

POLLEN ACCUMULATION ON THE HAWKMOTH PROBOSCIS VARIES SUBSTANTIALLY AMONG MOTH-POLLINATED FLOWERS

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Abstract—Using the pollen loads carried by floral visitors to infer their visitation behavior is a powerful technique to explore the foraging patterns of pollinators. Interpreting these pollen records, however, requires assumptions about pollen movement patterns. To compare visitor foraging across flower species, the largest assumption is that pollen is acquired and retained on the visitor at similar rates. However, even flowers with similar morphologies differ in pollen presentation traits such as grain number or stickiness. We investigated pollen accumulation on the proboscis of the hawkmoth *Manduca sexta*, testing the degree to which accumulation differed among flower species with differing morphologies and how pollen stickiness affected this accumulation. In no-choice floral visitation assays to six plant species visited by long-tongued hawkmoths in the wild, *M. sexta* individuals visited flowers 1, 2, or 5 times, after which pollen grains adhering to their proboscises were counted. We found that the six plant species varied orders of magnitude in the number of pollen grains deposited on the moth proboscises, with some placing thousands of grains after one visit and others placing none after five. Plant species with sticky pollen placed more pollen on the moths and had less pollen accumulation over successive visits than did plants with non-sticky pollen. Moths also carried fewer grains on their proboscises after 5 visits than after 2 visits, suggesting that both sticky and non-sticky pollen was lost during foraging. Together, our results suggest that interpretation of pollen load data should be cautious, especially when comparing across plant species.

Keywords—Pollination, pollen accumulation, pollen fate, pollen loss, hawkmoth

INTRODUCTION

Given the importance of ecosystem services provided by pollinators in both natural and agricultural settings (Ollerton 2017), the study of pollinator foraging behavior is critical to predicting their responses to both natural and anthropogenic perturbations. However, tracking the foraging decisions made by fast-flying floral visitors is challenging, especially in the natural floral communities in which these decisions are made. This is especially true for highly dispersive nocturnal pollinators such as hawkmoths (Sphingidae), due to rapid and unpredictable foraging bouts under dimly lit conditions (Baker 1961; Martins & Johnson 2007). While direct visit observations (Hopkins & Rausher 2012), interview choice assays (Campbell et al. 2016; Ogilvie & Thomson 2015) and even camera traps at flowers (Edwards et al. 2015; Johnson et al. 2020) can

provide key insights into pollinator behavior, assessing the relative usage of different floral resources and specialization requires finer resolution tracking of complete foraging bouts.

One common method of inferring the foraging decisions of pollinators over entire bouts or longer foraging periods is to collect and identify the pollen carried on their bodies (Burkle et al. 2013; Scheper et al. 2014). As pollinators are unlikely to pick up a given plant's pollen anywhere except from that plant's flowers, pollen loads serve as a forensic record of which plants pollinators had visited during previous foraging bouts and can be collected from many more individuals than would be possible to observe. This technique is especially useful for studying the foraging of hawkmoths (Alarcón et al. 2008; Haber & Frankie 1989; Kislev 1972; Nilsson et al. 1987; Smith et al. 2021a): hawkmoths do not groom themselves or consume

pollen (though pollen can still be lost; Smith et al. 2021b). As such, any pollen found on their proboscises is unlikely to be biased by decisions on which resources to collect, as can be seen in pollinators such as bees (e.g., Lunau et al. 2015). Drawing inferences beyond a given plant's presence from these loads, however, requires several assumptions, the largest of which is that pollen from different flowers is acquired at relatively comparable rates.

While this assumption may hold in rare cases, per-visit pollen acquisition rates are likely to vary substantially across species based on a variety of plant traits. For example, plants vary orders of magnitude in the number and size of pollen grains they produce, from hundreds of 0.2–0.7mm long pollen grains in *Zostera* (Ruckelshaus 1996) to many thousands of 10–12µm pollen grains in *Myosotis* spp. (Meudt 2016). Furthermore, many plants display traits that improve pollen deposition and retention on their visitors, such as a sticky pollenkitt (Pacini & Hesse 2005) or viscin threads (Cruden & Jensen 1979), that could significantly increase the number of pollen grains picked up during a single visit. Plants are also likely to differ in where they place pollen on their visitors based on floral dimensions such as nectar tube depth, corolla shape or stamen exertion (Huang & Shi 2013; Muchhala & Potts 2007). For example, in flowers pollinated by Lepidoptera (butterflies and moths), the majority of pollen grains can be deposited anywhere from the proboscis (Bryant et al. 1991) to the head (Maad & Nilsson 2004) to the wings (Cruden & Hermann-Parker 1979; Murphy 1984). Researchers, however, often collect pollen from only certain structures (e.g., the proboscis of hawkmoths or legs of bees), and may thereby miss grains deposited elsewhere even if some grains are detected at the collection site (Alarcón et al. 2008). Thus, while pollen loads may serve as a reliable record of *which* floral species had been visited, the degree to which pollen grain numbers can be used to draw inferences about the *relative frequency* of floral visitation remains unclear. While pollen removal and deposition location on pollinators has been examined in a wide variety of pollinator taxa (e.g., Butler & Johnson 2020; Harder & Thomson 1989; Muchhala & Thomson 2012; Newman & Anderson 2020), studies frequently focus on closely related plant species, and studies of pollen accumulation

on pollinators over multiple floral visits are rare. Therefore, the degree of variation in deposition or accumulation rates likely to be encountered in a given floral community or pollinator guild is largely unknown, despite its importance in determining the inferences that can be drawn from commonly used forensic pollen load analysis.

Guilds of hawkmoth-pollinated plants have evolved world-wide, showing convergent evolution for nocturnal anthesis, pale coloration, perfume-like aromas and deep nectar tubes or spurs (rev. by Johnson et al. 2017). However, because hawkmoth-pollinated plants often belong to very different angiosperm lineages, pollen morphology and pollen-placement mechanisms may differ substantively between species sharing hawkmoths as pollinators (Grant 1983; Haber & Frankie 1989). Here, we examine the number of pollen grains acquired by *Manduca sexta*, a widespread, long-tongued, nocturnal hawkmoth, on their proboscises over successive floral visits to six species of hawkmoth-pollinated plants from the Americas, showing diverse floral morphologies and traits. We assess two key questions. 1) To what degree do plants differ in pollen deposition on moth proboscises? Given the diversity of floral and pollen traits across the species studied, we predict that the plants should vary substantially in both the number of grains they deposit and in pollen accumulation over multiple visits by *M. sexta*. 2) How does pollen stickiness, which can alter adhesion and male fitness (Cruden & Jensen 1979) affect pollen deposition and pollen saturation on the proboscis? Here we present the answers to these questions, revealed through standardized, no-choice behavioral assays and blinded data analyses of the resulting pollen samples.

