RESEARCH ARTICLE
Short Title: Butterfly pollination of a Texas wildflower

Characterizing each step of pollination in *Phlox drummondii* reveals that a single butterfly species predominates in the pollinator assemblage

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Manuscript received 19 December 2022; revision accepted 19 March 2023

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Citation: Burgin, G. A., O. Bronzo-Munich, A. G. Garner, I. A. Acevedo, and R. Hopkins. 2023. Characterizing each step of pollination in *Phlox drummondii* reveals that a single butterfly species predominates in the pollinator assemblage. *American Journal of Botany* 110: XXXX
DOI: XXXX

Abstract

Premise: A central goal of pollination biology is to connect plants with the identity of their pollinator(s). While predictions based on floral syndrome traits are extremely useful, direct observation can reveal further details of a species' pollination biology. The wildflower *Phlox drummondii* has a floral syndrome consistent with Lepidoptera pollination. We tested this prediction using empirical data.
Methods: We observed each step of pollination in *P. drummondii*. First, we observed 55.5 h of floral visitation across the species range. We used temporal pollinator exclusion to determine the contribution of diurnal and nocturnal pollination to reproductive output. We then quantified *P. drummondii* pollen transfer by the dominant floral visitor, *Battus philenor*. Finally, we tested the effect of *B. philenor* visitation on *P. drummondii* reproduction by quantifying fruit set following single pollinator visits.
Results: *Battus philenor* is the primary pollinator of *P. drummondii*. Pollination is largely diurnal, and we observed a variety of lepidopteran visitors during the diurnal period. However, *B. philenor* was the most frequent visitor, representing 88.5% of all observed visits. Our results show that *B. philenor* is an extremely common visitor and also an effective pollinator by demonstrating that individuals transfer pollen between flowers and that a single visit can elicit fruit set.

Conclusions: Our data are consistent with the prediction of lepidopteran pollination and further reveal a single butterfly species, *B. philenor*, as the primary pollinator. Our study demonstrates the importance of empirical pollinator observations, adds to our understanding of pollination mechanics, and offers a specific case study of butterfly pollination.

KEYWORDS
Battus philenor, butterfly pollination, ecological specialization, *Phlox*, Polemoniaceae, pollination efficiency, pollination syndromes

Pollination in flowering plants is the process by which pollen moves from a flower's male reproductive organ (anthers) to a flower's female reproductive organ (stigma) and results in fertilization. This process occurs over a series of stepwise interactions between plants and their pollinating vectors. In plants that rely on animals for pollination, pollinators first visit a flower and contact the pollen-producing anthers. The pollinator then transfers pollen to a new flower, contacts the receptive stigma, and deposits pollen. To serve as an effective pollinator, an agent must enact each step of this process and, ultimately, affect the reproductive success of visited plants (Cox and Knox, 1988; Proctor et al., 1996). Given the fundamental importance of plant-pollinator interactions to reproductive, ecological, and evolutionary processes, detailing the steps of pollination contributes to our understanding of plant diversity and persistence (Stebbins, 1951; Kay and Sargent, 2009; Mitchell et al., 2009; van der Niet and Johnson, 2012). Although these kinds of data exist for some plant groups, our knowledge is far from complete. Notably, the distribution of these data is uneven across biological systems, with relatively fewer examples of butterfly pollination compared to other insects (e.g., bees; Weiss, 2001; Mertens et al., 2021; but see Epps et al., 2015; Daniels et al., 2020; Kiepiel and Johnson, 2021).

