to determine if seed production per fruit was affected by nectar augmentation. I also investigated pollen-limitation to determine whether pollinators delivered sufficient amounts of pollen to affect seed production.

METHODS

Study system

Nicotiana alata Link and Otto is a member of the monophyletic clade of section *Alatae* (Ippolito et al. 2000; Chase et al., 2003; Knapp et al., 2004). It has long, tubular, white flowers that emit a strong fragrance at night (Raguso et al., 2003), owing to its hawkmoth-pollination. It is self-incompatible (McClure et al., 1990) and native to southern Brazil and adjacent areas in Paraguay, Uruguay and eastern Argentina (Goodspeed, 1954). Typical habitats of *N. alata* are rocky slopes and stream sides, but it commonly spreads along roadsides. The source population of the seeds used in this study is Rio Pelotas, which can be found where the Pelotas River intersects highway BR-116 at the state lines of Santa Catarina and Rio Grande do Sul, Brazil. Plants of this population can be found emerging from the rocky slopes, along the river, and along the road.

Experimental methods

Multiple seeds from 176 full-sib families, produced for a heritability experiment, were grown at the Experimental Station of Campestre in the state of Rio Grande do Sul, Brazil, within 25 km of natural *N. alata* populations. Seeds were germinated in a hoop greenhouse until they were large enough to be transplanted outdoors. One hundred of the 176 different families were chosen based on seedling size to be planted for the fitness experiment. In December 2004, the plants were transplanted into a plowed plot (approximately 20 × 10 m). Although some biotic and abiotic factors can potentially differ between the farm plot and natural roadside populations, the environmental conditions were likely to be very similar between the two areas. Two sets of the 100 chosen families were planted adjacent to each other without separation. All plants were

placed 1 m apart from each other and surrounded with border plants to reduce edge effects. Many of the plants were large enough, with enough flowers, to be manipulated by January 12, 2005. At this time, the largest 60 of the 200 plants were chosen to serve as experimental plants. Each plant was randomly assigned to one of three different plant groups (A, B, and C). Care was taken to distribute an equivalent number of big, medium, and smaller plants to each of the groups.

Nectar was sampled from two to three flowers (as explained in Kaczorowski et al., 2005) from 20 different plants two days prior to the first experimental day to determine the average nectar volume and concentration for the plants at that time and place (volume in µl: 6.9 ± 0.50, concentration in % solids: 19.1 ± 0.33). I made artificial nectar with sucrose (table sugar) and bottled water at approximately the mean concentration (19%). I also used the mean nectar volume to determine the amount of artificial nectar to use in the two nectar augmentation treatments (5 and 10 µl). Augmentation treatments were determined by selecting a lower nectar quantity (5µl) that was slightly below the population mean and a higher quantity (10 µl) that, not only doubled the lower quantity, but also should have set most flower nectar volumes just greater than the maximum nectar volume found in the population (16.5 µl).

Manipulations of the experiment took place over three days during January 14-17, 2005. Manipulations were postponed for one day during this time due to cold, rainy weather. One flower on each plant was chosen as the hand-pollinated control. These flowers were emasculated (through small slits in the corolla tube) in the late afternoon, approximately 3-4 h before anthesis (2000-2100 GMT-2). Donor pollen from many non-experimental plants in the plot was collected into a single tube. Hand-pollinations were

performed with the donor pollen (1800-1900 GMT-2), and then the flowers were bagged to restrict floral visits ensuring that pollination was only effected by hand-deposited pollen. Nectar manipulations began at the same time as hand-pollinations, but required more time, usually until shortly after sundown. Up to four remaining flowers on each plant were manipulated according to their group, with either 10 µl of artificial nectar added, 5 µl of nectar added, or a control manipulation (probed with the pipette but with no nectar addition). Additional flowers beyond four per plant (not including the hand-pollination control) were removed to keep floral display relatively similar across experimental plants. All 20 plants from each of the three groups received a different treatment on each of the three experimental nights (i.e., group C plants received the 10 µl augmentation on night 1, the 5 µl augmentation on night 2, and the control manipulation on night 3). Experimental flowers from the previous night's run had their corolla limbs torn off the corolla tubes to discourage additional visits on subsequent nights. Flowers were also marked with different colored string tied around the pedicels to distinguish hand-pollination controls and manipulated flowers within the different nights. Time restrictions prohibited additional rounds of the experiment.

