Article

INCREASED RELIANCE ON DIURNAL POLLINATION IN A GEOGRAPHICALLY AND MORPHOLOGICALLY ATYPICAL SAND VERBENA

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Abstract—*Premise*—Pollinator-mediated selection drives floral morphologies to converge on sets of traits deemed "pollination syndromes". As a result of similarity due to common descent, pollination syndromes can be shared among closely related plants in cases where pollinators remain relatively constant over evolutionary time. In these cases, species that display trait deviations away from their ancestral states may indicate hidden pollinator shifts. Identifying when and where trait deviations reflect reproductive contributions from unexpected pollinators is important for understanding the boundaries and cohesion of pollination syndrome phenotypes.

Methods—The floral morphology of heart's delight, *Abronia ameliae*, unites a collection of traditional moth-pollination syndrome traits, typical for the genus, with several characters odd for moth pollination, including diurnally open, pink flowers and large, tall inflorescences. We predicted that the evolution of this combination of traits reflects a change in pollinators from other *Abronia*, specifically that this species is primarily diurnally-pollinated. We conducted pollinator-exclusion experiments in a natural population and a common garden to determine the independent reproductive contributions of diurnal and nocturnal pollinators to *A. ameliae* and characterized its volatile profile.

Results—We found that *A. ameliae* is indeed primarily diurnally pollinated: visitation by day-active butterflies and moths contributed to higher seed set than visitation by nocturnal moths. However, *A. ameliae* also emits nocturnal moth-associated volatile compounds and receives considerable nocturnal pollination.

Conclusions—We suggest that the unusual phenotypes found in *A. ameliae* flowers relative to the genus reflect a shift in reproductive contributions to incorporate mostly diurnal pollination and transition to a mixed pollination strategy that blends diurnal and nocturnal pollination phenotypes.

Keywords—*Abronia ameliae*; butterfly pollination; floral trait evolution; floral pigmentation; moth pollination; volatile organic compounds

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INTRODUCTION

Floral features that have repeatedly converged on similar morphologies indicative of a guild of pollinators, defined as pollination syndromes, are valuable in predicting a plant's primary pollinators (Rosas-Guerrero et al. 2014 and Ashworth et al. 2015; but see Ollerton et al. 2009 and Dellinger 2020). Pollination syndromes can be a useful heuristic to infer pollinators of flowers with clear phenotypes. However, identifying cases where species deviate from a typical set of pollination syndrome phenotypes can be important for understanding their utility and boundaries. Asking when and where trait deviations reflect shifts to new pollinators can be particularly powerful for understanding the complexity of floral phenotypes beyond simple discrete views of syndromes. Here, we characterize the floral features and pollination system of a species atypical for its moth-pollinated genus to ask whether shifts from ancestral floral trait combinations reflect changes to new or non-primary pollinator contributions.

Pollinator-mediated selection may be strong enough to separate flowers pollinated by even closely related and functionally-similar groups into discrete syndromes, such as long- and short-tongued fly syndromes (Robertson 1928; Fenster et al. 2004), or fragrance-, resin-, and pollen-collecting euglossine bee syndromes (Armbruster 1993). Moth and butterfly pollination syndromes too are defined distinctly (Faegri & Pijl 1979; Armbruster et al. 2000; Fenster et al. 2004), despite most Lepidoptera sharing many similarities in anatomy, resource requirements, and other physical and ecological traits (Ford 1946; Scoble 1992; Young 2010). The moth floral syndrome (phalaenophily) includes white or pale-colored flowers, nocturnal anthesis, ample and sweet floral fragrance, and lots of nectar; the butterfly syndrome (psychophily) describes diurnally-open, colorful (often pink or red) flowers that produce little fragrance and comparatively little nectar (Faegri & Pijl 1979; Dobson 1994). Many flowers that align with one syndrome are indeed pollinated by the expected, corresponding functional pollinator (e.g., the comet orchid's moth syndrome and its hawkmoth pollinator (Darwin 1862)). Clades containing switches between moth and butterfly pollinators with correlated switches in floral traits (e.g., Johnson et al. 1998, Goldblatt and Manning 2002, Balducci et al. 2019, Chen et al. 2021, Liu et al. 2022) further support discrete moth and butterfly pollination syndromes.

However, there is increasing recognition of consequential secondary pollinators of otherwise specialized flowers. Butterflies may increase reproductive success of primarily moth-pollinated species (e.g., Yan et al. 2016, Koptur et al. 2021, Jaeger et al. 2023) and vice versa (Grant & Grant 1965). Therefore, flowers presenting predominantly moth or butterfly syndrome traits do not necessarily exclude any fitness benefit from pollination by the 'non-preferred taxa.' Indeed, secondary visitors may substantially boost plant fitness by maximizing reproduction (Jennersten 1983; Jaeger et al. 2023), increasing pollen dispersal distance (Courtney et al. 1982), replacing herbivorous pollinators (Kessler et al. 2010), increasing per visit efficiency (Bertin & Willson 1980; Morse & Fritz 1983), or providing pollination assurance during years of low primary pollinator abundance (Ghazoul 2004; Reith & Zona 2016). Non-primary pollinators may thus select for floral trait changes that maximize their reproductive contributions – even altering those traits normally associated with the primary pollinator (Aigner 2001; Wenzell et al. 2024).

Collections of floral traits not clearly consistent with single pollination syndromes may thereby indicate stable states of minor to equal pollinator contributions to reproduction (e.g., bimodal system or dual specialization (Dellinger et al. 2019), mixed pollination system (Tan et al. 2023), or adaptive generalization (Aigner 2001; Ohashi et al. 2021)), ongoing transitions between pollinators (e.g., pollinator-shift model (Grant and Grant 1965, Stebbins 1970, Whittall and Hodges 2007)), or fluctuating selection. Still, as atypical floral morphologies abound in nature, many alternative explanations for slight deviations away from a pollination syndrome remain possible. Atypical flowers may also indicate non-pollinator floral selection (i.e., florivores or other antagonists (Strauss & Whittall 2006; Doubleday et al. 2013) or abiotic pressures (Levin & Brack 1995; Coberly & Rausher 2003; Caruso et al. 2019)), or any number of non-adaptive explanations for floral trait evolution (i.e., genetic drift, linked selection, etc.).