MATERIALS AND METHODS

STUDY PLANTS

For this study we selected six night-blooming plant species from different regions of the Americas that are visited by hawkmoths and show diverse floral traits. *Datura wrightii* (Solanaceae) is a primary host plant and nectar resource for *Manduca sexta* in the southwestern USA (Bronstein et al. 2009), with large, funnel-shaped white flowers that moths completely enter to access nectar (Grant 1983). *Ipomoea alba* (Convolvulaceae)

produces similarly large and fragrant white flowers with a more narrow nectar tube and exerted stamens, and its copious nectar rewards are sought by hawkmoths in its native Argentina (Galletto & Bernardello 2004) and in naturalized, invasive populations elsewhere (Haber & Frankie 1989; Johnson & Raguso 2015). The narrow, slightly flared nectar tubes (perianths) of *Mirabilis longiflora* (Nyctaginaceae) are similar in depth to those of *D. wrightii* and *I. alba*, and likewise are pollinated primarily by long-tongued hawkmoths (Grant and Grant 1983). In contrast, the related *M. jalapa*, a commonly cultivated plant, has shorter, trumpet-shaped flowers that are typically visited and pollinated by shorter-tongued hawkmoths in its native range in Mexico (Martinez del Rio & Burquez 1986). *Mandevilla macrosiphon* (Apocynaceae) presents narrow floral tubes similar to *I. alba* and *M. longiflora* in morphology, but displays a highly specialized mechanism of pollen placement common to other *Mandevilla* species, whereby a sticky secretion is applied to their visitors' long tongues before they contact the anther cones (de Araújo et al. 2014; Moré et al. 2007). Finally, *Oenothera harringtonii* (Onagraceae), endemic to Colorado, USA, is almost exclusively pollinated by short-tongued (*Hyles lineata*) and long-tongued (*Manduca quinquemaculata*) hawkmoths (Skogen et al. 2016). Despite all of these target species being primarily pollinated by hawkmoths, these plants vary substantially in their floral dimensions (Fig. 2) as well as other important floral traits. For example, both *O. harringtonii* and *M. macrosiphon* have mechanisms to adhere their pollen to visitors with sticky viscin threads (Hoch et al. 1993) and a sticky epidermal secretion respectively (de Araújo et al. 2014). Thus, these species have the potential to demonstrate a wide range of pollen placement and accumulation patterns on hawkmoth proboscises. Importantly, they are not all adapted to proboscis-placement as a primary pollen-transfer strategy.

All plants used in this experiment were grown from seeds. Seeds for *Datura wrightii* and *Mirabilis longiflora* were collected from the Santa Rita Experimental range in Santa Cruz Co., Arizona. Seeds of *Oenothera harringtonii* were collected from David Canyon, Comanche National Grassland, near La Junta, Otero Co. Colorado by K.A. Skogen, and seeds of *Mandevilla macrosiphon* were collected near Big Bend, Brewster Co. Texas by R.A. Levin.

Seeds of *M. jalapa* and *I. alba* were acquired from commercial seed packets (Burpee, Inc.). Once sown, all plants were grown under greenhouse conditions in Sun-Gro Metro-Mix 360, with day / night temperatures of 24°C / 21°C. Pressed vouchers were deposited for all plant species used at the L.H. Bailey Hortorium (BH), Cornell.

The floral dimensions of each species were measured from 10-15 individual flowers from at least 3 individual plants to the nearest mm using a metric ruler. For more methodological details and the measurements for each plant species, see Supplementary Section [S1].

STUDY ANIMALS

Manduca sexta is an abundant hawkmoth with a wide distribution in North, Central and South America that is a frequent visitor of many night-blooming flowers (e.g., Alarcón et al. 2008; Moré et al. 2006; Schlumpberger et al. 2009). With the assumption that it's floral foraging behavior is comparable to that of other large hawkmoths, *M. sexta* is often used as a surrogate model system for the large guild of long-tongued hawkmoths that are not currently tractable for laboratory studies (see Kaczorowski et al. 2012; Klahre et al. 2011). Adult *Manduca sexta* moths used in this experiment were obtained from a laboratory colony maintained at Cornell. Larvae were reared in the lab on a cornmeal diet (Bell & Joachim 1976; Goyret et al. 2009) on a long-day cycle (LD 16:8 h; 24°C; 40-50% RH). Pupae were removed from the colony and isolated in a 31 x 31 x 32 cm polypropylene mesh cage (BioQuip, Inc.) to eclose. Newly eclosed moths were kept under ambient conditions for 24 h prior to experimental flights.

FLOWER VISITATION

At dusk, unmated, flower-naïve moths were moved from their holding cage into release tubes constructed from soft garden screening (Loew's). While the moths acclimated, a single flowering plant was placed into a large (61 x 61 x 91 cm) polypropylene mesh cage (BioQuip, Inc.), such that the flower (or flowers) were unobstructed and oriented as they would be in nature (i.e., horizontally or upward facing for our plants). All of the plant species tested bloom at night, and present fully dehisced pollen in newly opened flowers. Only virgin flowers that opened on the night of testing were used; older blooms were

removed for plant species whose flowers remain on the plant longer than one day. For *D. wrightii* plants, which were too large to fit into the flight cage, a single flower was fed into the cage while the rest of the plant remained outside. Once active, moths were released into the cage individually and were allowed to visit flowers until a) a given number of visits was reached, b) they had landed three times, or c) 10 minutes had elapsed, at which point they were re-captured gently with the release tubes. Moths were allowed to visit flowers 1, 2, or 5 times ($N = 5$ moths per visit number (3) for each plant species (6) = 90 total moth replicates). Within flights of a given plant species, the number of times each moth was allowed to visit flowers was chosen

haphazardly, with a bias towards allowing more moth visits to ensure that the target sample sizes for 5-visit moths were reached. For representative videos of visits to each plant species, see Supplement [S2]. After each trial was completed, moths were re-captured and pollen on the proboscis (see Fig. 1) was removed using ~2 mm² cubes of fuchsin gel (Kearns & Inouye 1993), following the methods of Smith et al. (2021). These cubes were then melted onto clean microscope slides with a cover slip. All tools involved with producing slides were cleaned with alcohol swabs after each moth to prevent accidental pollen transfer.