A centuries-long goal of pollination biology has been to link plants with the identity of their pollinating agent(s) (Darwin, 1862; Proctor et al., 1996; Alison et al., 2022). Historically, investigations have focused largely on understanding variation in floral form, but more recent pursuits are invigorated by the role of pollinators in plant reproduction and fitness variation, dispersal across space and time, trait evolution, and macroevolutionary patterns of species diversification (Delpino, 1873–1874; Proctor et al., 1996; O'Meara et al., 2016; Lagomarsino et al., 2017; Rhodes et al., 2017; Smith and Kriebel, 2018; Wessinger, 2021; Burgin and Hopkins, 2022). One theme that has not wavered across historical and modern perspectives is the importance of empirical pollination data (Mitchell et al., 2009; Ollerton et al., 2009; Dellinger, 2020; Valverde-Espinoza et al., 2021). Observing and demonstrating effective pollination can be time consuming and logistically challenging. Yet detailed empirical data on the pollination process is essential to validating the foundational patterns on which pollination biology is built.

One of the most widely used frameworks in pollination biology is the pollination syndrome concept, which aims to unite floral trait diversity with its ecological function in pollination (Fenster et al., 2004; Dellinger, 2020). Pollination syndromes are suites of floral traits that have evolved across unrelated groups, presumably because of similarities in the selective regimes imposed by specific pollen vectors (e.g., bees vs. birds vs. wind; Stebbins, 1970; Faegri and Van Der Pijl, 1979). This framework has been continually refined over the past 150 yr, and extensive empirical observations confirm associations between floral trait variation and pollinator identity across taxonomic groups (Martén-Rodríguez et al., 2009; Rosas-Guerrero et al., 2014; Ashworth et al., 2015). Pollination syndromes have been broadly applied throughout the field of plant biology, from analysis of species diversification to issues of conservation

(Dellinger, 2020). Despite their wide utility, pollination syndromes are imperfect predictors of pollinator identity, and it is important to validate syndrome assumptions with detailed observations of plants and their pollinators (Ollerton et al., 2009; Dellinger, 2020; Valverde-Espinoza et al., 2021; Berardi et al., 2022; Sinnott-Armstrong et al., 2022). An intuitive set of experimental aims to test for pollination using empirical data was formalized in the 1980s (Cox and Knox, 1988). These "pollination postulates" require demonstration of vector participation in each step of pollination, from visitation to pollen transfer to fruit set. Through observation of each stepwise species interaction involved in the pollination process, the biological complexities underlying plant-pollinator interactions can be better understood.

The first step in the pollination process is visitation. One challenge in connecting plants with their pollinators is the recognition that visitation does not equate to pollination (King et al., 2013). Most flowering plants are pollinated by animals, and while these interactions can be mutually beneficial, they evolve under different selective pressures for each participant (Ollerton et al., 2011; van der Kooi et al., 2021). Because angiosperms rely on pollination for sexual reproduction, floral traits that improve visitation, pollen transfer, and fertilization are expected to be favored by natural selection (Stebbins, 1970). Animals, on the other hand, are seeking access to floral resources, including calorie-rich nectar or pollen (Vaudo et al., 2020). Traits that increase reward acquisition and utilization are predicted to evolve within floral visitors regardless of their impact on pollination success (Irwin et al., 2010). Put simply, flowering plants evolve to attract pollinators and pollinators evolve to extract resources. As a result, not all floral visitors are expected to cooperate equally in the pollination process.

Variation in floral visitors across space and time presents an additional challenge in connecting the observation of visitation to pollination (Herrera, 1988; Horvitz and Schemske, 1990). If visitation varies across space, observations made in one population may not extrapolate to the entire species range. Including multiple populations can identify potential variation in pollinator assemblage across space. If visitation varies across time, observers may miss the relevant window of visitation and/or pollination (Alison et al., 2022). The use of temporal exclusion experiments can be used to identify the relevant period for observations.

After visitation, the next step of the pollination process is pollen transfer. Sources of variation in pollen pickup include anatomical mismatch between vector and anther placement or inability to remove pollen from anthers (e.g., adaptations necessary for buzz pollination or pollinia removal; Anders Nilsson, 1992; Muchhala and Thomson, 2009; Newman et al., 2015; Vallejo-Marín, 2019). Quantifying the number of pollen grains on an animal's body following visitation can reveal pollen pickup. However, the fate of pollen on an animal's body is uncertain. Previous studies have demonstrated that in many plant-pollinator interactions, just a small percentage of the pollen picked up during visitation will eventually reach a receptive stigma (Johnson et al., 2009; Minnaar et al., 2018). Species may engage in pollen grooming, proboscis coiling, or long-distance travel that results in pollen loss before a subsequent floral visit can be made (Levin and Berube, 1972; Castellanos et al., 2003; Tong and Huang, 2018). Quantifying pollen number on stigmas following visitation can confirm pollen deposition.