Here, we investigate whether a marked transition from the ancestral floral phenotype is tied to an associated pollinator shift in a sand verbena. Most species of sand verbenas, the western North American sister genera *Abronia* and *Tripterocalyx*, are moth-pollinated (i.e., Tillett 1967, Keeler and Fredricks 1979, Williamson et al. 1994, Saunders and Sipes 2006, Douglas 2008, Jabis et al. 2011, Doubleday and Eckert 2018), including those within the same clade as *A. ameliae* (*A. fragrans*, *A. macrocarpa*, *A. angustifolia* and *A. nealleyi*) and its sister clade (*A. mellifera* and *A. ammophila*) (phylogeny from unpublished data and Nosratinia 2013 thesis). Congruent with a moth pollination syndrome, nearly two-thirds of sand verbena taxa have white or pale pink flowers which generally close within a couple hours of first light and reopen around dusk (e.g., Keeler & Fredricks 1979; Saunders & Sipes 2006; Douglas 2008, S. Jaeger and E. LoPresti, pers. obs). However, a pink, partially day-flowering population of an otherwise white-flowered, day-closing species, *A. fragrans*, receives most pollination nocturnally and yet also receives a small, significant increase in seed set from diurnal butterfly pollination (Jaeger et al. 2023). Here we experimentally determine the pollination system of heart's delight, *A. ameliae* (Nyctaginaceae), an enigmatic range-restricted species that has a series of traits suggestive of evolution away from the moth pollination of its close relatives. This South Texas Sand Sheet endemic represents both a geographic and phenotypic outlier in this clade, with extremely long pedicels supporting enormous pink inflorescences, containing often over 100 flowers that remain open throughout the entire night and day (Fig. 1A). Among sand verbenas, *A. ameliae* boasts a list of correlated superlatives – it has the most flowers and fruit per inflorescence, largest inflorescences (by weight), tallest stems (most species are prostrate), longest pedicels, and occurs in the southeastern-most region of the genus. Because of this collection of atypical characteristics, we suspected that *A. ameliae* is not solely moth pollinated; it has the color and size – with a distinct landing pad – suggestive of butterfly pollination. However, its strong, sweet evening and night floral volatile emissions suggests that it is not solely diurnally pollinated either (consistent with a "temporal switching multi-phenotypic strategy" (Ohashi et al. 2021)).

We could find no published examinations of the pollination biology of *A. ameliae* (or any investigations of any sort for this species). To determine whether the unusual floral phenotypes of the species are a result of a shift in pollinator preference to include diurnal species (rather than

Figure 1. Flowering *A. ameliae* plant (A). A few *A. ameliae* floral visitors in 2022, with proboscises inserted within *A. ameliae* flowers and covered in *A. ameliae* pollen (B-D). *Amblycirtes celia* (Celia's roadside-skipper) (B), *Erinnyis obscura* (obscure sphinx) (C), and *Danaus plexippus* (monarch butterfly) (D). Photos credit: Eric LoPresti.

solely nocturnal visitors), we experimentally investigated the contributions of different floral visitor guilds to *A. ameliae*'s pollination. We conducted these experiments in both a natural population and a common garden outside the species' native range. If *A. ameliae* floral traits determine the animal visitors that pollinate, we expect comparable reproduction by each pollinator guild both within and outside of *A. ameliae*'s natural habitat. We also characterized a key trait in floral visitor attraction, the floral volatile profile, of *A. ameliae* plants grown in a greenhouse. Finally, we discuss some possible explanations for *A. ameliae*'s curious pollination system and unique collection of floral characters.

MATERIALS AND METHODS

DIURNAL AND NOCTURNAL POLLINATOR CONTRIBUTIONS

Natural population in Falfurrias, Texas, 2022

We performed a pollinator-exclusion experiment in a natural *A. ameliae* population to quantify the independent pollination contributions and identities of nocturnal and diurnal pollinators in its native habitat. We conducted this study in a robust population (a few thousand plants) northwest of Falfurrias, Texas, when most of the population was blooming in mid-March 2022. With a random order, we alternately assigned each of three pollinator-exclusion treatments to nearly open inflorescence buds and covered all with small organza drawstring bags to exclude floral visitors. Diurnal treatment inflorescences were open to pollinators during the day and bagged overnight; nocturnal treatment inflorescences were open to pollinators overnight and bagged during the day; and control inflorescences were never bagged, but a bag was hung from the pedicel to mimic the bags hung while the other two treatments were open. Given the demonstrated self-incompatibility of *A. ameliae* in the lab and greenhouse (LoPresti, unpublished data), we did not apply a closed inflorescence (always-bagged) treatment to measure seed set by self-pollination. Most plants received at least two or three replicates of each treatment. We opened/closed bags during every dawn and dusk from the day of first flower until senescence of the inflorescence (recorded for each inflorescence; usually 5-7 days). Upon senescence, we permanently bagged each inflorescence to prevent seed loss during fruit development and retrieved all bagged infructescences ~3 weeks after the experiment start date when fruits were ripe. The base of the *Abronia* flower perianth (the anthocarp) persists to house either a single achene or is empty if no fruit is produced; therefore, we recorded pollination success per flower if a seed was present.

While we tagged 226 inflorescences in this population, the actual sample size was lower due to herbivory (by leafcutter ants: *Atta texana*), several plants dying, some bagged inflorescences breaking in the wind, and exclusion of all inflorescences that were still in flower when we concluded the experiment (24-March) and thus did not receive equitable pollination opportunity. These 107 surviving inflorescences on 82 plants comprised 6807 individual flowers. We analyzed pollination success in R (version 4.4.1) using a binomial response variable (fruit present or fruit absent) in generalized linear mixed-models (lme4 package, version 1.1-35.5). Our null model included plant ID as a random effect (as many plants had multiple replicates of each treatment), and the full model additionally included the pollinator exclusion treatment and flowering period (number of days each inflorescence was open) as fixed effects. We used Akaike Information Criterion (bbmle package, version 1.0.25.1) for model comparison. We compared bagging treatments using a Tukey's post-hoc comparison of means (emmeans package, version 1.10.4).