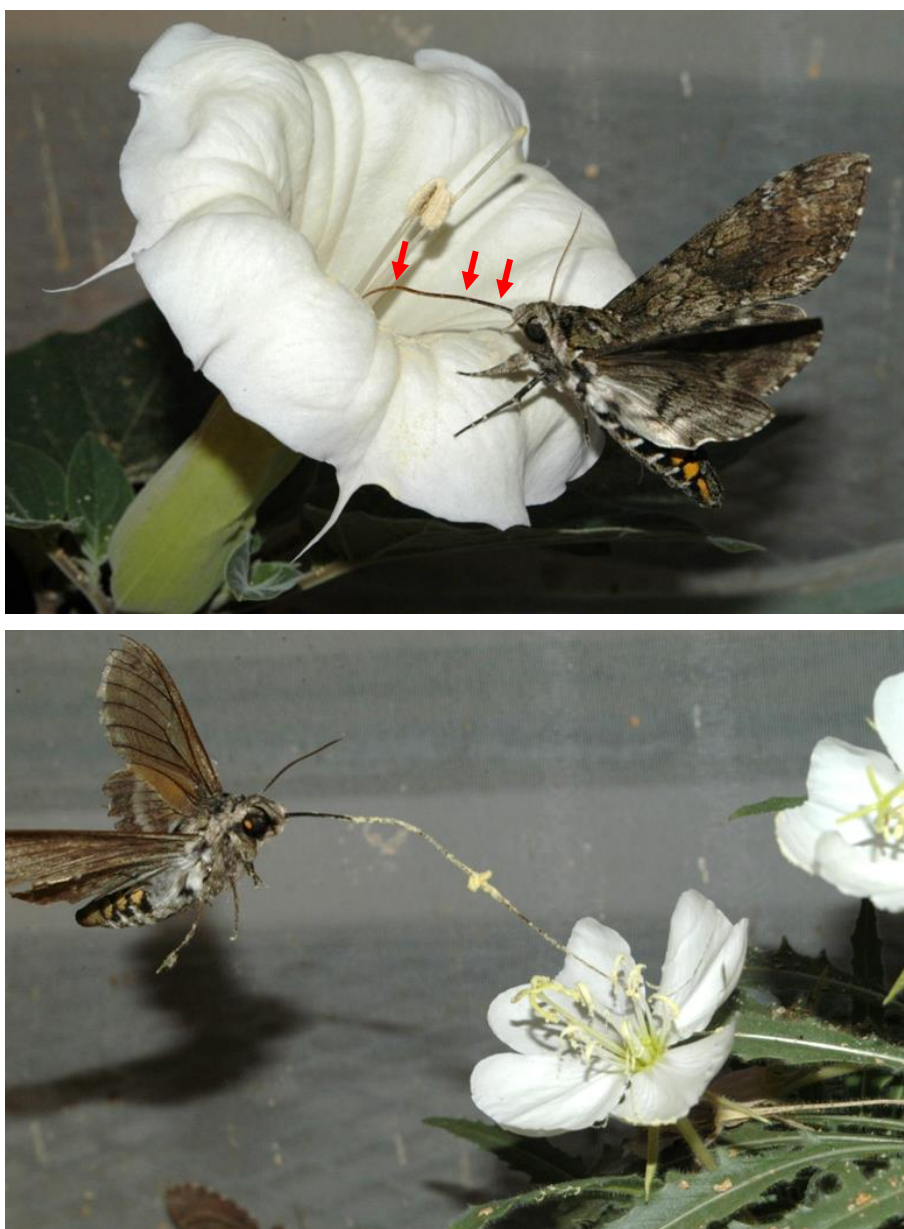


Figure 1. Pollen being deposited on the proboscises of *Manduca sexta* while visiting *Datura wrightii* (top) and *Oenothera harringtonii* (bottom). While the yellow pollen of *O. harringtonii* is especially obvious, pollen can be seen on the proboscis of both moth individuals with the naked eye (highlighted with arrows on the moth visiting *D. wrightii*). *Manduca sexta* proboscises contact *Datura wrightii* anthers and stigmas as they approach and probe the flowers.

POLLEN COUNTING

Pollen grains on slides from all species except *O. harringtonii* were individually counted at 10X-40X magnification using a light microscope (Nikon Eclipse 80i). For *Oenothera* pollen, which was too densely packed on the slides for individual grains to reliably be distinguished and counted, pollen numbers were estimated based on the size of the pollen clusters and their opacity, itself determined by the depth of the pollen in that cluster. For each cluster, the depth was determined by moving the microscope's focal plane up and down to distinguish layers. Due to the flattened shape of the grains and variation in their orientation, clusters varied between 1.5 and 3.5 grains deep. Areas with similar opacity, and therefore depth, were measured with a scale and multiplied by the depth to estimate pollen grain numbers.

The total number of pollen grains presented by *Datura wrightii* and *Mandevilla macrosiphon* were estimated by vortexing fresh anthers in 70% ethanol, counting the grains contained in an aliquot of this mixture, and estimating the total number through multiplication. For *D. wrightii*, one anther (of 5 total) was vortexed in 200 μ L of ethanol, and grains were counted from 10 μ L. For *M. macrosiphon*, all anthers were vortexed in 100 μ L of ethanol, and grains were again counted from 10 μ L. The pollen presented by *Ipomoea alba*, *Oenothera harringtonii*, and *Mirabilis jalapa* were counted directly from fuchsin gel cubes that had removed all pollen from a subset of anthers, as the ethanol dilutions were not feasible for these species. For *O. harringtonii*, vortexing caused the viscin threads to clump pollen grains together rather than diluting them evenly. All *O. harringtonii* grains from one fresh anther (of 8 total) were removed with 2 gel cubes and were counted as described above. For *I. alba*, grains were removed from a pressed specimen; gel cubes were used to collect grains that had been dislodged from the anthers. For *M. jalapa*, the number of grains on fresh anthers was simply small enough that direct counting of every grain on a fuchsin gel cube was more accurate than estimates from dilutions. *Mirabilis longiflora* was not counted; all grains on pressed specimens were absent at the time of counting.

