

Do Morning Butterfly Visitors Benefit a Night-Flowering Hawkmoth Pollinated Plant?

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ABSTRACT

The white, tubular, fragrant flowers of *Guettarda scabra* (Rubiaceae), rough-leaved velvetseed, open in the evening and are visited by hawkmoths (Sphingidae). Flowers last for one day, and recent observations reveal that butterflies also visit these flowers. Hawkmoths hover over the flower and lower their proboscis into the corolla to collect the nectar. Butterflies land on the petals before inserting their proboscis and may transport pollen on their bodies as well as their mouthparts. We conducted an experiment to determine the importance of each of these guilds for pollination of *G. scabra*. We excluded day-time visitors from some inflorescences and night-time visitors from others on the same plants (with two controls: some open all the time and some bagged all the time). We maintained this regimen during the entire flowering period of the selected inflorescences over two months and compared fruit set among the treatments. The control-open inflorescences and the night-open inflorescences had substantially higher fruit set than day-open and control-bagged inflorescences. Mean fruit set of day-open plus night-open inflorescences approximated that of control-open inflorescences, and although the fruit set of day-open flowers was small, it differed from bagged controls. Fruit set in *G. scabra* is determined almost entirely by hawkmoths, but butterflies may be useful as secondary pollinators. As plants flower in months when afternoon and evening rains can extend into the night, morning pollinators may be important. This study provides additional evidence that diurnal pollinators can contribute to the reproduction of predominantly nocturnal pollinated plants.

Key words: butterflies, *Guettarda scabra*, hawkmoths, Lepidoptera, pine rocklands, pollination, velvetseed

INTRODUCTION

Pollination syndromes, collections of floral traits typical of particular pollinator groups, suggest that plants are specialized for particular pollinators, often associated with plant speciation (Waser et al. 1996, Dar et al. 2006, Valdivia and Niemeyer 2006, Johnson et al. 2011). Indeed, there are many times where certain pollination systems are classified as being specialized (Grant 1949, Gilbert 1975, Pérez et al. 2006), but increasingly, they are found to be more generalized, with a variety of visitors performing pollination (Waser et al. 1996, Molina-Freaner et al. 2004, Balmford et al. 2006, Dar et al. 2006).

Plants with flowers accessible to a variety of visitors, flowers open both day and night, or multiple flower opening times (Koptur 1983), may get pollinated when extreme temperatures or heavy rains prevent visits to flowers during some parts of the day. A number of diurnal (day-open) versus nocturnal (night-open) comparisons have supported the nocturnal moth pollination syndrome, despite observations of plentiful daytime visitors (Young 2002, Reynolds et al. 2009); others have shown that pollination systems are more mixed than originally thought (Goldblatt and Manning 2002, Dar et al. 2006, Valdivia and Niemeyer 2006, Maruyama et al. 2010, Johnson et al. 2011).

Flowers with features that suggest diurnal or nocturnal pollination are perhaps not as specialized as those features imply (Waser et al. 1996, Groman and Pellmyr 1999, Young 2002, Valdivia and

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Niemeyer 2006, Maruyama et al. 2010). For example, the pollination system in *Faramea cyanea* (Rubiaceae) is mixed, with bee pollinators during the day and moth pollinators at night, despite the presence of moth attractive floral traits (Maruyama et al. 2010). Many diverse species of pollinators visit these plants, providing they can access the floral rewards, regardless of the flower morphology (Groman and Pellmyr 1999, Valdivia and Niemeyer 2006, Johnson et al. 2011). *Guettarda scabra* (L.) Vent. (Rubiaceae), rough-leaved velvetseed, is one such plant where the flowers may have both diurnal and nocturnal pollinators, though they have nectar in a narrow floral tube accessible only to long-tongued visitors.

Flowers of *G. scabra* are borne in paired cymes (Figure 1A), and on each inflorescence one or two flowers begin opening in the late afternoon and are mostly open by dusk (Figure 1B). Plants are self-compatible, with some fruits produced by automatic self-pollination (Richards and Koptur 1993). Nocturnal flower visitors are various species of hawkmoths (Koptur 2020). Hawkmoth visitors will hover over the flower and lower their proboscides into the flower. Pollen from the anthers collects on the proboscis and is then carried to the next flower where it may be deposited onto the stigma. In the morning, butterfly visitors have been observed to land and lower their proboscis into the flower (Figures 2A, B) to collect nectar (Koptur 2020). Both perianth whorls are attached above the inferior ovary that may develop into a fruit (Figure 1E, F) in a pollinated (and successfully fertilized) flower.