Fruits from all experimental and hand-pollinated flowers were collected separately on February 1, 2005. Most fruits appeared fully developed at the time of collection, but they had not yet opened; thus, no seeds were lost at the time of collection. Many of the experimental flowers lacked fruits by the time of collection, possibly due to damage or lack of pollination. These missing fruits were not included in the estimations of seed production per fruit because I could not distinguish between loss due to lack of pollination and loss due to pistil damage or herbivory. I observed an apparent increase in

florivorous-beetle frequency on augmented flowers the day immediately following manipulations. Additionally, I observed birds foraging on these beetles, which could have also induced floral damage. To determine whether exclusion of missing fruits was warranted, I compared the percentage of fruits set (of the number of flowers manipulated) across plant groups for each of the experimental days and overall (PROC GLM in SAS 9.1). There was no significant difference in percentage of fruits set between plant groups for any of the experimental days or overall ($P >> 0.05$; day 1: $F_{2,52} = 0.81$, day 2: $F_{2,56} = 0.87$, day 3: $F_{2,57} = 0.66$, overall: $F_{2,57} = 0.76$). Therefore, I feel justified in excluding missing fruits from estimates of average seed production per fruit.

Seed counts could not be taken immediately and many seeds were lost (a visual estimate of approximately 10%) from the paper envelopes, which became unglued while in storage. This seed loss may have exerted a bias on seed counts. The seed counts, though questionable in accuracy, serve as an estimate of female fitness. Male fitness estimation was attempted by collecting pollen (anthers) from experimental flowers and bagged control flowers. However, this component of the experiment had to be abandoned early due to high winds, extensive staminal herbivory after manipulation, insufficient storage materials, and time constraints.

Seed production per fruit was estimated by weighing two sets of 20 seeds, and then estimates of average seed weight were used to calculate the number of seeds expected for the total weight of seeds per fruit. Seed counts from hand-pollinated flowers were averaged over plants because hand pollinations were performed each of the three experimental nights, although some fruits were lost, presumably due to pistil damage or herbivory, and were not included in the estimates.

Analysis

To determine whether nectar augmentation significantly affected seed production per fruit, plant group nested within day was used to analyze seed production per fruit (PROC GLM in SAS 9.1). To determine whether seed production per fruit was limited by natural pollination (or to ensure that pollinators were efficient at depositing pollen during visitation to experimental flowers), I used pollination type (hand vs. natural [experimental controls]) to analyze seed production per fruit (PROC TTEST in SAS 9.1). The equality of variances was simultaneously tested in this analysis (Folded *F'* statistic). Because seed production variances were not significantly different between the hand- and naturally-pollinated flowers ($F'_{53, 57} = 1.09$, $P = 0.7553$), the pooled variance estimator method was used to determine the probability of differential seed production per fruit between these two pollination types.

RESULTS

Seed production per fruit was not significantly affected by nectar augmentation (Fig. 1; Table 1). Seed production per fruit was not pollen limited during natural pollination, as there was no significant difference in seed production between fruits produced from hand-pollinations and natural-pollinations (Fig. 2; Table 2).

DISCUSSION

Nectar augmentation did not significantly affect seed production per fruit in this study (Fig 1, Table 1). Unfortunately, I was unable to record pollinator observations during this study due to the inability of two people to adequately observe multiple

experimental plants interspersed with non-experimental plants across a large area in the dark. Additionally, I only quantified fitness through seed production per fruit, which is only one female fitness component for plants, while other components of fitness (i.e., pollen transfer) could have been significantly affected. It is possible that nectar augmentation affected some aspect of hawkmoth foraging behavior. If pollinator behavior modifications resulted from nectar augmentation, they may have significantly affected other fitness components, such as pollen transfer, while not affecting seed production (Pleasants and Chaplin, 1983; Mitchell, 1993; Hodges, 1995). However, some studies have failed to find a significant effect of certain pollinator behaviors (i.e., probe duration) on pollen transfer (Galen and Stanton, 1989; Mitchell and Waser, 1992). Furthermore, increased visitation can have negative effects on reproductive success by increasing geitonogamy, pollen transfer within flowers of a plant (Galen and Plowright, 1985; Hodges, 1995). However, *N. alata* is self-incompatible (McClure et al., 1990) and therefore geitonogamy should not affect the reproductive success of these plants.