The final step of the pollination process is fertilization. Vectors may visit flowers of a different plant species or flowers on the same self-incompatible plant, resulting in pollen transfer without fertilization (Diller et al., 2022). If floral visitors deposit an insufficient number of pollen grains to elicit fertilization, pollination does not occur (Németh and Smith-Huerta, 2003; Koski et al., 2018). If visitation and stigma receptivity do not overlap temporally, fertilization following pollen deposition will not occur (Bingham and Orthner, 1998). Comparing pollination with and without vector exclusion can address whether a floral visitor affects reproductive output.

Quantifying each step from visitation through pollen pickup, pollen deposition, and fertilization provides inference-free documentation of a plant's pollination biology. Detailed investigations into a variety of systems can further validate the assumptions underlying pollination syndromes and reveal new hypotheses about how plant-pollination interactions may evolve in response to changing environments.

Here, we offer an example of pollination biology with minimal reliance on inference or assumptions. Floral traits of the Texas wildflower *Phlox drummondii* are consistent with a generalized lepidopteran pollination syndrome (Figure 1; Grant and Grant, 1965; Faegri and Van Der Pijl, 1979). Flowers are pale blue/purple throughout most of the range, are nectar producing, and the sexual organs are fully inserted into a narrow corolla tube of medium length (13–17 mm; Wherry, 1955). We empirically validated the prediction of lepidopteran pollination through direct observation. First, we described the pollinator assemblage by observing visitation throughout the day/evening in three populations across the species range. We then compared the contribution of diurnal and nocturnal visitation to fruit set and identified the relevant window for pollination in this species. These data reveal that diurnal pollination contributes significantly to reproductive output, whereas nocturnal pollination and autonomous selfing are minimal. Among diurnal visitors, a single butterfly species, *Battus philenor*, is the most frequent visitor by far. To understand whether *B. philenor* is not only a common visitor but also an effective one, we conducted a series of follow-up experiments. We characterized pollen transfer by quantifying pollen on *B. philenor* proboscises and *P. drummondii* stigmas following visitation. Finally, we determined whether *B. philenor* visitation affects reproductive output in *P. drummondii* by exposing greenhouse-grown plants to *B. philenor* and monitoring fruit set. Our study addresses each step of the pollination process with empirical field-based data and confidently identifies *B. philenor* as the primary pollinator ofthe wildflower, *P. drummondii*.

<h1>MATERIALS AND METHODS

<h2>Study species

Phlox drummondii (Polemoniaceae) is an annual herb native to central Texas, USA, that occurs along roadsides, fields, and pastures. Seeds germinate in early spring, and individuals flower and fruit from March through June. Flowers are radially symmetric, with a diameter of 18–24 mm (Figure 1A, B; Wherry, 1955). Floral buds open in the morning (0700–0900 hours) in both field and greenhouse conditions. If unpollinated, open flowers last approximately 2–4 d before wilting. Fruits begin developing 1–2 d after pollination, and each flower can produce a single fruit. *Phlox drummondii* has an active self-incompatibility system and displays low rates of autonomous selfing (~1%; Roda and Hopkins, 2019).

Previous studies within the *Phlox* genus demonstrate visitation by a broad group of lepidopteran pollinators (Strakosh and Ferguson, 2005; Wiggam and Ferguson, 2005). Like other members of its genus, *P. drummondii* exhibits floral traits consistent with a lepidopteran pollination syndrome (Grant and Grant, 1965; Stebbins, 1970; Faegri and Van Der Pijl, 1979). Flowers produce nectar at the base of a narrow, fused, tubular corolla ranging in length from 13 mm to 17 mm (Wherry, 1955). Sexual organs (stigma and anthers) are fully inserted within the corolla tube. Brightly colored petals are light-blue/purple throughout most of the species range and emit a faint fragrance (Hopkins and Rausher, 2012).