Common garden in Okemos Michigan, 2020

If *A. ameliae* flowers signal to specific pollinator guilds, we expect the reproductive contributions of diurnal and nocturnal pollinators to remain consistent between habitats where *A. ameliae* does and does not naturally occur. In May 2020, we grew *A. ameliae* in a Michigan State University greenhouse from seed provided by Lady Bird Johnson Wildflower Center, collected from the same population we used in the field experiment two years later. We planted these *A. ameliae* plants and individuals of 16 other greenhouse-grown *Abronia* species into a raised bed in a garden in Okemos, Michigan, containing 50:50 sand/topsoil. Once plants began to flower, we censused pollinators daily when not raining, windy, or excessively cold. As this greenhouse is far outside of *A. ameliae's* native range (the nearest populations are >1000 km away), all floral visitors were naïve to any *Abronia*. Due to the significant geographic separation from any wild-growing *Abronia* species, these floral visitors were also unable to facilitate any hybridization with native sand verbenas. To determine the contributions of nocturnal and diurnal pollinators to seed set, we implemented the same bagging treatments: day open (n=24), night open (n=24), always open (n=35) inflorescences. We followed the same seed collection and seed set scoring methods conducted in the natural Texas population. We analyzed pollination success using a generalized linear mixed-model where seed set was the binomial response variable, treatment was a fixed effect, and plant ID was a random effect. We used AIC to compare this model to a null model including only the random variable plant ID. Treatments were compared using a Tukey's post-hoc comparison of means.

FLORAL VISITORS IN THE FIELD

To determine the general community of floral visitors, we made extensive observations of insect visitation in the field. We recorded floral visitors for several hours each day, including while setting up the experiment but not during daily dawn-dusk bagging, as this was a time-sensitive process. Observations in the natural Texas population occurred during the March 2022 field study as well as during another visit to this population in March 2021. We recorded all floral visitors and visitation time. We collected (photographed and/or physically collected) any visitors we could not identify visually. The rates of visitation to any given inflorescence or even individual plants were low enough that ascertaining pollination rates was logistically infeasible; therefore, our data shows the overall community visiting these plants.

FLORAL VOLATILES IN THE GREENHOUSE

We characterized *Abronia ameliae* floral volatile organic compounds (VOCs) as part of a broader comparative study of VOCs across sand verbenas that required use of the same methods and greenhouse growing conditions for all species. We collected VOCs from 11 *A. ameliae* plants grown from seed in greenhouses at UC-Davis and Michigan State University. VOCs were collected using dynamic headspace sampling. We also sampled volatiles from the leaves of a single *A. ameliae* plant to determine which compounds might have vegetative origins. At approximately 6:00 pm on the day of sampling, inflorescences were fully enclosed in a nylon oven bag (Reynolds) and given 30 minutes to equilibrate. We chose to collect floral VOCs overnight based on the observation that *A. ameliae* was more fragrant during nocturnal compared to diurnal periods (LoPresti, pers. obs.), and also for direct comparison with other *Abronia* species as part of a broader phylogenetic comparative study of floral VOC emissions. Headspace sampling was conducted at a rate of 1 L/min using a vacuum system and an inline pressure gauge. Volatiles were collected continuously overnight for 12 hours and were adsorbed onto activated coconut charcoal scent traps (ORBO 32 Small, Suppelco). At the end of sampling, scent trap vials were capped and frozen at -20ºC for later processing, and the sampled inflorescence was cut and weighed to nearest 0.01 mg.

We used 400 µL of 99% dichloromethane (Sigma-Aldrich) as an extraction solvent to elute scent traps (Raguso & Pellmyr 1998; Zhang et al. 2023), with no further concentration steps. We added 5 µL of a 90 ng/µL solution of tetrahydronaphthalene (Sigma-Aldrich) as an internal standard to each sample for use in quantification. To analyze floral volatiles, we conducted gas chromatography/mass spectrometry using a 7890B Agilent GC coupled to an Agilent 5977A (Agilent Technologies, Santa Clara, CA, USA) mass spectrometer at the University of California, Davis. The instrument was fitted with a 30 m x 25 mm x 0.25 µm HP-5 Ultra Inert column. Samples were injected at a 1 µL sample volume in splitless mode using an autosampler, with the split valve closed for 1 minute post-injection, and with the inlet and transfer line held at a constant temperature of 250°C. The initial oven temperature was 40°C, held for 3 minutes, followed by a temperature ramp of 5°C/min up to 210°C, followed by a subsequent ramp of 20°C/min to 300°C, followed by a final hold at 300°C for one minute. Helium was used as the carrier gas at a flow rate of 1.2 mL/min. Electron impact mass spectra were obtained by scanning between 30-550 *m/z*.

GC-MS data were processed using MassHunter GC/MS Acquisition software version B.07.00 and MSD ChemStation Enhanced Data Analysis

Software version F.01.00 (Agilent). Peaks were initially called automatically using the RTE integrator and a detection threshold limit of 0.5% of the largest peak. Chromatograms were manually annotated to include peaks that were visible but fell below this detection threshold (Fig. S1), and also to remove contaminant compounds that appeared in control injections containing only dichloromethane. Peak alignment was based on retention times.

We assigned tentative identifications to compounds by comparing mass spectra and retention times to published databases (Adams 2007, NIST mass spectral library) as well as comparisons to authentic standards for a small subset of compounds (Table S1). Remaining compound IDs should be considered tentative, and we did not attempt to classify compounds that were potentially present as mixtures of structural isomers such as lilac aldehydes and alcohols (Dötterl et al. 2006). We follow the recommendations of Eisen et al. (2022) and only report compound identities when quality scores for matches to mass spectral libraries were above 90%. To analyze floral VOC emissions, we subtracted emission rates for leaf compounds. We then used the integrated peak area of the internal standard (tetralin, 450 ng) and the fresh mass of the sampled inflorescence to calculate volatile emission rates (ng/g hr^{-1}) for each compound. After removing compounds that were only detected in a single sample or that had very low emission rates (<5 ng/g hr^{-1}), we retained 40 unique compounds from the 11 *A. ameliae* plants (Table S1), which were assigned to biosynthetic classes according to the categorizations used in Knudsen et al. (2006).