This study examined the effects of both diurnal and nocturnal visitors to flowers of *G. scabra* to determine which are effective in pollination and producing fruit. By exposing flowers to only daytime or nighttime visitors, and then comparing fruit set (the number of fruits over the total number of flowers per inflorescence) we can measure how much pollination is accomplished by diurnal versus nocturnal visitors.

MATERIALS AND METHODS

Study Species

In pine rocklands of the Everglades, rough-leaved velvetseed is one of the most abundant and conspicuous woody plants. *Guettarda scabra* (L.) Vent. (Rubiaceae) is a native perennial shrub of both pine rockland and hardwood hammock habitats in subtropical regions of southern Florida (Davis et al. 2009), the northernmost part of its tropical distribution (Richards and Koptur 1993). Its flowers (ca. 2 cm long) are white, with a tubular corolla, and a very sweet, pleasant fragrance. The flowering season is May–July, with the fruits developing in July and August, green until they are mature (when they become red and velvety) throughout the fall (Richards and Koptur 1993).

Though plants reach reproductive size at 1 m or less in height, they can grow to 6 m in hardwood hammocks (Koptur and Garcia 2017). Pine rockland *G. scabra* plants bear many leaves that mature to be tough and smaller in the bright sun of the pine rockland, whereas their counterparts in hammocks produce fewer leaves that are larger (ibid.). The plants are dry-season deciduous, and most individuals spend a month or more leafless from December through March, though some retain their old leaves while producing new leaves, then dropping the older ones (Koptur unpublished data). Flower buds are evident within two months of new leaves being produced. The species was categorized as “tropical summer-flowering” (Tomlinson 1980), and plants at Larry and Penny Thompson Park begin flowering in late April, while those in Everglades National Park begin blooming in late May, with the last individuals finishing flowering in July; peak flowering is early June at Larry and Penny Thompson Park, and late June in Everglades National Park (Loope 1980, Gunderson et al. 1983).

Study habitat

Pine rocklands are much reduced from their original extent in south Florida due to extensive human development on this higher ground since the early 1900s (Koptur 2006). Pine rocklands are a fire-successional habitat, and persistence and flowering of many species depend on periodic fires (Snyder et al. 1990). Fire stimulates flowering in many pine rockland plants (Loope 1980, Gunderson et al.