DATA ANALYSIS

All analyses were performed using R version 3.6.3 (R Core Team 2020).

Question 1: To assess whether plant species differed in their pollen deposition on moth proboscises, we ran a Poisson generalized linear model (GLMM) using the *glmm* command in the *lme4* package (Bates et al. 2015), with pollen grain number as the response variable and plant species, visit treatment, and their interaction as fixed effects. Due to the small number of visit treatments and the fact that the numbers were not continuous (i.e., 3- and 4-visit treatments were not included), visit number was treated as a categorical variable rather than a continuous variable. Testing date and previous visits (i.e., the number of flower visits that had occurred on a given set of flowers before a moth was introduced) were included as random effects. Additionally including moth size (proboscis length) as a random effect increased AIC values and did not qualitatively affect the results; therefore the GLMM without proboscis length is reported below. Furthermore, moths that had acquired zero pollen grains were excluded; including these moths did not qualitatively change the results. To better examine the pollen acquisition pattern for each species, post-hoc models were run for each plant treatment independently. In these models, visit number was the only fixed effect, and all *p*-values were adjusted using a false discovery rate (FDR) correction.

Question 2: To assess whether pollen stickiness affected pollen deposition, we ran a Poisson GLM with pollen counts as the response and pollen stickiness, visit treatment and their interaction as fixed effects. For the purposes of this model, pollen stickiness was treated as a binary variable, with *M. macrosiphon* and *O. harringtonii* considered sticky and all other plant species considered not sticky.

RESULTS

POLLEN GRAINS PRESENTED BY FLOWERS

The number of pollen grains presented to floral visitors differed substantially among plant species. In our greenhouse, each *Datura wrightii* flower produced an estimated 348,200 pollen grains, *Ipomoea alba* produced an estimated 1,625; *Mirabilis jalapa* produced an estimated 275; *Mandevilla macrosiphon* produced an estimated 1,100; and *Oenothera harringtonii* produced an estimated 10,968.

Question 1: Variation in deposition

The number of pollen grains present on moth proboscises varied significantly, over three orders of magnitude, among plant species (GLM, see Tab. 1, Fig. 2). Moths that visited flowers of *Oenothera harringtonii* carried the most pollen grains (mean grains \pm SE: 1807.7 ± 297.4), followed by *D. wrightii* (mean 833.1 ± 185.9 grains), *M. macrosiphon* (452 ± 101.7 grains) and *I. alba* (12 ± 4.9 grains). Only one moth carried a single pollen grain of *Mirabilis jalapa* on its proboscis, and none of the moths that visited *M. longiflora* carried any pollen on their proboscis. The number of pollen grains also differed significantly between the visit treatments, with moths in the 2-visit and 5-visit treatments carrying more pollen grains than 1-visit moths across all plants. Visit number and plant species also interacted significantly, such that the shapes of the pollen accumulation curves for each species differed (Fig. 3). In planned post-hoc analyses of the four plant species whose pollen was detected on moth proboscises, 2-visit moths carried more grains than 1-visit moths for all plants (GLMM $P > 0.001$ for *I. alba*, *D. wrightii*, *O. harringtonii* and *M. macrosiphon*), and carried more grains than 5-visit moths for all but *M. macrosiphon* (GLMM $P > 0.001$). For *D. wrightii*, *O. harringtonii* and *M. macrosiphon* (but not *I. alba*), 1-visit moths carried fewer pollen grains than 5-visit moths (GLM $P > 0.001$).

Question 2: the effect of pollen traits

Moths carried significantly more grains from plants with sticky pollen (Tab. 2). Pollen stickiness

also interacted significantly with visit treatment such that 1-visit moths carried relatively more grains compared with 2-visit and 5-visit moths in sticky plants. Thus, the relative differences between the visit treatments were smaller in sticky plants (Fig. 3).

DISCUSSION

Studying the pollen loads of foraging pollinators can allow the examination of many complete foraging records, but interpreting those records relies on several important assumptions. Here, we tested one such assumption by examining the degree to which the number of pollen grains picked up by the moths is likely to vary between plant species. We found that the studied plant species varied orders of magnitude in the number of pollen grains placed on the moths' proboscises, that the shape of accumulation curves likewise varied between plants, and that plant traits such as pollen stickiness can have large impacts on the pickup and retention of pollen grains.

Together, these results clearly suggest that the number of pollen grains detected in a pollen load is a poor predictor of the number of visits or foraging effort allocated by an individual forager towards a given flower species. In some cases, even the presence or absence of pollen may be deceiving, as neither of the *Mirabilis* species we tested deposited almost any pollen grains on moth

Table 1. Model output for comparisons of number of pollen grains placed on the hawkmoth proboscis across four plant species. Previous visits to experimental flowers and testing date were included as random effects.

Model		Output			
Fixed effects:		Estimate	Std.Error	z-value	Pr(> z)
Grain number ~	(Intercept)	5.59942	0.37807	14.81	< 2e-16
PlantSp *	2 visits	1.85352	0.06901	26.859	< 2e-16
VisitNum	5 visits	0.86702	0.08653	10.02	< 2e-16
	Ipomoea	-4.69122	0.5738	-8.176	2.94e-16
	Mandevilla	-1.25644	0.59041	-2.128	0.0333
	Oenothera	0.64534	0.12350	5.225	1.74e-7
	Ipomoea*2 visits	-0.16230	0.31173	-0.521	0.6026
	Ipomoea*5 visits	-0.86573	0.35361	-2.448	0.0144
	Mandevilla*2 visits	-0.82682	0.08356	-9.895	< 2e-16
	Mandevilla*5 visits	-0.10086	0.09742	-1.035	0.3005
	Oenothera*2 visits	-1.11119	0.07703	-14.425	< 2e-16
	Oenothera*5 visits	-0.46444	0.09747	-4.765	1.89e-6