<h2>Observing floral visitation

To characterize the diversity and frequency of floral visitors, we conducted 55.5 h of direct pollinator observations. We observed visitation throughout the day/evening (0700–2030 hours). Coinciding with peak bloom, observations were conducted from 23 April to 4 May 2022. We identified three focal populations for pollinator observations throughout central Texas (Appendix S1). These focal study sites were located on rural roadsides or in a protected meadow at the Brackenridge Field Laboratory, University of Texas at Austin. At each site, we delimited a patch area ranging from 30 m^2 to 140 m^2. Observers stood just outside the patch boundary for 1 h observation periods and noted every animal interacting with flowers inside the study patch. Visitors were recorded if any part of the animal's body was inserted into the corolla tube opening. Species identity was assessed visually. We conducted a handful of additional observation hours near range edges to assess potential variation in pollinator assemblage across space (Appendix S1).

<h2>Temporal pollinator exclusion

We used temporal pollinator exclusion to assess the contributions of diurnal pollination, nocturnal pollination, and autonomous selfing toward overall reproductive output. In April 2022, a population of *P. drummondii* was identified at the Brackenridge Field Laboratory (30.2832 N, −97.7801 W), near the geographic center of the species range (Appendix S1). Throughout an area of ~100 m^2, 86 experimental plants were haphazardly chosen and assigned to one of four pollinator treatments: total exclusion (n = 15), diurnal exclusion (n = 23), nocturnal exclusion (n = 23), and no exclusion (n = 25). All open flowers and fruits were removed before covering plants in fine-mesh cages to exclude pollinators of all sizes. At 0700 hours each morning, fine-mesh cages were removed from the nocturnal exclusion group and placed on the diurnal exclusion group. At 1900 hours each evening, fine-mesh cages were removed from the diurnal exclusion group and placed on the nocturnal exclusion group. The total exclusion group remained covered, while the no exclusion group remained uncovered. After 5 d, new but unopened buds were removed and all flowers that opened during the experiment were counted (as measured by remaining calyx number). All plants were then covered with exclusion cages and monitored for fruit set. We counted the number of fruits on days 5 and 8 after beginning treatment. On day 8, we observed minor plant death (n = 84 live plants remaining) and caterpillar herbivory resulting in the removal of entire inflorescences, leading to some uncertainty in final reproductive success. We implemented a binomial mixed model using the R package lme4 version 1.1-30 to model fruit set in our exclusion conditions (Bates et al., 2015). Each individual flower that opened during the experiment was represented in the data set as either "fruit present" or "fruit absent." A total of 124 flowers were recorded in the total exclusion treatment, 309 in the diurnal exclusion treatment, 235 in the diurnal treatment, and 250 in the no exclusion treatment. Because our data set includes multiple flowers from the same individual, we included plant individual as a random effect in the model. To compare fruit set across treatment groups, we conducted pairwise post hoc Tukey-Kramer tests using the glht() function in the R package multcomp version 1.4-20 (Hothorn et al., 2008).

<h2>Quantifying pollen transfer

To determine whether visitors transfer pollen between flowers, we assessed both pollen pickup and deposition by insect visitors. We characterized pollen pickup by capturing insects foraging in natural populations of *P. drummondii* and quantifying pollen grains on proboscises. These insects were captured outside of patches used for observation and represented a small fraction of all insects observed in the area. Because *B. philenor* was by far the most frequent visitor, most insects captured and included in our analysis were *B. philenor* (*n* = 24). We collected at least one representative individual of each pollinator type observed visiting *P. drummondii* (*n* = 1 *Hyles lineata*; *n* = 4 skipper species; *n* = 2 *Phoebis sennae*) except for the common buckeye (*Junonia coenia*), which was only seen making a single floral visit throughout all observation periods.