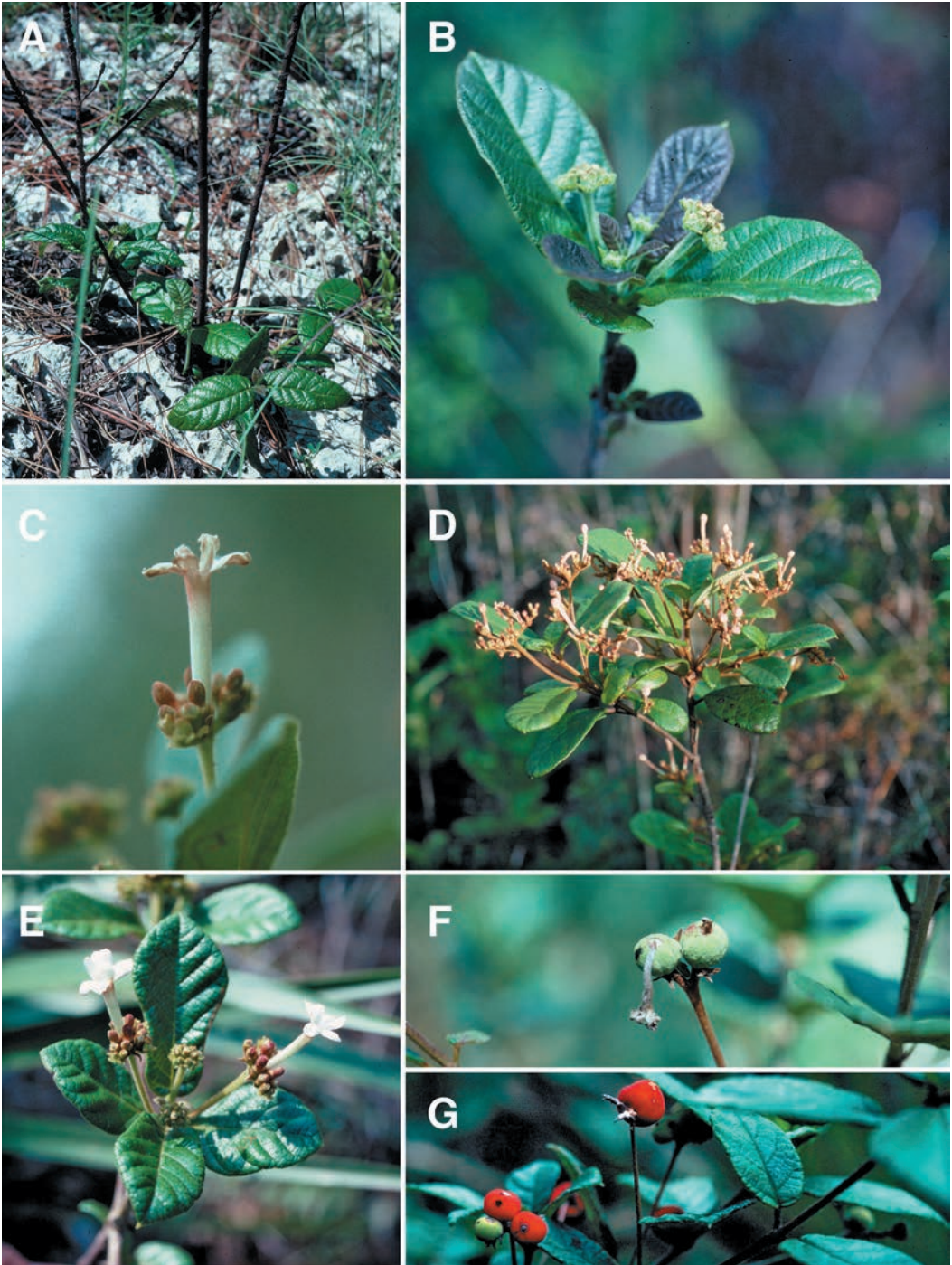


Figure 1. Leaves, flowers, and fruits of rough-leaved velvetseed, *Guettarda scabra*, at Larry and Penny Thompson Park in Miami-Dade County, Florida. **A.** Resprouting stems after a fire in pine rocklands. Leaves are opposite, decussate and scabrous (with a rough surface). **B.** Buds in the dichasial cymose inflorescences. **C.** A single flower open in the morning. **D.** Late afternoon elongated buds that will open that evening. **E.** Open flowers on opposite inflorescences, **F.** Green fruit, one with old flower still attached. **G.** Red velvety fruits that complete the common name of the species.

1983), but unlike perennial wildflowers (Spier and Snyder 1998, Liu et al. 2005, Cardel and Koptur 2010, Barrios et al. 2011) and understory palms (Koptur and Khorsand 2018, Koptur and Barrios 2020) that bloom the following year, resprouting *G. scabra* individuals do not flower until two years after a fire.

Pollinator observations

We encountered a variety of insects associated with the flowers of *G. scabra* in both Everglades National Park (Koptur 2020) and the study site of this experiment, described below. Observations were made watching plants near our experimental plants, in morning hours between 7 a.m. and noon, and evening hours between 7 and 10 p.m., in 15-minute increments for a total of approximately 10 hours for each time period. Our earlier observations at Everglades National Park showed that *Guettarda scabra* flowers open late in the afternoon, becoming fully open to visitors by dusk (Richards and Koptur 1993). With their sweet fragrance, and their pinkish-white flowers with long corolla tubes, it was no surprise to find them visited by moths (Koptur 2020). Many plants had butterflies visiting their flowers in the morning hours, more easily observed than hawkmoths at night, but apparently quite frequent. We retained voucher specimens from the county park in the insect collection of the Plant Ecology Lab at FIU, for eventual deposit in the Florida Collection of Arthropods in Gainesville, Florida.

Pollinator exclusion experiment

We studied *G. scabra* in pine rocklands of Larry and Penny Thompson Memorial Park, one of the largest extant pine rockland fragments in Miami-Dade County, Florida. In May 2014, we chose three locations and selected individual plants more than 1 m away from another chosen individual plant, choosing 20 plants in each location, for a total of 60 plants overall. GPS coordinates of the center of the three locations are: 25°36'7.52"N, 80°24'31.96"W; 25°35'58.08"N, 80°24'26.08"W; and 25°36'0.83"N, 80°24'6.53"W.