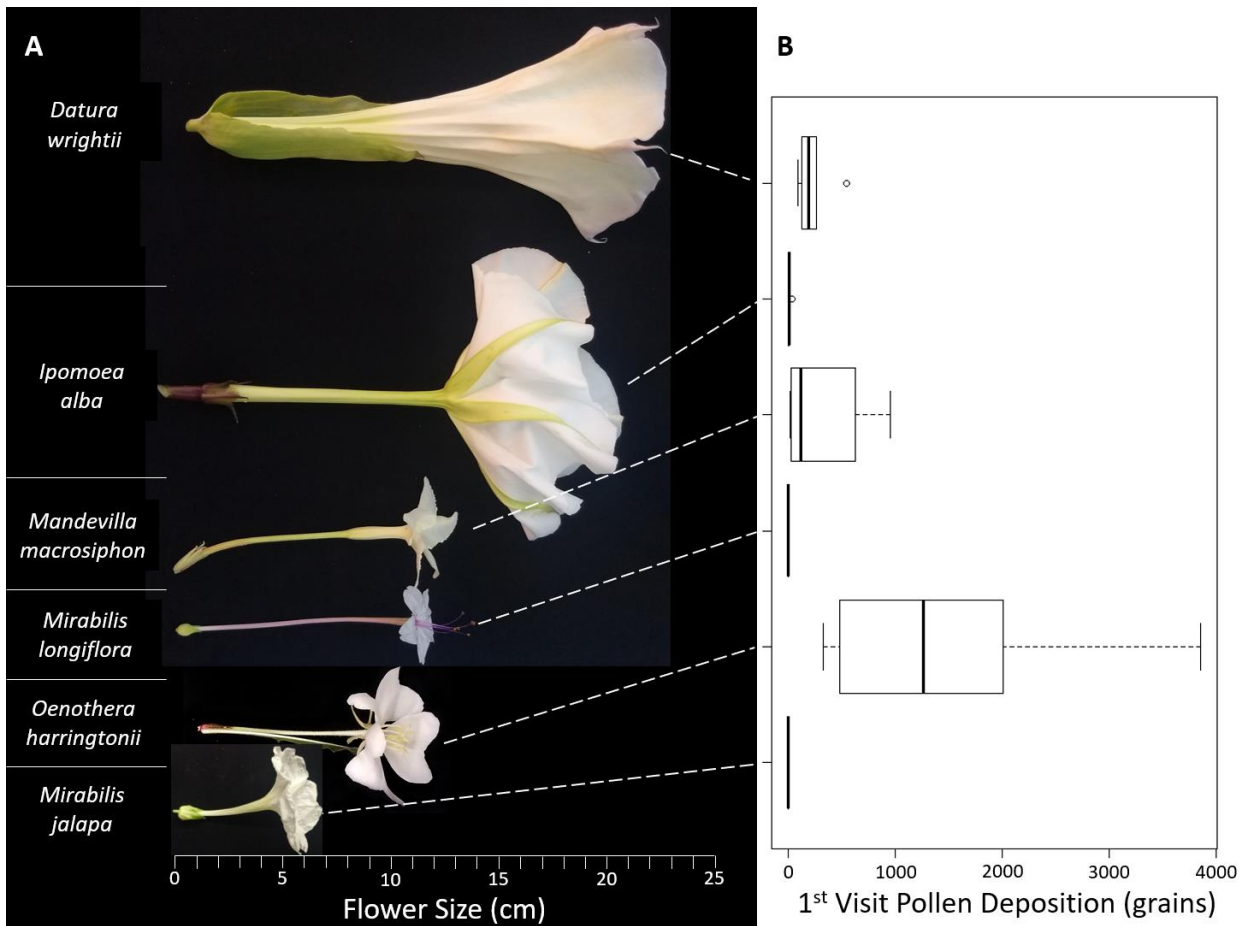


Figure 2. First-visit accumulation of pollen grains on the proboscises of *Manduca sexta* after visiting one of six plant species. **A:** Plant species arranged from largest to smallest based on nectar tube depth. **B:** Box-plot of pollen grain numbers present on moth proboscises after a single visit. The heavy bar represents the mean and the box bounds the 1st and 3rd quartiles. The yellow line on the scale bar represents the mean length of *M. sexta* proboscises in this study (mean ± SEM = 6.65 ± 0.74cm).

proboscises even after five visits. This absence of grains was likely due at least in part to the pollen of these species primarily contacting other sites on their visitors, such as the highly exerted anthers of *M. longiflora* (Supplement S1) contacting the head and eyes of our moths. However, moth proboscises did contact the anthers of these species during probing and nectaring, leaving the

possibility that the large, smooth pollen grains simply adhered poorly to that structure despite prior reports of pollen from this species being found on the proboscis (Alarcón et al. 2008; Grant & Grant 1983). Collecting pollen from other areas on the moth’s body may help alleviate this issue, when feasible (Moré et al. 2006).

Table 2. Model output for comparisons of number of pollen grains placed on hawkmoth proboscises the between plants with sticky and non-sticky pollen. Previous visits to experimental flowers and testing date were included as random effects.

Model		Output			
		Estimate	Std.Error	z-value	p-value
<i>Fixed effects:</i>					
Grain number ~	(Intercept)	4.12461	0.51585	7.996	1.29e-15
Sticky *	Sticky	1.78374	0.06657	26.797	<2e-16
VisitNum	2 visits	0.76242	0.08316	9.168	<2e-16
	5 visits	0.61973	0.11836	5.236	1.64e-7
	Sticky*2 visits	-0.91377	0.06928	-13.19	<2e-16
	Sticky*5 visits	-0.20692	0.08780	-2.357	0.0184