Seed production could be limited by resource availability (Galen et al., 1985; Zimmerman and Pyke, 1988; Ehrlen, 1992; Asikainen and Mutikainen, 2005) or the amount of pollen received (Galen, 1985; Burd, 1994, 1995; Juenger and Bergelson, 1997; Mothershead and Marquis, 2000). I attempted to account for resource limitation by applying different nectar augmentation treatments to individual plants across different nights so the effects from resource limitation, as well as the effect of plant genotype and environment, would be randomized over augmentation treatments. However, resources can be reallocated among flowers based on pollination success, which can potentially overestimate the treatment effects, although no treatment effects were detected. I also

removed flowers on many plants to keep floral display relatively equivalent among all plants in the experiment. This could also affect the resource allocation, and nectar production, to flowers that remain on the plant. I also verified that seed production per fruit was not pollen limited by showing that there was no significant difference in seed production between hand-pollinated and naturally-pollinated control flowers (Fig. 2, Table 2). Regardless of the insignificant effect of nectar augmentation on seed production, other effects may have been detected had methods been different.

Had individual plants been augmented with the same amount of nectar every night of the experiment, pollinators could have potentially learned which plants were more rewarding and selection may have been stronger. Although hawkmoths have the ability to learn which flowers or plants to visit based on spatial cues (Balkenius et al., 2004), there is no evidence that suggests that hawkmoth foraging in the field is based primarily on these spatial cues. However, available data does suggest that hawkmoths are likely to visit more flowers on plants that have more nectar (Hodges, 1995). Therefore, the rotation of nectar augmentation treatments on individual plants each night was still expected to affect hawkmoth visitation by causing them to immediately visit more flowers on plants that they encounter with higher nectar quantities. However, I did not analyze the percentage of manipulated flowers that set fruit across treatments because fruit loss occurred in all treatments, including the hand-pollinated flowers. Although fruit loss could have been due to lack of pollination in the naturally-pollinated flowers, loss of hand-pollinated fruits and personal observation suggests that pistil damage or general herbivory contributed to at least some of the fruit loss. Perhaps if there were more flowers in the field during the experiment, damage to flowers and plants may have been reduced.

Time constraints forced me to apply the treatments to relatively small plants with relatively few flowers. When the treatments were applied, less than half of the plants in the plot had a sufficient number of flowers for the experiment. It is likely that additional time for the experiment would have allowed the plants to acquire a larger floral display, which could have attracted more pollinators and potentially increased selective pressure. However, because seed production per fruit was not pollen limited, the pollinators that were present in the field during the experiment were sufficiently pollinating the flowers they visited. Additionally, there was no significant difference in seed production between the hand-pollination controls and the naturally-pollinated (0 μl) controls, which were not significantly different from the other nectar augmentation treatments (5 and 10 μl). This suggests flowers from all experimental plants were visited sufficiently. It is likely that there were few enough flowers in the experimental plot that pollinators visited most of those available. Perhaps if floral display was larger, pollinators would have had more flowers to select from.

Other weaknesses of this study include the estimated 10% seed loss, which may have biased results, and time constraints restricting additional replications of the experiment. Each experimental plant had each of the three nectar treatments applied only once, where, ideally, they would have had each treatment applied at least three times to strengthen the experiment. Additionally, I chose to discourage pollinator visits to flowers that were manipulated on prior nights by removing corolla limbs from flowers the day after they were augmented. However, this method did not completely restrict visitation and therefore previously manipulated flowers could have been visited by pollinators when the plants were under a different nectar augmentation treatment. The damage to

corollas through limb removal (or emasculation) is not expected to have an effect on seed production. Reduced or damaged corolla tissue has been shown to decrease plant seed set through lower pollinator visitation (Mothershead and Marquis, 2000; Leavitt and Robertson, 2006), although it is not expected to physically affect seed production unless reproductive parts are damaged (Leavitt and Robertson, 2006). However, removing the corolla limbs could have made reproductive parts more vulnerable to damage.