From each group of 20 plants at each location, we chose plants with inflorescences just beginning to bloom. On each of these plants we designated branches of inflorescences to receive each of the four treatments (below), which were designated a “set”. We had 15 sets of treatments per site (45 sets overall). The four treatments were: day-open (D) (flowers open to visitors from 7 a.m.–7 p.m.); night-open (N) (flowers open to visitors from 7 p.m.–7 a.m.); control-open (CO) (flowers were not bagged, always open to visitors); and control-bagged (CB) (flowers themselves opened, but always in a bag, visitors excluded). We used pollination bags of 100% polyester fine-mesh fabric that would allow access to air and light but that visitors could not penetrate with their proboscides (Figure 3).

For each replicate, we chose a branch with inflorescences of buds developing for the seasonal bloom, but none yet open. We randomly assigned each branch of inflorescences to one of the four treatments and labeled each below its base using laboratory tape and indelible marker. We did not observe the actual opening time of all the flowers on the inflorescences in this experiment, but treated all the same. Because two of our plants did not bloom concurrently with the rest, we had duplicate sets on two individuals at one site to provide equal sample sizes. The overall sample size was at least 45 inflorescences in each treatment and control group; individual plants varied as to the numbers of inflorescences at the end of a branch.

We visited the sites every morning at 7 a.m. and every night at 7 p.m. to open or close the bags, depending on the time of day, every day from May 24–July 18, 2014. At 7 a.m. we would visit each site to open the day-open treatment bags and close the night-open treatment bags. At 7 p.m. we would return to the sites to close the day-open treatment bags and open the night-open treatment bags. Each bag we opened was secured on the stem below and near the inflorescence, so that each inflorescence had its same bag throughout the experiment. These treatments were maintained throughout the flowering season. Usually one or two flowers per inflorescence opened each day. Once all the flowers on an inflorescence had bloomed and senesced, it was re-bagged to protect and retain the developing fruits (we also bagged the formerly always-open inflorescences).

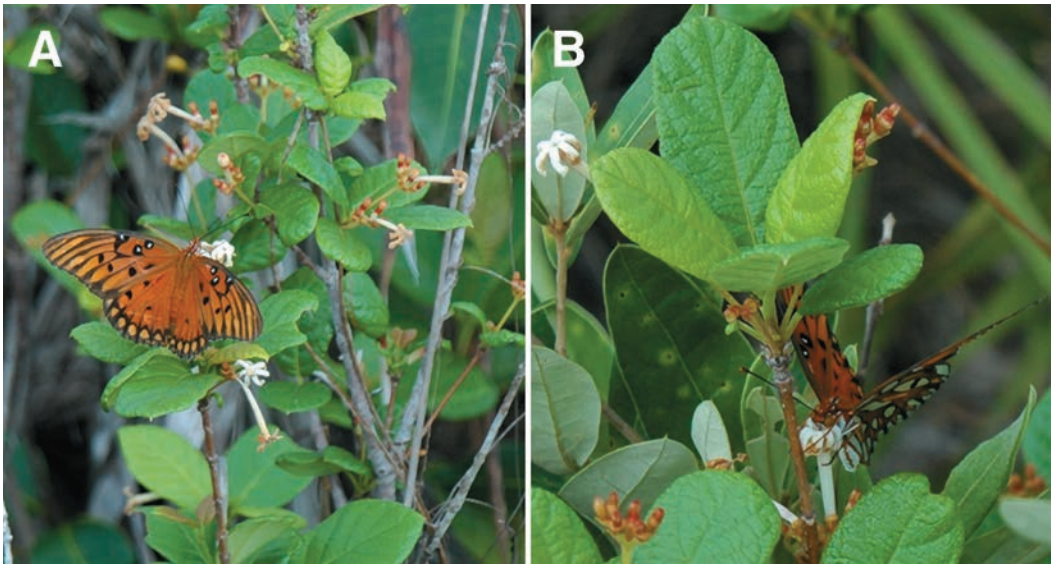


Figure 2. Gulf fritillary butterfly (*Agraulis vanillae*) visits to *Guettarda scabra* flowers in the morning. **A.** Butterfly dorsal view, with its leg on the mouth of a flower as its proboscis enters the corolla tube. **B.** Ventral view of butterfly clamping outside of corolla tube with its face in the flower.