RESULTS

DIURNAL AND NOCTURNAL POLLINATOR CONTRIBUTIONS

Natural population in Falfurrias, Texas, 2022

Consistent with the hypothesis that *A. ameliae* has transitioned to include diurnal pollination, we found day-open and always-open flowers had significantly higher pollination success than night-pollinated flowers (day-open differs from night-open: $P < 0.005$, SE = 0.1356; always-open differs from night-open: $P < 0.0144$, SE = 0.1255; Fig. 2). We also found that day-open flowers did not differ significantly from always-open flowers ($P > 0.05$, SE = 0.1336; Fig. 2), consistent with a scenario in which *A. ameliae* can receive total pollination success from daytime pollinators alone. The best-fitting model (Table S2) included inflorescence bagging treatment and flowering period as significant predictors (delta AIC = 7.8 compared to the null model); inflorescences which were open for more days had higher pollination success ($\beta = 0.18 \pm 0.08$, $z = 2.17$, $P = 0.03$). A model fitting treatment and flowering period as interactive predictors fit no better than the additive model (likelihood ratio test, $X^2 = 2.9332$, df = 2, $P = 0.2307$).

Common garden in Okemos Michigan, 2020

We found qualitatively the same results in the natural population as in the native population. Day-open and always-open flowers did not significantly differ in pollination ($P = 0.3177$, SE = 0.0674), but both received significantly more pollination than night-open flowers (day-open differs from night open ($P < 0.001$, SE = 0.7421; $P < 0.001$, SE = 0.0736, respectively) (Fig. 2). The best-fitting model (Table S2) included inflorescence bagging treatment as a significant predictor (delta AIC = 146.9 compared to the null model).

FLORAL VISITORS IN FIELD

The observed communities of floral visitors to the natural Texas *A. ameliae* population in 2021 and 2022 are summarized in Table 1. Butterflies and sphingid moths were especially common floral visitors: probable pollinators (floral visitors with proboscises long enough to contact the inserted *A. ameliae* stigma and covered in *A. ameliae* pollen) are included in Fig. 1B-D and Fig. S2. Qualitatively, butterflies and day-active moths were abundant visitors in Michigan as well, though nocturnal visitors were observed. The most common visitor to *A. ameliae* during the day in the Michigan common garden was the diurnal sphingid, *Hemaris thysbe*. In both locations, butterflies, bees, and flies were only observed visiting flowers diurnally.

FLORAL VOLATILES IN THE GREENHOUSE

Abronia ameliae produced a complex combination of floral volatiles (Fig. 3, 4), with components associated with both moth and non-moth visitors. The total floral bouquet is summarized by volatile compound class in Fig. 4, and for a full list of compounds, compound classes, emission rates, and mean squared error see Table S1. Volatile emissions of *A. ameliae* were predominantly benzenoid/phenylpropanoid-

Table 1. Floral visitors observed in the natural Texas population in 2021 and 2022. Bold X: specimen in LoPresti Lab collection. Lower case x: photographed (credit, Eric LoPresti). 1: Forrest Whittaker, pers. comm.

	2021	2022
Lepidoptera - Butterflies		
Hesperiidae		
Amblycirtes celia	x	x
Atalopedes campestris	**x**	**x**
Chioides catillus		**x**
Erynnis funerealis		x
Lerema accius		X
Lerodea eufala	x	**x**
Polites vibex	**x**	x
Wallengrenia otho		**X**
Lycaenidae		
Strymon melinus		**X**
Nymphalidae		
Agraulis vanillae		x
Chlosyne lacinia		X
Danaus plexippus		x
Euptoieta claudia		**X**
Junonia coenia		**x**
J. nigrosuffusa		**X**
Vanessa atalanta		**X**
V. virginiensis		**X**
Papilionidae		
Battus philenor	X	**x**
Papilio cresphontes[1]		
Pieridae		
Colias eurytheme		X
Pontia protodice		**X**
Riodinidae		
Calephelis nemesis		**x**

	2021	2022
Lepidoptera - Moths		
Geometridae		
Eubaphe unicolor		**x**
spp. (2 morphospecies)		**X**
Pyraloidea		
spp. (4 morphospecies)		**X**
Noctuidae		
Anicla infecta		**X**
Megalographa biloba		**X**
Schinia indiana	**x**	
spp. (4 morphospecies)		X
Sphingidae		
Erinnyis obscura		x
Hyles lineata		**x**
Xylophanes tersa		**x**
Coleoptera		
Buprestidae	**X**	
Melyridae		
Collops sp.	**x**	x
Diptera		
Bombylidae sp.	**x**	
Syrphidae		
Copestylum mexicanum		**x**
spp. (2 morphospecies)		x
Hymenoptera		
Apidae		
Apis mellifera	**x**	**x**
Halictidae		
spp. (2 morphospecies)	**x**	x

derived aromatic compounds (Fig. 4), particularly benzaldehyde, benzyl acetate, methyleugenol, benzyl alcohol, and p-methylanisole, consistent with nocturnal pollination. Benzyl acetate and benzyl alcohol, two of the most abundant compounds produced by *A. ameliae* (Fig. 3), have been linked to pollination by hawkmoths in other systems (e.g., Knudsen and Tollsten 1993, Raguso et al. 1996, Johnson et al. 2020). The floral bouquet also contained a comparatively high proportion of lilac alcohols and aldehydes (~15% of total bouquet) relative to other *Abronia* species (generally 0%, and ~3-5% in a few species), compounds generally associated with visitation by Noctuid moths (Dötterl et al. 2006). *Abronia ameliae* has no completely unique compounds, and much lower nocturnal floral VOC emission rates per unit mass than some other truly nocturnal, white-flowered *Abronia* (e.g., *A. elliptica*, *A. glabrifolia*, *A. fragrans*) (unpublished data).