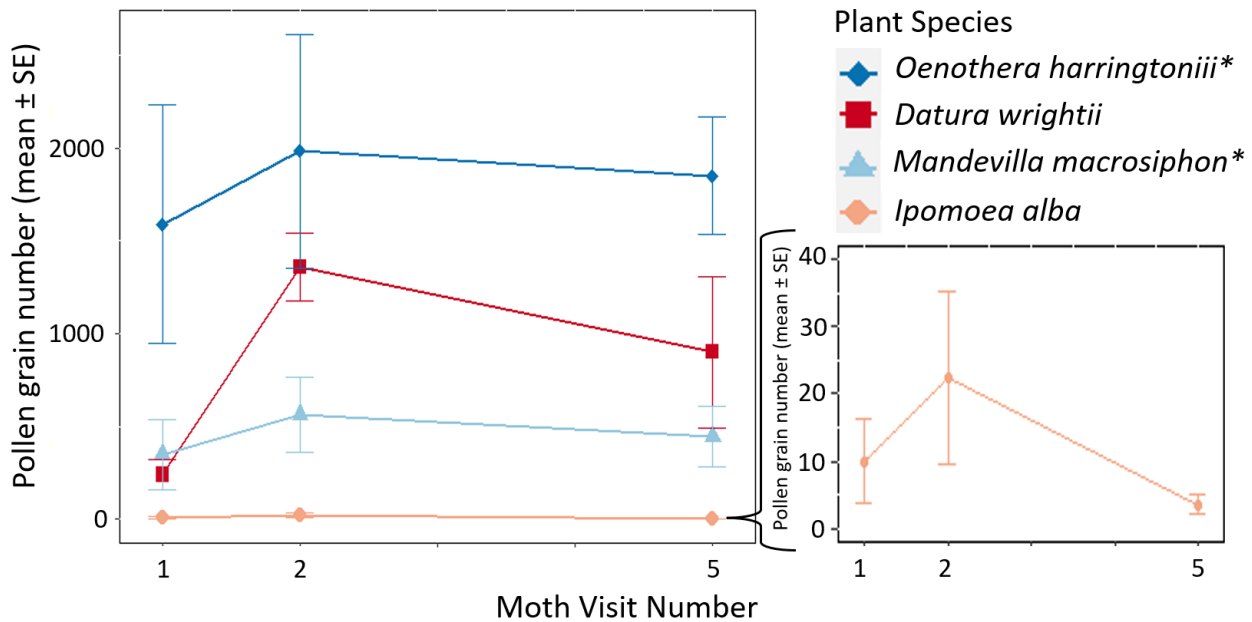


Figure 3. Accumulation of pollen on the proboscises of *Manduca sexta* moths by four plant species after 1, 2, or 5 successive floral visits. Due to the smaller number of pollen grains accumulated by moths visiting *I. alba*, data for this species is presented on both the main graph and the expansion below the legend. Plant species represented with blue and marked with an * had sticky pollen adhesion mechanisms

It is also clear, and unsurprising, that pollen traits affect both the number of pollen grains deposited and the accumulation of grains over subsequent visits. In particular, the viscin threads of *Oenothera* and sticky floral secretion of *Mandevilla* were correlated with higher pollen grain numbers than the less sticky *Ipomoea* and *Datura*. This effect was most apparent in the smaller differences between the 1-visit and 2-visit treatments for *O. harringtonii* and *M. macrosiphon*, which suggests that a single visit was nearly sufficient to saturate the moth's proboscis with pollen. Such high efficiency pollen transfer would be especially valuable for plant species that do not occur in highly dense populations, either due to their growth form and life history or to habitat fragmentation (see Suzan et al. 1994). These species also placed a large proportion of the total grains they produced on their visitors: a single visit removed ~15% and ~40% of total pollen from *Oenothera* and *Mandevilla*, respectively. While not quite as efficient as plants with pollinia or other pollen packaging mechanisms (e.g., orchids, milkweeds; Nilsson 1983; Woodson 1954) where a single pollinator visit can remove a pollinium containing thousands of pollen grains, these high removal proportions suggest that sticky pollen may reduce both pollen discounting rates and the

potential missed mating opportunities associated with producing less pollen.

Pollen grain production is likely a large determinant as well: in addition to being sticky, our *O. harringtonii* flowers produced ~11000 pollen grains and therefore had many more grains available for transfer than *Mirabilis jalapa*, which produced fewer than 300 grains. While the total amount of *M. longiflora* pollen available was not directly counted in this study, it likely produced a similar number to its congener (~150-800, based on pollen:ovule ratios and a single ovule in this genus; Cruden, 1973). These low numbers likely interacted with other factors such as placement location and pollen morphology, resulting in the low (or absent) pollen grains moths picked up from these species. The high number of pollen grains produced by *D. wrightii* (~350,000) was also likely a major contributor to the fact that *D. wrightii* species placed the 2nd highest number of grains on moth proboscises without being especially sticky.

In addition to assumptions about pollen acquisition, interpreting pollen loads often assumes that once pollen is acquired, it is not lost. Intriguingly, our data also cast doubt on this assumption, as all the plant species that placed any pollen on the moths' proboscises saw declines in

the number of grains present between the 2nd and 5th visit. For example, 5-visit moths visiting *Datura* carried ~66% of the pollen carried by 2-visit moths, and on *I. alba* 5-visit moths carried only ~16% of the grains carried by 2-visit moths. These patterns most likely suggest that pollen is being lost from the proboscis between the 2nd and 5th visit. While the specific causes of this loss are not clear from this study, we speculate that the majority is due to passive loss (Inouye et al. 1994) resulting from proboscis curling. In hawkmoths, curling the proboscis after feeding can result in substantial pollen movement and loss from this structure (Smith et al. 2021). Anecdotally, repeated curling events were more likely to have occurred for 5-visit moths: after 1-3 visits to focal flowers in quick succession (during which there may not have been even 1 proboscis curl), moths often explored the rest of the cage and examined other parts of the plant before returning for the 4th and 5th visit. Intriguingly, while the loss was somewhat dampened in *O. harringtonii* and *M. macrosiphon*, the fact that that even sticky pollen was lost suggests that loss due to proboscis curling may be a relatively general phenomenon for plants placing loose pollen (rather than pollinia) on hawkmoth proboscises.

CONCLUSIONS

Our results suggest that pollen accumulation on hawkmoth proboscises is highly variable, even across diverse flowers presumably adapted to hawkmoth pollination, and therefore that comparisons across plant species should be made with care. This is not to say, however, that grain numbers in pollen loads do not provide valuable information. For example, comparisons *within* species may be more reliable: for most of our plants, the degree of variation across treatments (visit number) within species was relatively low compared with the differences among plant species. Thus, while determining the relative foraging effort of single wild-caught moths on *Datura* versus *Oenothera* may not be possible based on pollen grain numbers, it may be possible to compare the number of *Datura* grains carried by two different individuals to explore a number of questions, such as their relative foraging effort to that plant or the impacts of variation in tongue length on pollen transfer. Further examination of these patterns of pollen accumulation and loss

from floral visitors would be valuable in more accurately interpreting animal-carried pollen loads.

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APPENDICES

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

Appendix I. Floral morphology measurements of the six plant species included in this study.

Appendix II. Representative videos of hawkmoth visits to flowers of the study plants.