Despite the problems associated with this study, I feel that the lack of effect in seed production with nectar augmentation is likely to be valid for this population at the time of the experiment. Therefore, it is unlikely that nectar production could be evolving in this population at this time. I do feel, however, that with more data or different methods, I may have been able to detect an effect of nectar on some aspect of fitness. Additional studies that include more fitness components and more replications with pollinator observation are necessary for a better understanding of how nectar production affects plant fitness in *Nicotiana*.

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Table 1. Results from the ANOVA for the effects of plant group (A, B, and C) within day. Different nectar treatments were applied to each of the three plant groups on a given day, so that plant group within day accounts for differences across nectar treatments.

Source	DF	MS	*F*	*P*
Plant group (day)	8	86506.27	1.09	0.3749

Table 2. Seed production (mean number of seeds per fruit ± SE) and t-test results for differences between hand- and naturally-pollinated flowers. Seed production variances were not significantly different, thus the pooled variance estimator method was used to determine significance.

Source	Hand-pollinated	Naturally-pollinated	df	*t*	*P*
Seed production (# seeds/fruit)	734.9 ± 36.2	717.2 ± 39.1	110	0.33	0.7409

Figure 1. Seed set per fruit for *Nicotiana alata* plants by the amount of nectar addition and day. All data points within each box plot are an average of the seed set per fruit for all fruits from a treatment on a plant. The mean volume of nectar expected for flowers within a nectar treatment is also included, based on preliminary sampling two days prior to the start of the experiment and addition of nectar augmentation amount. Plant sample sizes are denoted in parentheses. 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.

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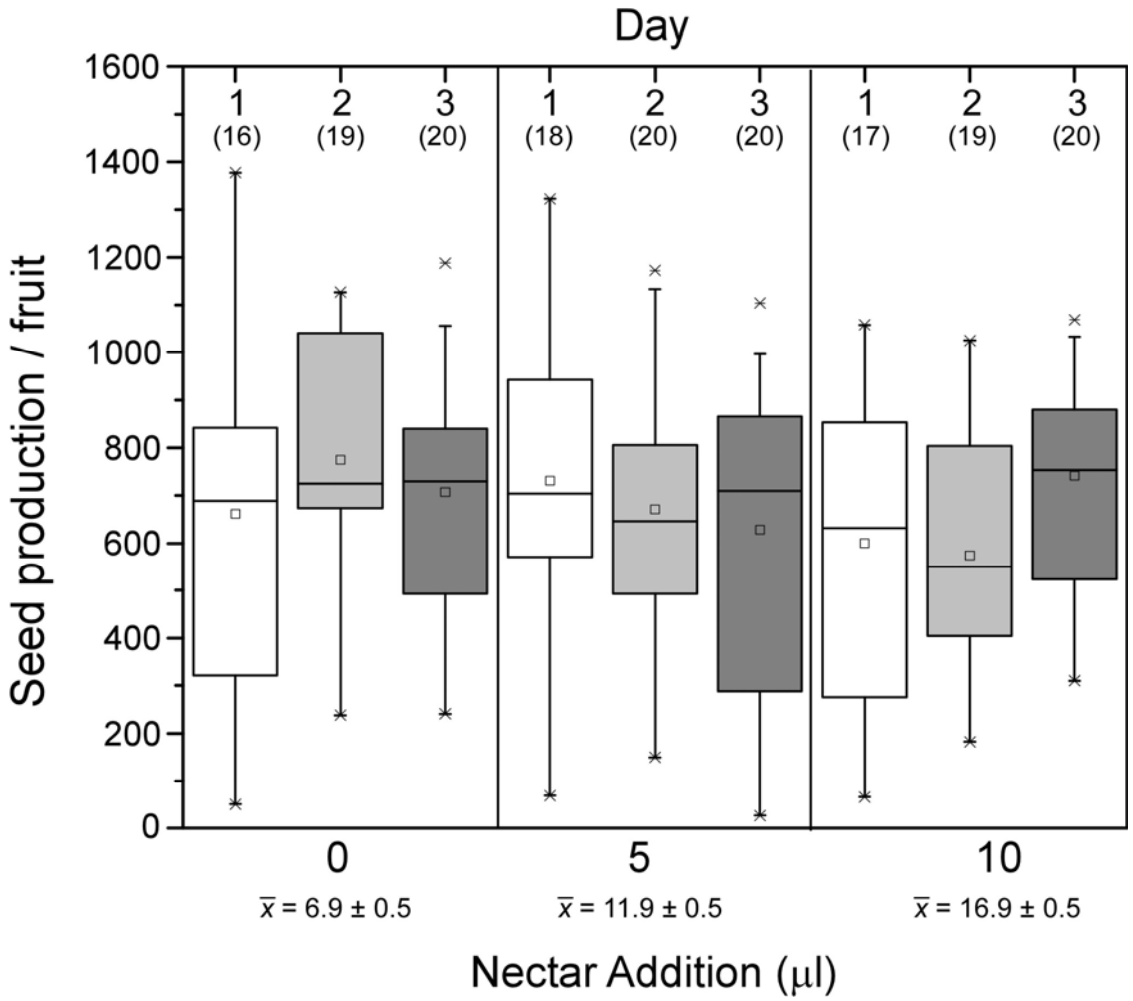
Day
1
(16)
2
(19)
3
(20)
1
(18)
2
(20)
3
(20)
1
(17)
2
(19)
3
(20)
Seed production / fruit
1600
1400
1200
1000
800
600
400
200
0
0
5
10
$\bar{x}$ = 6.9 ± 0.5
$\bar{x}$ = 11.9 ± 0.5
$\bar{x}$ = 16.9 ± 0.5
Nectar Addition (µl)