The number of inflorescences per treatment and the number of flowers per inflorescence were counted before fruit development obscured that information. We recorded fruit set after one month and monitored fruit until four months after the last flowering. *Guettarda scabra* fruits have a stony endocarp within which several seeds are produced (Tomlinson 1980), requiring a hack-saw to determine how many seeds are present. As we planned to germinate the seeds/fruits, we did not undertake this destructive method to count the seeds. Therefore, only fruit set is reported. We used non-parametric Kruskal-Wallis and Mann-Whitney tests to compare fruit set among the four treatments. In addition, we also used the Bonferroni method to control for Type I error for pairwise comparisons.

RESULTS

Flower behavior and visitor observations

Most flowers on our study plants of *G. scabra* at Larry and Penny Thompson Park started opening (buds cracking) from 5:30 to 6:45 p.m., becoming fully open after 7 p.m., lasting 12–18 hours. A few flowers on some plants appeared to open in the morning (buds cracking between dawn and 6:45 a.m., flowers fully open after 7 p.m.), lasting less than 12 hours before their corolla lobes began to turn brown. We often observed butterflies at the flowers in morning hours, most commonly the gulf fritillary, *Agraulis vanillae* (L.). We observed several hawkmoth visitors in the evening hours at this park, but only managed to catch a single individual of one species, *Xylophanes tersa* (L.) (Tuttle 2007).

The flowers would then abscise by late afternoon leaving only the calyx and ovary. If fertilized, the ovary developed into a fruit; otherwise, both eventually fell off within about two weeks.

Fruits from exclusion experiment

Fruit set (the proportion of flowers that set fruit) was compared among the four treatments. The control open treatment had the highest fruit set among the four treatments (Figure 4). The night-open treatment had the second highest fruit set among the four treatments. The day-open treatment had a significantly lower fruit set when compared to the night-open and control open treatments. Finally, the control bagged treatment had a lower fruit set than the other three treatments and was