REFERENCES

- Alarcón R, Davidowitz G, & Bronstein JL (2008) Nectar usage in a southern Arizona hawkmoth community. *Ecological Entomology* 33(4):503–509. <https://doi.org/10.1111/j.1365-2311.2008.00996.x>
- Baker HG (1961) The adaptation of flowering plants to nocturnal and crepuscular pollinators. *The Quarterly Review of Biology* 36(1):64–73.
- Bates D, Maechler M, Bolker B, Walker S (2015) Fitting linear mixed-effects models using lme4. *Journal of Statistical Software* 67(1):1–48. doi:10.18637/jss.v067.i01.
- Bell RA, Joachim FG (1976) Techniques for rearing laboratory colonies of tobacco hornworms and pink bollworms. *Annals of the Entomological Society of America* 69(2):365–373. <https://doi.org/10.1093/aesa/69.2.365>
- Bronstein JL, Huxman T, Horvath B, Farabee M, Davidowitz G (2009) Reproductive biology of *Datura wrightii*: the benefits of a herbivorous pollinator. *Annals of Botany* 103(9):1435–1443. <https://doi.org/10.1093/aob/mcp053>
- Bryant VM, Pendleton M, Murry RE, Lingren PD, Raulston, JR (1991) Techniques for studying pollen adhering to nectar feeding corn earworm (Lepidoptera: Noctuidae) moths using scanning electron microscopy. *Journal of Economic Entomology* 84(1):237–240. <https://doi.org/10.1093/jee/84.1.237>
- Burkle LA, Marlin JC, Knight TM (2013) Plant-pollinator interactions over 120 years: loss of species, co-occurrence, and function. *Science* 339(March):1611–1616.

- Butler HC, Johnson SD (2020) Butterfly-wing pollination in *Scadoxus* and other South African Amaryllidaceae. *Botanical Journal of the Linnean Society* 193(3):363–374. <https://doi.org/10.1093/botlinnean/boaa016>
- Campbell DR, Jürgens A, Johnson SD (2016) Reproductive isolation between *Zaluzianskya* species: the influence of volatiles and flower orientation on hawkmoth foraging choices. *New Phytologist* 210(1):333–342. <https://doi.org/10.1111/nph.13746>
- Cruden RW (1973) Reproductive biology of weedy and cultivated *Mirabilis* (Nyctaginaceae). *American Journal of Botany* 60(8):802. <https://doi.org/10.2307/2441173>
- Cruden RW, Hermann-Parker SM (1979) Butterfly pollination of *Caesalpinia pulcherrima*, with observations on a psychophilous syndrome. *Journal of Ecology* 67(1):155–168.
- Cruden RW, Jensen KG (1979) Viscin threads, pollination efficiency and low pollen-ovule ratios. *American Journal of Botany* 66(8):875–879.
- de Araújo LDA, Quirino ZGM, Machado IC (2014) High specialisation in the pollination system of *Mandevilla tenuifolia* (J.C. Mikan) Woodson (Apocynaceae) drives the effectiveness of butterflies as pollinators. *Plant Biology* 16(5):947–955. <https://doi.org/10.1111/plb.12152>
- Edwards J, Smith GP, McEntee MHF (2015) Long-term time-lapse video provides near complete records of floral visitation. *Journal of Pollination Ecology* 16(13):91–100.
- Goyret J, Kelber A, Pfaff M, Raguso RA (2009) Flexible responses to visual and olfactory stimuli by foraging *Manduca sexta*: larval nutrition affects adult behaviour. *Proceedings of the Royal Society B: Biological Sciences* 276(1668):2739–2745. <https://doi.org/10.1098/rspb.2009.0456>
- Grant V (1983) Behavior of hawkmoths on flowers of *Datura meteloides*. *Biological Gazette* 144(2):280–284.
- Grant V, Grant KA (1983) Hawkmoth pollination of *Mirabilis* (Nyctaginaceae). *Bulletin of the Torrey Botanical Club* 80:1298–1299. <https://doi.org/10.2307/2483395>
- Haber WA, Frankie GW (1989) A tropical hawkmoth community: Costa Rican dry forest Sphingidae. *Biotropica* 21(2):155–172.
- Harder LD, Thomson JD (1989) Evolutionary options for maximizing pollen dispersal of animal-pollinated plants. *The American Naturalist* 133(3):323–344.
- Hoch PC, Crisci JV, Tobe H, Berry PE (1993) A cladistic analysis of the plant family Onagraceae. *Systematic Botany* 18(1):31–47.
- Hopkins R, Rausher MD (2012) Pollinator-mediated selection on flower color allele drives reinforcement. *Science*, 335(6072):1090–1092. <https://doi.org/10.1126/science.1215198>
- Huang SQ, Shi XQ (2013). Floral isolation in *Pedicularis*: how do congeners with shared pollinators minimize reproductive interference? *New Phytologist* 199(3):858–865. <https://doi.org/10.1111/nph.12327>
- Inouye DW, Gill DE, Dudash MR, Fenster CB (1994) A model and lexicon for pollen fate. *American Journal of Botany* 81(12):1517–1530.
- Johnson SD, Balducci MG, Bijl A, Castañeda-Zárate M, Cozien RJ, Ortmann CR, van der Niet T (2020) From dusk till dawn: camera traps reveal the diel patterns of flower feeding by hawkmoths. *Ecological Entomology* 45(3):751–755. <https://doi.org/10.1111/een.12827>
- Johnson SD, Moré M, Amorim FW, Haber WA, Frankie GW, Stanley DA, Cocucci AA, Raguso RA (2017) The long and the short of it: a global analysis of hawkmoth pollination niches and interaction networks. *Functional Ecology* 31(1):101–115. <https://doi.org/10.1111/1365-2435.12753>
- Johnson SD, Raguso RA (2015) The long-tongued hawkmoth pollinator niche for native and invasive plants in Africa. *Annals of Botany* 117(1): 25–36. <https://doi.org/10.1093/aob/mcv137>
- Kaczorowski RL, Seliger AR, Gaskett AC, Wigsten SK, Raguso RA (2012). Corolla shape vs. size in flower choice by a nocturnal hawkmoth pollinator. *Functional Ecology*, 26(3), 577–587. <https://doi.org/10.1111/j.1365-2435.2012.01982.x>
- Kislev ME (1972) A study of hawkmoth pollination, a palynological analysis of the proboscis. *Israel Journal of Botany* 21:57–75.
- Klahre U, Gurba A, Hermann K, Saxenhofer M, Bossolini E, Guerin PM, Kuhlemeier C (2011) Pollinator choice in *Petunia* depends on two major genetic loci for floral scent production. *Current Biology*, 21(9):730–739. <https://doi.org/10.1016/j.cub.2011.03.059>
- Lunau K, Piorek V, Krohn O, Pacini E (2015) Just spines—mechanical defense of malvaceous pollen against collection by corbiculate bees. *Apidologie* 46(2):144–149. <https://doi.org/10.1007/s13592-014-0310-5>
- Maad J, Nilsson LA (2004) On the mechanism of floral shifts in speciation: gained pollination efficiency from tongue- to eye-attachment of pollinia in *Platanthera* (Orchidaceae). *Biological Journal of the Linnean Society* 83(4):481–495. <https://doi.org/10.1111/j.1095-8312.2004.00406.x>
- Martinez C, Burquez A (1986) Nectar production and temperature dependent pollination in *Mirabilis jalapa* L. *Biotropica* 18(1):28–31.
- Martins DJ, Johnson SD (2007) Hawkmoth pollination of aerangoid orchids in Kenya, with special reference to nectar sugar concentration gradients in the floral spurs. *American Journal of Botany*, 94(4):650–659. <https://doi.org/10.3732/ajb.94.4.650>