Figure 2. Seed set per fruit for *Nicotiana alata* plants from natural and hand pollinations. Natural pollination seed set was calculated as the average seed count per fruit from all flowers on the plant that received the control treatment (no nectar added). Hand pollination seed set was calculated as the average seed count per fruit for all hand pollinations performed on a plant across the three nights of the experiment. Plant sample sizes are denoted in parentheses.

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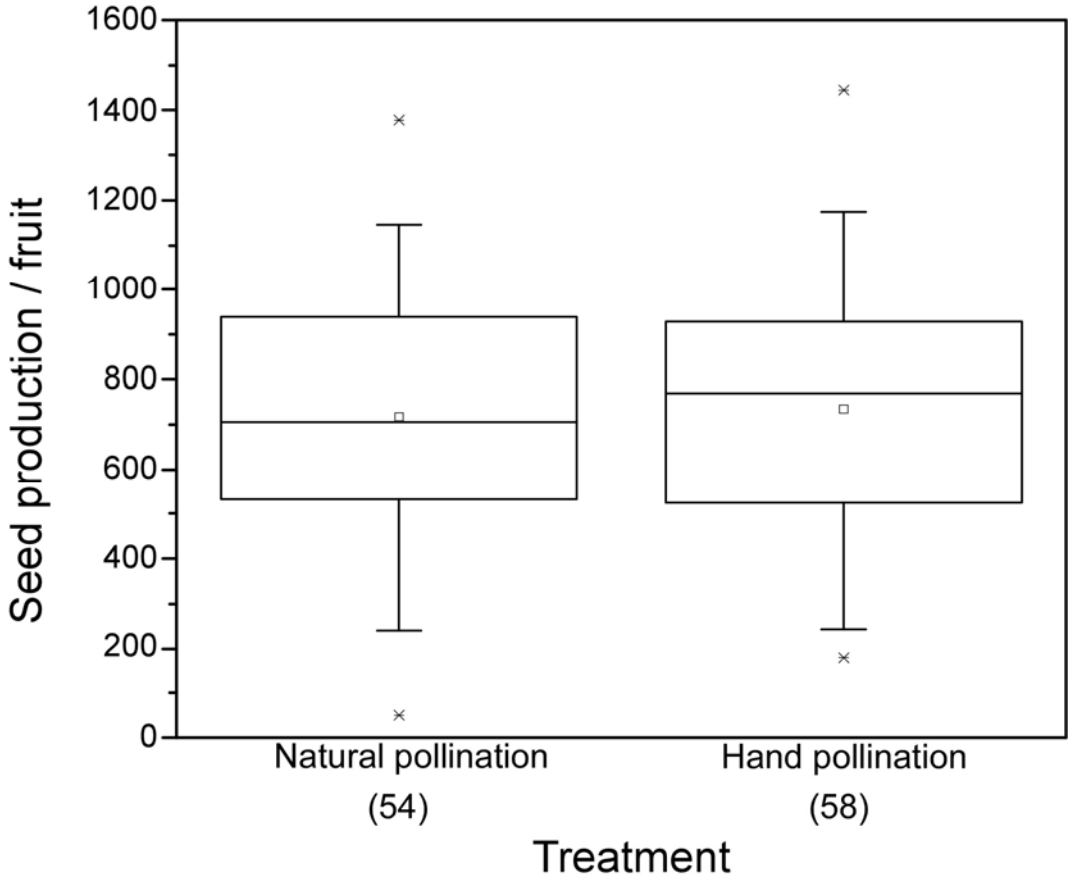
1600
1400
1200
1000
800
600
400
200
0
Seed production / fruit
Natural pollination
(54)
Hand pollination
(58)
Treatment