DISCUSSION

Many animal-pollinated plants possess flowers with traits conforming to a pollination syndrome, but some include atypical traits that deviate from

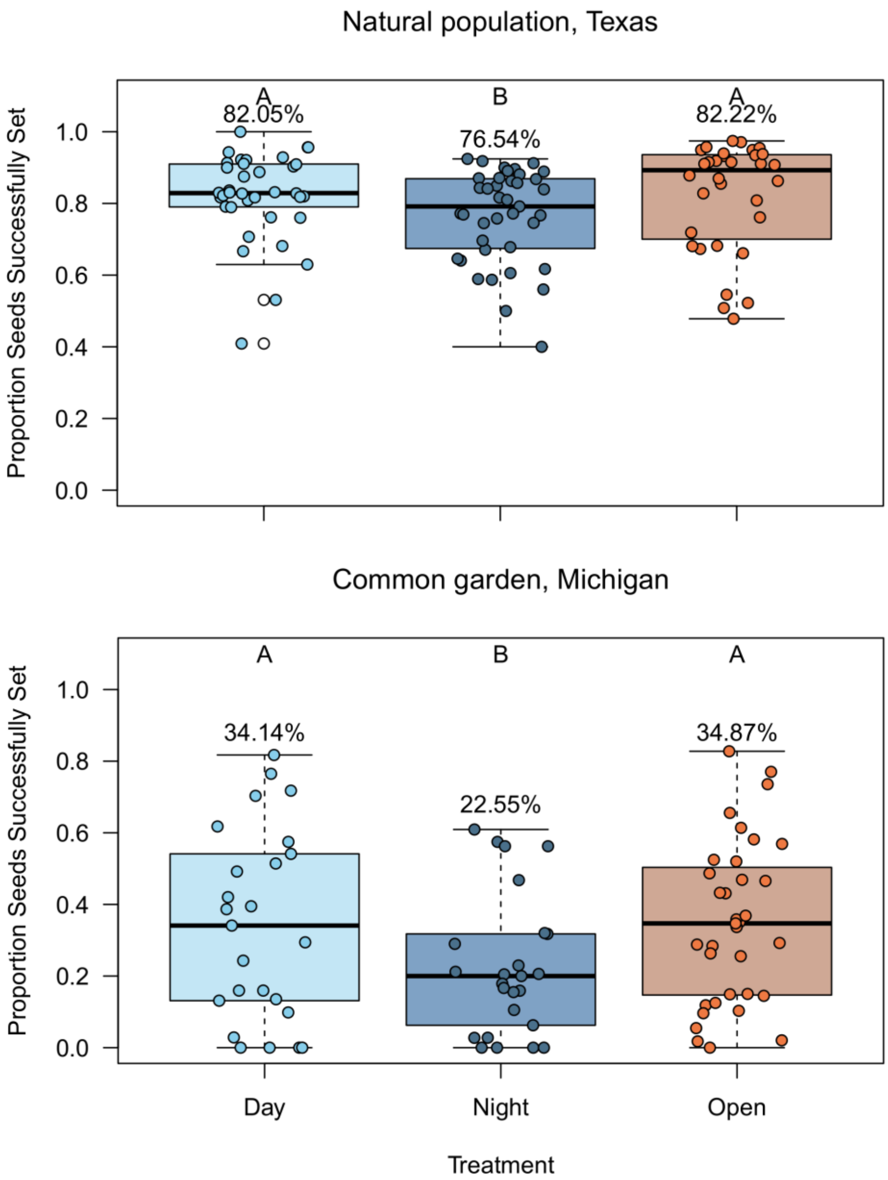

Figure 2. Inflorescence seed set (proportion of flowers pollinated, or fruits with seeds present/total fruits) in the natural Texas *A. ameliae* population (2022) and in the common garden in Michigan (2020) across inflorescence bagging treatments (day-open, night-open, and always-open). Unfilled points designate outlier data from the boxplots; filled points are the overlaid raw data. In both populations, a post-hoc Tukey test revealed that day and always treatments each differed significantly from the night treatments, but day and always treatments did not differ from each other. Letters denote significant difference in Tukey's post-hoc comparison of means.

close relatives and do not neatly fit into typical syndromes. These unique trait assemblages may represent selection by additional or alternative pollinators that drive a departure in pollination biology. Alternatively, species with atypical floral morphologies may also be the result of non-pollinator selection forces, such as other biotic factors (florivores, pathogens), abiotic selection, or non-adaptive forces such as genetic drift or linkage. Here, we investigated whether *A. ameliae*'s marked floral differences from closely related, moth-pollinated *Abronia* species are associated with non-standard pollination biology for the genus. As hypothesized, *A. ameliae* exhibited unusual pollination patterns for the genus. While *Abronia* species are typically night pollinated, diurnal pollinators contributed most to seed set in *A. ameliae* across two experimental locations, revealing that diurnal pollinators reliably play a more substantial role in the reproductive success of *A. ameliae* than nocturnal pollinators. Similar responses of pollinators both within and outside of *A. ameliae*'s native range further suggest that the floral characteristics are driving the observation of diurnal pollination, as the plants are attractive to even naïve pollinators. We conclude that *A. ameliae*'s atypical floral traits – day-open and pigmented flowers, large inflorescences, and long peduncles – are likely associated with diurnal pollination, and we propose that these phenotypes may be adaptations for increasing apparency to visually-oriented pollinators, as has been found in