- Meudt HM (2016) Pollen morphology and its taxonomic utility in the Southern Hemisphere bracteate-prostrate forget-me-nots (*Myosotis*, Boraginaceae). *New Zealand Journal of Botany* 54(4):475–497. <https://doi.org/10.1080/0028825X.2016.1229343>
- Moré M, Sérsic AN, Cocucci AA (2006) Specialized use of pollen vectors by *Caesalpinia gilliesii*, a legume species with brush-type flowers. *Biological Journal of the Linnean Society* 88(4):579–592. <https://doi.org/10.1111/j.1095-8312.2006.00644.x>
- Moré M, Sérsic AN, Cocucci AA (2007) Restriction of pollinator assemblage through flower length and width in three long-tongued hawkmoth-pollinated species of *Mandevilla* (Apocynaceae, Apocynoideae). *Annals of the Missouri Botanical Garden* 94(2):485–504.
- Muchhala N, Potts MD (2007) Character displacement among bat-pollinated flowers of the genus *Burmeistera*: analysis of mechanism, process and pattern. *Proceedings of the Royal Society B: Biological Sciences* 274(1626):2731–2737. <https://doi.org/10.1098/rspb.2007.0670>
- Muchhala N, Thomson JD (2012) Interspecific competition in pollination systems: costs to male fitness via pollen misplacement. *Functional Ecology* 26(2):476–482. <https://doi.org/10.1111/j.1365-2435.2011.01950.x>
- Murphy DD (1984) Butterflies and their nectar plants: the role of the checkerspot butterfly *Euphydryas editha* as a pollen vector. *Oikos* 43(1):113–117.
- Newman E, Anderson B (2020) Character displacement drives floral variation in *Pelargonium* (Geraniaceae) communities. *Evolution* 74(2):283–296. <https://doi.org/10.1111/evo.13908>
- Nilsson LA (1983) Processes of isolation and introgressive interplay between *Platanthera bifolia* (L.) and *P. chlorantha* (Custer) Reichb. (Orchidaceae). *Botanical Journal of the Linnean Society* 87(4):325–350. <https://doi.org/10.1111/j.1095-8339.1983.tb00997.x>
- Nilsson LA, Jonsson L, Ralison L, Randrianjohany E (1987) Angraecoid orchids and hawkmoths in central Madagascar: specialized pollination systems and generalist foragers. *Biotropica* 19(4):310–318.
- Ogilvie JE, Thomson JD (2015) Male bumble bees are important pollinators of a late-blooming plant. *Arthropod-Plant Interactions* 9(2):205–213. <https://doi.org/10.1007/s11829-015-9368-x>
- Ollerton J (2017) Pollinator diversity: distribution, ecological function, and conservation. *Annual Review of Ecology, Evolution, and Systematics* 48(1):353–376. <https://doi.org/10.1146/annurev-ecolsys-110316-022919>
- Pacini E, Hesse M (2005). Pollenkitt - Its composition, forms and functions. *Flora* 200(5):399–415. <https://doi.org/10.1016/j.flora.2005.02.006>
- R Core Team (2020) R: A language and environment for statistical computing (3.6.3). R Foundation for Statistical Computing. <https://www.r-project.org/>
- Ruckelshaus MH (1996) Estimation of genetic neighborhood parameters from pollen and seed dispersal in the marine angiosperm *Zostera marina* L. *Evolution* 50(2):856–864. <https://doi.org/10.1111/j.1558-5646.1996.tb03894.x>
- Scheper J, Reemer M, van Kats R, Ozinga WA, van der Linden GTJ, Schaminée JHJ (2014) Museum specimens reveal loss of pollen host plants as key factor driving wild bee decline in The Netherlands. *PNAS* 111(49):3–8. <https://doi.org/10.1073/pnas.1412973111>
- Schlumpberger BO, Cocucci AA, More M, Sersic AN, Raguso RA (2009) Extreme variation in floral characters and its consequences for pollinator attraction among populations of an Andean cactus. *Annals of Botany* 103(9):1489–1500. <https://doi.org/10.1093/aob/mcp075>
- Skogen KA, Jogesh T, Hilpman ET, Todd SL, Rhodes MK, Still SM, Fant JB (2016) Land-use change has no detectable effect on reproduction of a disturbance-adapted, hawkmoth-pollinated plant species. *American Journal of Botany* 1(11):1950–1963. <https://doi.org/10.3732/ajb.1600302>
- Smith GP, Davidowitz G, Alarcón R, Papaj DR, Bronstein JL (2021a) Sex differences in the foraging behavior of a generalist hawkmoth. *Insect Science* 1–11. <https://doi.org/10.1111/1744-7917.12926>
- Smith GP, Davidowitz G, Raguso RA, Bronstein JL (2021b) Proboscis curling in a pollinator causes extensive pollen movement and loss. *Ecological Entomology* 1–8. <https://doi.org/10.1111/een.13105>
- Suzan H, Nabhan GP, Patten DT (1994) Nurse plant and floral biology of a rare night-blooming *Cereus*, *Peniocereus striatus* (Brandege) F. Buxbaum. *Conservation Biology* 8(2):461–470. <https://doi.org/10.1046/j.1523-1739.1994.08020461.x>
- Woodson RE (1954) The North American species of *Asclepias* L. *Annals of the Missouri Botanical Garden*, 41(1):1-211. <https://doi.org/10.2307/2394652>