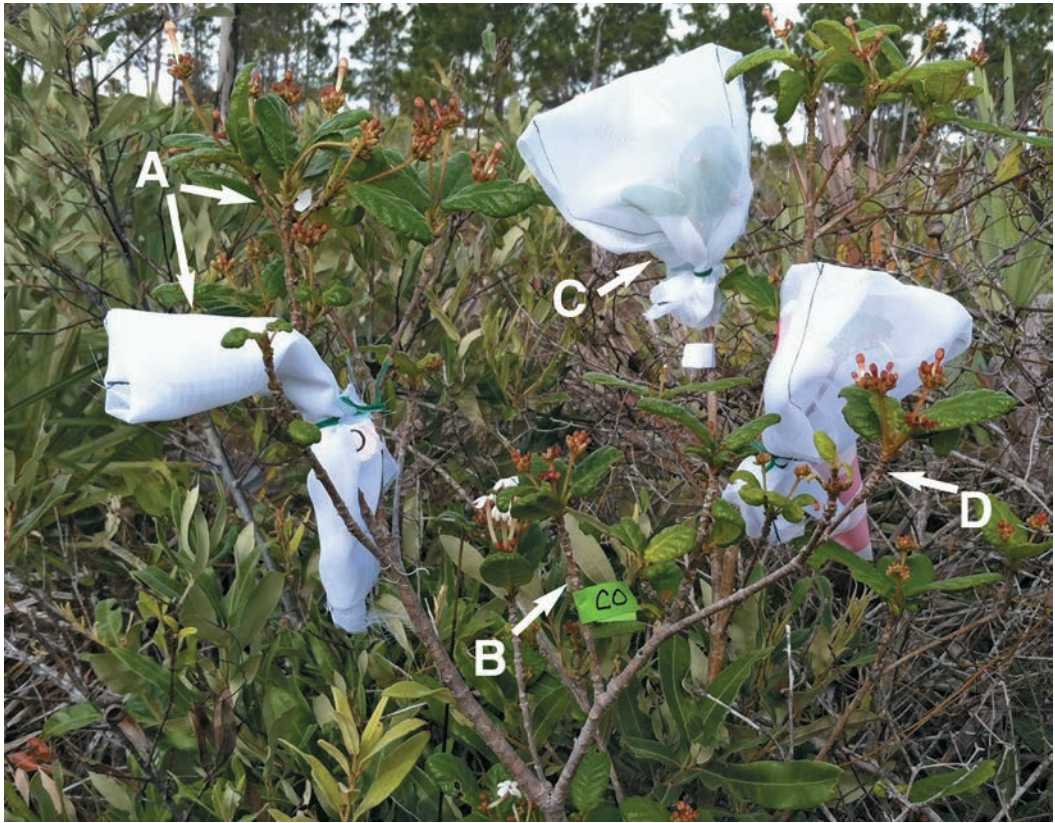


Figure 3. *Guettarda scabra* at Larry and Penny Thompson Park, Miami, Florida. An experimental unit was one individual plant with all 4 treatments: **A.** Day-open–flowers open to visitors during daylight hours (bag clipped to branch beneath it); **B.** Control-open–flowers open to visitors at all times; **C.** Night-open–flowers open to visitors during night hours (bagged here as this photo was taken during the day); **D.** Control-bagged–never open to visitors (red & white striped tape around bag closure).

significantly different from all (Figure 4). In short, flowers open day and night set the most fruit; the night-open flowers set much more fruit than day-open flowers, though day-open flowers set more than the bagged controls.

DISCUSSION

The pale-colored, long-tubed, sweet-smelling flowers of *Guettarda scabra* fit the moth-pollination syndrome, and our previous observations concluded that hawkmoths were the pollinators of this species (Richards and Koptur 1993). However, our subsequent observations of daytime butterfly visitors, and evidence provided here by our visitor exclusion experiments, show that morning-active Lepidoptera are also involved in the pollination of this species. The flowers of *G. scabra* are pollinated both diurnally and nocturnally, indicating that the pollination system of this species is somewhat less specialized and more mixed (Maruyama et al. 2010) than was originally assumed. Extreme specialization on hawkmoth pollinators has been shown to limit reproduction in species surviving only in habitats where hawkmoths are few (Johnson et al. 2004, Amorim et al. 2014). As our experiments took place in a pine rockland habitat fragment, an urban county park, hawkmoths may be less numerous here than in less disturbed situations and more contiguous pine rockland as in Everglades National Park.