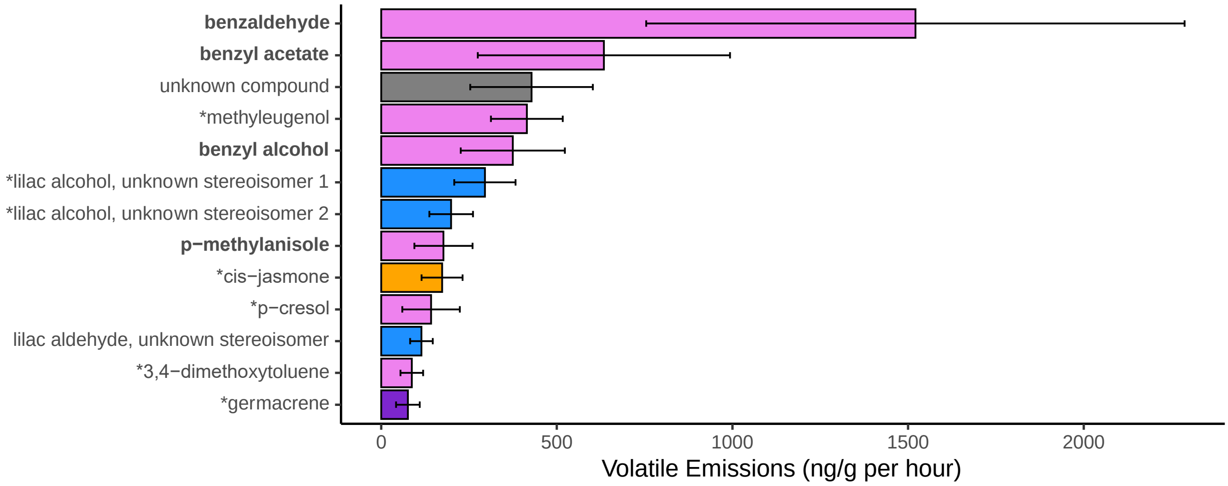

Figure 3. Mean *A. ameliae* nocturnal volatile emission rates, expressed as nanograms of tetralin equivelents per gram of fresh floral tissue per hour. Error bars correspond to +/- one MSE. The 13 most abundant floral volatile compounds are shown. The identities of compounds shown in bold (benzaldehyde, benzyl acetate, benzyl alcohol, and p-methylanisole) were confirmed with authentic standards. Remaining compound IDs were based on matches to mass spectral libraries and should be considered tentative; compounds with library match quality scores of >90% are indicated with an asterisk. See Table S1 for the full list of emitted compounds.

other plants (Gómez 2003; Parachnowitsch & Kessler 2010; Hirota et al. 2013; Knauer & Schiestl 2017).

Although butterflies and diurnal moths were the primary pollinators of *A. ameliae*, nocturnal pollinators also provide pollination services to *A. ameliae*. Nocturnally open inflorescences set considerable seed both in the common garden and the field, though still significantly less than diurnally and always open inflorescences. Apart from *A. ameliae*'s atypical floral features, many floral traits are still consistent with a moth-pollination syndrome, including nocturnal flowering and the abundance and composition of floral volatiles associated with nocturnal pollinators (though diurnal volatile emissions were not collected for comparison). As volatile organic compounds may respond to selection more quickly than morphological traits (Gervasi & Schiestl 2017), continued selection by nocturnal pollinators could be responsible for the continued production of scent consistent with moth pollination while other morphological traits generalize. Given that our experiment was timed to coincide with the peak of flowering and occurred in a single year, it is likely that in certain seasons or years, the contributions of nocturnal pollinators may be higher, or vice versa, than measured in this study.

The reproductive contributions of different pollinator groups can depend greatly on individual floral visitor abundances, each with its own degree of mechanical fit to flowers or foraging behaviors (Primack & Silander 1975; Morse & Fritz 1983; Adler & Irwin 2006). In *A. ameliae*, further investigation of visitation rates, pollen deposition, or other metrics of individual pollinator efficacies might reveal that one or two abundant or efficient hawkmoth species contribute all nocturnal pollination, while tens of butterfly species independently contribute small proportions of the total diurnal seed set. Still, our results clearly show that diurnal pollinators contributed more to seed set than nocturnal visitors during the study period, regardless of the mechanisms underlying this difference.

Does *A. ameliae*'s unique floral morphology represent an in-progress transition to exclusively diurnal pollination? Perhaps this trait combination arose from some balance between pollination and other selective pressures? Or perhaps this floral morphology represents a generalist lepidopteran pollination syndrome? While we cannot conclusively determine the evolutionary pressures that created the highly distinct, modern-day *A. ameliae* and the pollination patterns we found, we discuss the evidence for and against multiple interpretations.

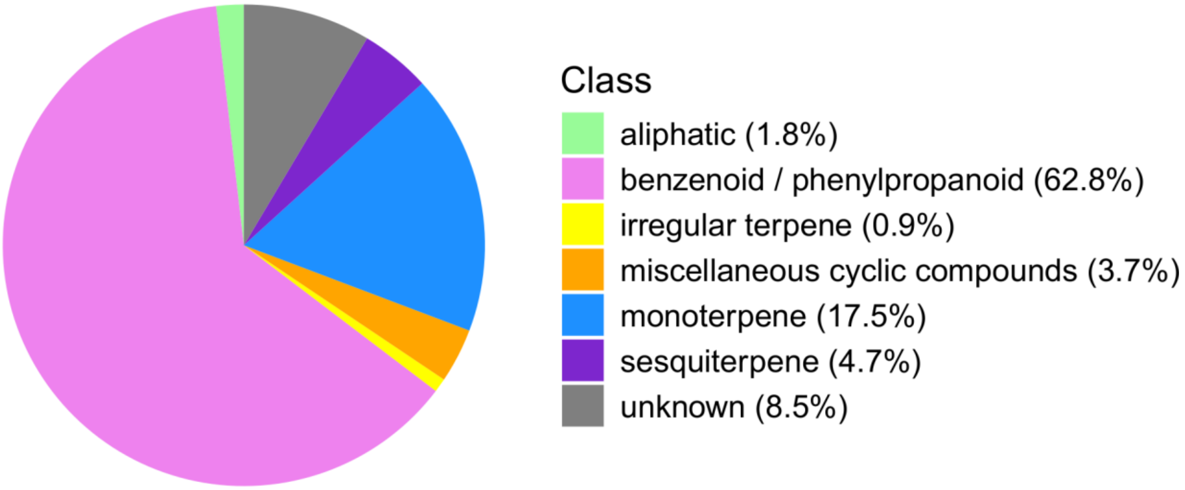

Figure 4. *Abronia ameliae* floral volatile emissions grouped by compound class. Percentages correspond to the proportion of total profile within each compound class.

EVIDENCE FOR A SHIFT TOWARDS DIURNAL POLLINATION IN *ABRONIA AMELIAE*

Given the morphology of most sand verbena species, *A. ameliae* is a clear outlier. Field pollination data across the clade is lacking, yet it is highly likely that moth-pollination is the ancestral state for *Abronia*. This assertion is backed up by three lines of evidence: (1) most species close their flowers diurnally, limiting the possibility, or quantity, of diurnal pollination (but see Jaeger et al., 2023); (2) all species investigated in depth, representing about a quarter of all sand verbenas, are primarily moth-pollinated (Tillett 1967; Keeler & Fredricks 1979; Williamson et al. 1994; Saunders & Sipes 2006; Jabis et al. 2011; Doubleday & Eckert 2018; Jaeger et al. 2023); and (3) most species broadly fit the moth-pollination syndrome of long narrow tubes, pale flowers, and strong scent. Broadly, the atypical floral traits of *A. ameliae* align with those of the butterfly floral syndrome. However, a comprehensive, agreed on set of characteristics for a solely butterfly-pollinated plant, like that for hummingbird, bee, or moth pollination, is yet to be widely adopted (see Anýž et al. 2019 for a short review).