CHAPTER 6

CONCLUSIONS

This thesis has focused on whether existing nectar variation among species suggests pollinators may have influenced nectar evolution in *Nicotiana* section *Alatae*, and the potential for nectar traits in *Nicotiana* to continue to evolve under selective pressure by pollinators. For chapter 2, I investigated whether nectar traits of greenhouse-grown plants were variable within and among all species of *Nicotiana* section *Alatae*. I also examined whether nectar traits varied in association with primary pollinators and mating system. In chapter 3, I investigated how natural environmental variation contributed to phenotypic variation in nectar traits of naturally-growing plants for all but two species of *Nicotiana* section *Alatae*. In addition to identifying sources of environmental variation in natural populations, I also compared nectar traits from plants grown naturally to plants grown in the greenhouse. In chapter 4, I examined whether nectar traits in *Nicotiana alata* were significantly heritable and whether correlations between and among nectar and floral morphology traits had a significant genetic component. In chapter 5, I investigated whether *N. alata* plant fitness could be affected by nectar augmentation.

Nectar traits were highly variable both within and among species of *Nicotiana* section *Alatae* grown in the greenhouse (chapter 2). Environmental variation is relatively low and equivalent for plants growing in the greenhouse, which allowed for an estimation of the genetic differentiation in nectar traits among species. I found that many nectar

traits tended to vary in association with primary pollinators. This variation makes it possible for pollinators to select for or discriminate against certain types of nectar, but it also suggests that nectar traits may have evolved from past selection pressures from pollinators. However, nectar traits may have evolved in association with other floral traits with or without the aid of pollinators. Nectar rewards also tended to be lower, or otherwise different, for the autogamous (self-pollinating) species. These plants do not need to attract pollinators and are not likely to be affected by pollinator selection. Therefore, random mutations or inbreeding effects that reduce or alter nectar rewards to reduce the energy costs in the autogamous species are more likely to be perpetuated. Although these results were found for species growing in the more environmentally stable conditions of the greenhouse, evolution occurs in natural populations; therefore nectar traits were also examined in plants growing in natural populations.

Nectar traits were also highly variable within and among natural populations and species of *Nicotiana* section *Alatae* (chapter 3). Various natural sources of environmental variation were identified. Nectar traits in the field were significantly affected by weather conditions, pollinators, and floral antagonists. Nectar traits from plants in natural populations were significantly different from those of plants growing in the greenhouse. Despite this significant difference, nectar traits of plants in natural populations also varied in association with primary pollinators. Therefore, natural population results also support the theory that nectar traits may have evolved from past selective pressure by pollinators.

Chapters 2 and 3 established that there was significant variation among *Nicotiana* section *Alatae* species, with and without natural variation, which corresponded to the

primary pollinators. This suggests that pollinators could have had a role in nectar evolution within these species. However, this is a correlative conclusion; it is impossible to know definitively if pollinators actually caused nectar trait evolution since these changes occurred long ago. The purpose of chapters 4 and 5 were to determine whether nectar traits currently have a heritable component and whether pollinators may still be a selective force in *Nicotiana alata*.

Nectar traits had a significant heritable component in *N. alata* and some traits were significantly correlated genetically (chapter 4). Nectar volume and energy content, as well as corolla tube length, had significant additive variances corresponding to significant heritability for these traits. Significant heritability in these nectar traits suggests that selective pressure on these nectar traits could affect their evolution, and perhaps previous selection may have resulted in the nectar traits found currently. In addition, only correlations between corolla limb width and mouth diameter and between nectar volume and energy had a significant genetic basis. Because nectar energy content is the product of nectar volume and concentration, it is not surprising that nectar volume and energy are genetically correlated. But because floral morphology traits could aid or restrict access to the nectar by pollinators (Campbell et al., 1996; Lange et al., 2000; Ree, 2005; Darrault and Schlindwein, 2005), and potentially add to the apparency of the flowers to pollinators, it was expected that nectar traits would be genetically correlated with at least one of the floral morphology traits measured. However, no significant genetic correlations were detected between nectar and floral morphology traits. Therefore, it is unlikely that nectar traits will evolve in association with floral morphology traits in *N. alata* unless selection is applied to both traits simultaneously.