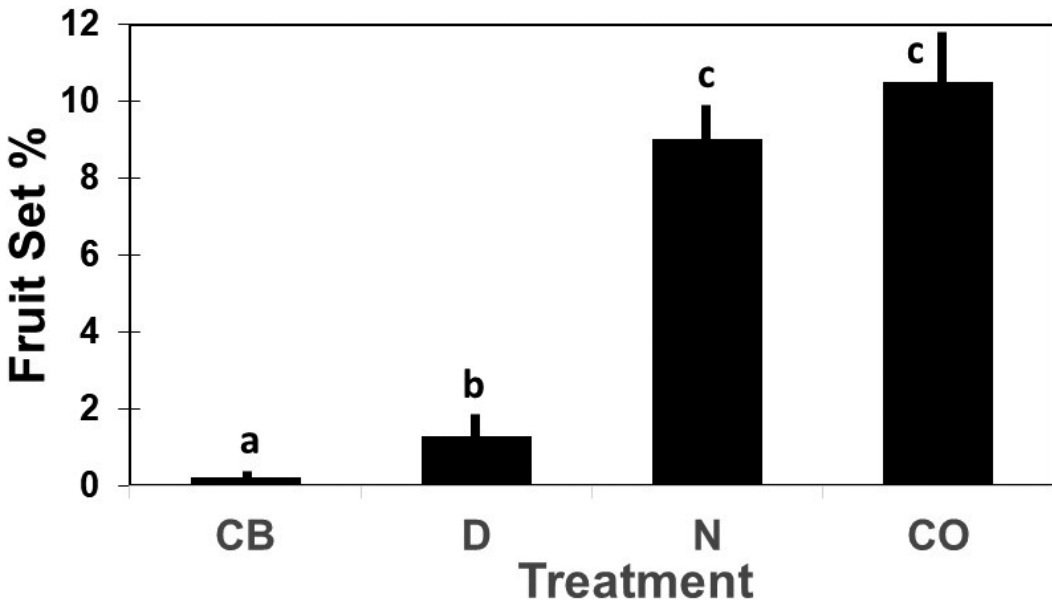


Figure 4. Pollination treatment vs. fruit set. Mean (+ S.E.) percent fruit set (the number of fruits over the total number of flowers found on an inflorescence) of *Guettarda scabra* ($n=45$ plants; 544 inflorescences in total; 138 per treatment group). Treatments: control-bagged (CB) (flowers themselves opened, but always in a bag, visitors excluded); day-open (D) (flowers open to visitors from 7 a.m.–7 p.m.); night-open (N) (flowers open to visitors from 7 p.m.–7 a.m.); control-open (CO) (flowers were not bagged, always open to visitors). Different letters above bars indicate significant differences ($p<0.05$) as determined from posthoc Bonferroni after Kruskal-Wallis test.

We undertook this experiment to determine if the flowers of *G. scabra* might be multipurpose, though there have been very few documented shifts to diurnal Lepidoptera (Fenster et al. 2004). Our exclusion experiment demonstrated that visitors are necessary for pollination in this species; in addition, those inflorescences open to visitors 24 hours a day (the control-open treatment) set the most fruit, somewhat more than night-open inflorescences. The hypothesis that nocturnal pollination is the more effective form of pollination for this species is supported: flowers open only at night to visitors set more than three times the fruit of those open only during the day. Fruit set from day-open flowers was small, but substantially more than that of bagged flowers. Our data from the populations we studied support the nocturnal moth syndrome with some pollination services provided by diurnal visitors.

Other species of the genus *Guettarda* (Croat 1978, Novo 2010, Quirino et al. 2014, Lawton et al. 1993, Hilje et al. 2015) also have white flowers (e.g., *G. angelica* in the Brazilian caatinga; *G. crispiflora* subsp. *poasana* in cloud forests of Monteverde CR; *G. folicacea* in the tropical wet forest on Barro Colorado Island, Panama; *G. macrosperma* in dry forests of Costa Rica; *G. platypoda* in the *restinga* of Pernambuco, Brazil; and *G. speciosa* in Asia) that are fragrant, have nectar, and nocturnal anthesis. The flowers in these other species also last for one night only, “an adaptation to pollination by night-active hawk-moths” (Puff 2007). The nectar of *G. speciosa* is sucrose-dominated (Freeman et al. 1991), typical of flowers visited by both hawkmoths and butterflies (Baker and Baker 1983). *Guettarda macrosperma* is described as having “lepidopterophily” (Hilje et al. 2015), a term that could include both diurnal and nocturnal visitors: butterflies and moths. Only nocturnal moths (including two hawkmoths) have been observed visiting flowers of *G. platypoda* (Novo et al. 2018).

We interpret the near-complete lack of fruit set in our always-bagged treatment as evidence this population is pollinator-dependent, but this result might have resulted from negative effects of bagging on the microclimate inside the bags. However, fine nylon mesh bags are the type of bag that

least affect flower nectar production and presumably other floral features (Wyatt et al. 1992), and we have used this type of bag in other field experiments in this habitat with no apparent ill effects, including earlier breeding system experiments on this species (Richards and Koptur 1993). It may also be that dawn and dusk are especially important times for pollinators to visit flowers, and we disrupted these times with our bagging and unbagging, a shortcoming of the design of this experiment.

One possible explanation for highest fruit set in the always-open (unbagged) flowers of *G. scabra* is that since the majority of flowers are dusk-opening and lasted for 12–18 hours before the corolla lobes began to brown, those flowers may have more visitors when compared to dawn-opening flowers, which are open for a shorter time, lasting less than 12 hours. The dusk-opening flowers remain fresh through the next morning, with white corolla lobes and still fragrant, whereas the dawn-opening flowers were likely faded by the time dusk came. This is likely due to the cooler, moist night air keeping flowers fresh longer than the extreme heat experienced during the day. This increased visitation rate to dusk-opening flowers, open through the night and the following morning, likely leads to greater fruit set in the inflorescences that were open only at night.