The Grant-Stebbins definition of the butterfly pollination syndrome as pink/red flowers (Grant & Grant 1965; Stebbins 1970) is perhaps the most generally accepted perspective, but others have reported butterfly sensitivities and/or preferences for yellow, orange, mauve (Swihart 1969; Struwe 1972; Willmer 2011; Sinha et al. 2023), blue, purple (Ilse & Vaidya 1956), magenta (Swihart 1969), violet (Sinha et al. 2023), and ultraviolet flowers (Arikawa 2017). This syndrome has thus been variously redefined by different or more inclusive categories of vivid floral colors (Cruden & Hermann-Parker 1979; Faegri & Pijl 1979; Hingston & Mc Quillan 2000), and would be interesting to examine in a modern framework of spectral properties including hue, chroma, and brightness (Stevens et al. 2023). The presence of nectar guides, nectar quantities, and strength of floral scent, among other floral traits, in the butterfly pollination syndrome are similarly disputed (Anýž et al. 2019). Nevertheless, a long corolla tube, daytime flowering, and a landing platform are characters that are reasonably uniform across descriptions of the butterfly syndrome, though these traits fail to differentiate entirely from certain other syndromes. Any of the diurnally open *Abronia* species may therefore fit this definition, but moth pollination was the predominant strategy in the two day-open species that have been investigated so far (*A. alpina* (Jabis et al. 2011), and *A. umbellata* (Doubleday and Eckert 2018)). In contrast to those species, *A. fragrans* contains pink-flowered populations with flowers that open in the early evening (~18:00), close in mid-morning (~10:00), and may receive <10% of pollination from butterflies during those short periods (Jaeger et al. 2023). *Abronia ameliae* flowers further exaggerate these traits, with enormous, bright inflorescences that remain open throughout the day that butterflies commonly land on. Therefore, despite the discordance surrounding the butterfly pollination syndrome designation, *A. ameliae*'s atypical floral traits appear to meet most criteria of the butterfly syndrome (which may more broadly include diurnal moths, as well), and indeed, we found in this study that *A. ameliae* is primarily diurnally pollinated.

LOCAL FACTORS – IS TEXAS A GOOD PLACE TO BE A BUTTERFLY-POLLINATED PLANT?

We suggest another important factor that may play into butterfly pollination of *A. ameliae*: location. While butterflies broadly occur from sea level to high mountains and in most habitats on all continents, less Antarctica, they are not uniformly abundant everywhere. However, South Texas is known as a particularly butterfly-rich area (Robbins & Opler 1997; Reaka-Kudla 1997) and has at least one other well-documented secondary shift to butterfly pollination (*Phlox drummondii*, Grant & Grant 1965; Wiggam & Ferguson 2005; Burgin et al. 2023). It is possible that butterfly pollination is less reliable year-to-year, or within season, in many other areas – including high mountains and deserts – inhabited by other *Abronia* species, which seem to be moth-pollinated (Tillett 1967; Keeler & Fredricks 1979; Williamson et al. 1994; Saunders & Sipes 2006; Jabis et al. 2011; Doubleday & Eckert 2018; Jaeger et al. 2023). Butterflies are usually less speciose than bees and moths (Michener 1979; Kristensen et al. 2007) and have far narrower thermal activity constraints (Kingsolver 1985; Heinrich 1987; Goller & Esch 1990). Therefore, a shift towards some, or complete, butterfly pollination could be more likely in very specific regions, such as areas with unusually high butterfly diversity and activity, including the narrow range of South Texas occupied by *A. ameliae*. Note that in areas with fewer or less reliable butterflies, they may still be important secondary pollinators, as we found in the shortgrass prairie *A. fragrans* in north Texas (Jaeger et al. 2023).

PERHAPS THIS TRAIT COMBINATION AROSE FROM BOTH POLLINATION AND OTHER SELECTIVE PRESSURES?

Inferring past selective pressures from current morphology or ecological function is a particularly fraught exercise. However, we see no particularly compelling reason to invoke non-pollinator selection in the case of the *A. ameliae* floral syndrome. We observed very few florivores, all caterpillars of *Hyles lineata,* which is also a very common pollinator. Further, selective pressures on floral size by such ovipositing moth species would probably push the flower to be smaller, less apparent, and produce fewer volatile emissions (quantity and/or variety of compounds associated with attracting ovipositing hawkmoths), as suggested in another *Abronia* species (Doubleday et al. 2013). Instead, *A. ameliae* exhibits gigantism and conspicuous inflorescences, and is often visited by *Hyles lineata*. The most devastating herbivores we witnessed were leaf-cutter ants, *Atta texana*, which completely defoliated and seemingly killed (at least the aboveground tissue of) several plants in the natural population (Fig. S3), though we never observed them consuming flowers. We also observed workers entrapped on the sticky surfaces of the plant (Fig. S3), suggesting that this herbivorous ant species could exert a selective pressure on this physically defensive trait (LoPresti 2016).

Physiological pressures, such as limiting nectar loss during hot days, also seem unlikely to have driven this floral morphology. Unlike most of its close relatives, *A. ameliae* does not close its flowers diurnally in its warm, windy native Texas habitat. With its large leaves, upright form, and enormous inflorescences (greater number of flowers contributing greater overall size), *A. ameliae* also lacks many obvious drought-stress adaptations seen in other species (i.e., *A. angustifolia*, *A. bigelovii*, *A. nana*). Betalain pigmentation, however, may confer tolerance to drought, provide photoprotection, or offer other benefits in stressful habitats (Jain & Gould 2015), and is present in high quantities in *A. ameliae*'s pink flowers (unpublished data). Thus, while *A. ameliae*'s bright floral color may increase attractiveness of flowers to diurnal pollinators, we cannot say for certain that greater floral pigment concentrations (as well as any of these atypical floral traits) might not have evolved under alternative selective pressures or other mechanisms such as drift.