However, current correlations may differ significantly from prior correlations. Additionally, significant genotype by environment interactions were detected between the Missouri field plot and the subset of families grown in Brazil, which suggests that the heritability and genetic correlations estimated in the Missouri field plot could differ from those that may be found in natural populations. Regardless, significant heritability of nectar traits in field-grown plants suggests that nectar traits can have a significant heritable component in the presence of considerable environmental variation (e.g., density effects, variable weather conditions). Although the significant nectar trait variation and heritability suggest that nectar traits have the potential to evolve, these nectar traits also need to affect plant fitness for them to affect evolutionary change.

With the methods used, I was unable to detect a significant effect of nectar augmentation on seed production per fruit (chapter 5). I also determined that the lack of effect was not due to a lack of pollination. Therefore, it is unlikely that nectar production would affect evolutionary change in nectar traits at the time and place of the experiment. However, it is possible that had I used different methods, locations or times, results may have differed. Even though seed production was not significantly affected by nectar augmentation, other components of plant fitness, such as pollen removal or dispersal, may have been. Also, had individual plants been augmented with the same amount of nectar every night of the experiment, pollinators could have potentially learned which plants were more rewarding and selection may have been stronger. Furthermore, additional pollinators could have been attracted to the plants, potentially increasing selection, had there been more time for the plants to acquire a larger floral display. However, there was no significant difference between the two nectar supplement

treatments and the natural-pollinated control treatment, which was not significantly different from the hand-pollination controls, suggesting that pollinator frequency was great enough that most available flowers were visited. Therefore, a larger floral display may have been more important in providing ample flowers for the pollinators to select from. Pollinator observations would have allowed me to determine whether nectar augmentation had an effect on pollinator behavior that was not evident through seed production. Pollinator frequency and behavior is likely to differ spatially and temporally (Cane and Payne, 1993; Price et al., 2005; Sahli and Conner, 2007), which could change the effect they have on plant fitness.

Significant variation within and among species of *Nicotiana* section *Alatae* in the greenhouse and in natural populations, and the significant heritability of nectar traits in *N. alata*, suggest that nectar traits in *Nicotiana* still have the potential to evolve. This increases the likelihood that current nectar profiles are due to prior selective pressure on nectar traits, especially since no genetic correlations were detected between nectar traits and floral morphology traits. However, the genetic correlations could have evolved during the radiation of *Alatae*.

Nectar traits can also be affected by factors that were not investigated for this thesis, but may also have contributed to *Nicotiana* nectar traits (e.g., time of season, time of day, plant size, and soil moisture; see Zimmerman, 1988). Although the nectar trait investigated (nectar production) did not effect the plant fitness component investigated (seed production), plant fitness may have been affected by nectar traits in the past or currently in different places or under different conditions. More fitness studies are needed to confirm this possibility.

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VITA

Rainee L. Kaczorowski was born on January 19, 1976 in Cleveland, Ohio. In 1994, she graduated from Valley Forge High School, located in the Cleveland suburb of Parma, Ohio. Her undergraduate education was distributed equally with a year and a half spent at three different schools, Muskingum College in New Concord, Ohio, Western Illinois University in Macomb, Illinois, and, finally, the University of Missouri in Columbia, Missouri, where she earned her B.S. in Biology in December of 1999. After a semester of non-degree graduate student status, she entered the doctorate program in the Division of Biological Sciences at the University of Missouri-Columbia in the fall of 2000. There she worked under the guidance of Dr. Timothy P. Holtsford, spending much of the time studying nectar traits in *Nicotiana* in Missouri, but with occasional trips to native populations of *Nicotiana* in South America. She received her Ph.D. in 2007 and plans to begin a post-doctoral position with Dr. Robert Raguso at Cornell University.