Another explanation could be that moth visitors might be more effective at transferring pollen to the stigmas of receptive flowers than diurnal visitors (Gimenez-Benavides et al. 2007, Valdivia and Niemeyer 2006). Though we first anticipated that perhaps butterflies might transfer more pollen not only on their mouthparts, but also their body parts as they perch to feed, whereas hawkmoths only insert their proboscis while hovering over the flower, it does not appear that butterflies are more important and/or successful pollinators of this species. In addition, hawkmoths may be able to transport the pollen farther than butterflies, possibly due to their larger bodies and stronger flight ability, and this could also lead to higher fruit set as a result of greater outcrossing (Valdivia and Niemeyer 2006, Gimenez-Benavides et al. 2007, Johnson et al. 2011).

In his consideration of plants and nocturnal and crepuscular pollinators, Baker (1961) noted that although moths might be excellent pollinators, they are sometimes not present when the flowers are in bloom; less ideal pollinators that are present may be very important in fruit and seed production. He also highlighted some flowers adapted to multiple kinds of pollinators; it may be that, in some circumstances, *G. scabra* is one of these. Offspring fitness may be affected by crossing distance between flowers resulting from differences in flight distances of butterfly and hawkmoth visitors (Bertin and Willson, 1980). The quality of the pollen deposited onto the stigmas of *G. scabra* may also be affected by the flight distance; it is likely that, if hawkmoths carry the pollen farther, the resulting increase in offspring fitness might select for traits more suited to hawkmoth pollinators (Willmott and Búrquez 1996).

Guettarda scabra blooms in the early summer, a time when afternoon thunderstorms are common, and rains may last long into the night. It may be that on those nights it is impossible for hawkmoths to fly and visit the flowers, so that morning visits from butterflies can mean those flowers are not “wasted”. Though many mornings during our study were overcast and very humid, morning rain is much less common than afternoon and evening rain in the subtropical south Florida summer, and so having a few flowers open in the morning might further insure some pollinator visits.

Summarizing data on trait shifts in flowering plants observed to be pollinated by more than one group of pollinators, Fenster et al. (2004) found many documented changes in floral color and morphology, but many fewer in reward and fragrance. A collaborative group (Ollerton et al. 2009) performed a global test of the predictive value of the pollinator syndrome hypothesis in diverse communities on three continents, describing flowers and then observing visitors to many different species in six communities. They found that the array of flower visitors is normally beyond those that the “syndrome” predicts, cautioning that pollinators of a flowering plant cannot be predicted based on flower characteristics alone. Rosas-Guerrero et al. (2014) tested the fit of pollination syndromes with effective pollinators and concluded that the syndrome hypothesis may have some value, as flower traits have been influenced by the most effective pollinators among the palette of flower visitors. They also discuss the importance of secondary pollinators, though they concluded that generalization is overestimated (Rosas-Guerrero et al. 2011) without information on the effectiveness of floral

visitors. Pollinator effectiveness is important because frequency of visitations does not necessarily indicate how effective that visitor is as a pollinator (Barrios et al. 2016). In fact, some may be nectar-robbars, collecting nectar from the flower without contacting the pollen or stigma, and subsequently not contributing to pollination (Molina-Freaner et al. 2004).

It is also important to determine the proportion of flowers that open at dawn versus dusk, and how long individual flowers remain open and active, in this and other *G. scabra* populations. By observing the proportion of dawn-opening and dusk-opening flowers, we can determine if there is a preponderance of one flower opening time. Additionally, comparisons of how the pollination system varies among populations in different pine rockland sites, specifically how habitat fragmentation affects the pollinator fauna and pollination of *G. scabra*, warrant further investigation.

Our study revealed the primary importance of nocturnal pollination in the reproductive biology of *G. scabra*. We suggest that although hawkmoths are evidently the primary pollinators of *Guettarda scabra*, butterflies may be secondary pollinators, potentially contributing to fruit set, important only when hawkmoths are not available. A study of common milkweed found nocturnal visitors to be higher quality in terms of fruits per visit, but diurnal visitors contribute more to overall fruit production, because of much higher visitation (Jennersten and Morse 1991). Further work on *G. scabra* should include comparing the effectiveness of moths and butterflies as pollinators, measuring their contributions to plant fitness (as in Fenster et al. 2009). Certainly, it is misleading to assume every pollination interaction to be specialized, and activities of flower visitors are not bound by rules suggested in pollination syndromes. Further investigation into the pollination biology of this attractive, native, flowering plant of the pine rocklands may reveal if both diurnal and nocturnal visitors are essential to its reproduction and persistence in a variable climate.

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