PERHAPS *ABRONIA AMELIAE* HAS A GENERALIST LEPIDOPTERAN POLLINATION SYNDROME?

This syndrome of pink flowers, open nocturnally and diurnally, with strong nocturnal fragrance, plenty of nectar, and a landing pad, perhaps suggests not an "intermediate stage of double function" (Stebbins 1970) as the syndrome shifts between pollinator groups but a distinct generalist lepidopteran syndrome. Differentiating this possibility from an incipient shift is impossible. However, it is worthwhile to consider that *A. ameliae* maintains potentially costly traits that seem to advertise separately to each lepidopteran group. *Abronia ameliae* has both very high scent emission at night, when nocturnal moths but not butterflies are active, and highly pigmented flowers open during the day, when butterflies and diurnal moths are most active (sphingid moths possess color vision and utilize different modalities for diurnal and nocturnal pollination (Kelber et al. 2002; Balkenius et al. 2006)). This trait combination may be particularly adaptive if it provides reproductive assurance to the plant – pollination assurance by diurnal pollinators in years of low moth abundance or vice versa.

While our data are consistent with butterflies and day-active moths being the most important pollinators of *A. ameliae*, the relatively high seed set in the night-only pollination treatments and the prevalence of nocturnal floral volatile emissions suggest that moth visitation is likely still important in this species. The compounds emitted in the highest amounts included benzaldehyde, benzyl acetate, and benzyl alcohol, all of which are classic examples of hawkmoth attractants (Raguso et al. 1996). Interestingly, *A. ameliae* also produced the highest relative abundance of putative lilac alcohol and aldehyde compounds of any sampled *Abronia* species (unpublished data); these compounds are strongly perceived by moths and have been implicated in Noctuid moth pollination of *Silene* (Dötterl & Jürgens 2005) and *Platanthera* (Tollsten & Bergström 1993) and fungus gnat pollination of *Asimitellaria* (Okamoto et al. 2015), but do not appear to elicit antennal responses in bees (Jürgens et al. 2014). While GC-EAD studies of floral volatile compounds are less common in diurnal compared to nocturnal lepidopterans, Nymphalid and Pierid butterflies show evidence for strong antennal responses to benzenoid compounds (Andersson et al. 2002; Andersson 2003; Andersson & Dobson 2003). Like other *Abronia* species (Doubleday et al. 2013) – and confamilials in the aptly-named four o'clock family, Nyctaginaceae – *A. ameliae* flowers begin emitting scent at dusk, which might indicate a role for floral VOC production in butterfly attraction during the final hours of diurnal visitor activity. Thus, floral VOCs that may have evolved in the context of moth attraction may still function as pollinator attractants even in shifts to primarily diurnal visitation. However, because we did not collect diurnal floral VOC emissions, any potential role of floral scent in mediating attraction of day-active visitors of *A. ameliae* is conjecture.

A. ameliae and other sand verbenas species are ideal for further investigating the evolution of pollination traits. While species with only one or a few traits differing from close relatives are ideal for determining the selective environment on these traits (Ohashi et al. 2021), *A. ameliae* is an extreme morphological outlier in the genus, with most measured floral traits differing greatly from its close relatives. Determining individual traits that contribute to the pollination system would require breaking up this trait combination, which is an attainable goal (*A. ameliae* is easy to hybridize in the lab; some examples are shown in Fig. S4). There are also several other sand verbena species with colorful and/or diurnally open flowers besides *A. ameliae*, which may represent shifts away from moth-pollination either partially or wholly. These species, including *A. latifolia, A. pogonantha, A. angustifolia* (specifically disjunct, annual, day-flowering populations near Phoenix, AZ), *A. maritima*, and *Tripterocalyx crux-maltae* (which is likely bee-fly pollinated – LoPresti, pers. obs.) can be crossed to break up trait-combinations as well (as intergeneric crosses between *Abronia* and *Tripterocalyx* are possible).

CONCLUSIONS

Consistent with our predictions of mostly diurnal pollination, we found *A. ameliae* to be predominately pollinated by various butterflies and diurnal sphingid moths. However, *A. ameliae* also produces a complex volatile profile primarily constituted of moth-attractive compounds and receives substantial nocturnal pollination. Therefore, this species exhibits multiple potential strategies to maximize reproductive success, even though it deviates from expected syndromes. *Abronia ameliae*'s floral traits and pollination biology demonstrates the complexity of pollination systems in nature and challenges strict conventional pollination syndrome designations.

Thus, whether *A. ameliae* represents a case of an ongoing pollinator shift, a result of non-pollinator selection, a balance of effective pollinators, or alternative explanations, we present its current morphology as an example of a generalist lepidopteran pollination syndrome. Our study also suggests that investigating the pollination strategies of plants with atypical floral morphologies may provide insights into the roles of plant-pollinator dynamics and related factors in driving the evolution of these traits.

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AUTHOR CONTRIBUTION

EFL and SLJ devised the study and conducted the field observations and pollination experiments; EFL, SLJ, and CMA quantified seed set. EFL, MGW, and MGF devised the volatile organic compound (VOC) analyses and MGF collected the VOC data. ETH and SLJ analyzed the data. SLJ wrote the manuscript with input from all authors.

DISCLOSURE STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

All data and scripts are archived within a single Figshare project titled “*Abronia ameliae* data and scripts”. Pollination data and script are available at https://doi.org/10.6084/m9.figshare.28541690 (Jaeger et al. 2025a); VOC script at https://doi.org/10.6084/m9.figshare.28560659 (Jaeger et al. 2025b); GC-MS readouts at https://doi.org/10.6084/m9.figshare.28560599 (Jaeger et al. 2025c); VOC output data at https://doi.org/10.6084/m9.figshare.28548119 (Jaeger et al. 2025d); and VOC input data at https://doi.org/10.6084/m9.figshare.28548086 (Jaeger et al. 2025e).

APPENDICES

Additional supporting information may be found in the online version of this article:

Appendix I. *Abronia ameliae* volatile organic compounds data, floral visitors, herbivores, hand-crossed hybrids, and pollination model details.

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