After capture, insects were immediately immobilized in a glassine envelope and placed on ice. Specimens were stored at −20°C. Following 24 h in a humidifying chamber to increase pliability, we pinned each specimen through the thorax onto glassine-covered Styrofoam. The proboscis was carefully unrolled with a clean pin, and length was measured. To remove pollen from the proboscis for counting, the dorsal, ventral, and lateral sides of the unrolled proboscis were rubbed with a gelatin-glycerin cube (Kearns and Inouye, 1993). We melted the gelatin-glycerin cube onto a microscope slide and imaged the entirety of the melted gelatin in brightfield at 2.5× magnification using an Axio Imager (Zeiss, Jena, Germany). Total pollen grain number was quantified automatically using ImageJ and confirmed by manual count (Schindelin et al., 2012).

Based on the literature and extensive experience observing *Phlox* pollen using a light microscope, we identified pollen grains matching *Phlox* pollen's distinctive morphology in size and shape (Wherry, 1955; Buthod and Skvarla, 2014; Suni and Hopkins, 2018; Roda and Hopkins, 2019). Pollen grains consistent with *Phlox* pollen morphology were included in our quantifications. Another *Phlox*, *P. cuspidata*, is found geographically near, although not at, some of our study sites. Because of shared pollen morphology, distinguishing between pollen of these species is challenging. However, we do not expect this to present an issue in our analyses, given that *P. cuspidata* is highly selfing with low pollinator visitation (Levin, 1989).

We assessed *B. philenor* pollen deposition per visit by allowing wild-captured individuals to visit flowers in succession on greenhouse-grown plants maintained indoors. After each single visit, corollas were removed and stigmas were collected, fixed in 80% isopropanol, and stored at 4°C (*n* = 20 unvisited; *n* = 28 visited by *B. philenor*). We removed corollas and collected stigmas from unvisited flowers as a control. Stigmas were then mounted in glycerol media on a slide and imaged in brightfield at 10× magnification using an Axioscope 7 (Zeiss). Pollen grain number per image was quantified using ImageJ (Schindelin et al., 2012). We used a linear model implemented in R package lme4 to test for an effect of *B. philenor* visitation on the number of pollen grains on stigmas.

<h2>Quantifying pollination efficiency

To assess whether *B. philenor* visitation affects reproductive output, we performed a controlled foraging experiment. Plants were grown from seed in the greenhouse and maintained indoors to exclude pollinators. We captured *B. philenor* foraging on natural populations of *P. drummondii* and allowed them to forage on a "pollen donor plant" while inside of a mesh cage. Because *B. philenor* is a generalist, wild-foraging insects likely carry some heterospecific pollen, which may affect overall pollination efficiency. To capture this effect in our measurement of efficiency, insects were not cleaned prior to controlled foraging.

Next, we presented the butterfly with a single, new plant and noted the first three flowers visited in succession by the butterfly (geitonogamous visitation). Corollas were removed following *B. philenor* visitation and calyxes were labeled with tape. On the same plant, we removed corollas from three unvisited flowers as a control for autonomous selfing. Additionally, we manually applied pollen from the "pollen donor plant" to stigmas of three open flowers. Pollen donor and experimentally visited plants were from independent genetic lines grown in our lab, to reduce the likelihood of sharing incompatibility genotypes. We included 18 plants in our experiment. Following pollinator exposure, plants were maintained in the greenhouse and monitored for fruit set for 1 wk. We used a binomial mixed model using R package lme4 to model fruit set across the five pollination treatments (manual outcross, autonomous selfing, and first, second, and third flower visited by *B. philenor*; Bates et al., 2015). We included plant individual as a random effect in the model. To compare fruit set across treatment groups, we conducted pairwise post hoc Tukey-Kramer tests using the glht() function in the R package multcomp.

Although quantifying pollen transfer and single-visit efficiency data from each visitor type would be informative, the low visitation rates of non-*Battus* insects made capturing other visitors extremely challenging. We were unable to gain adequate sample sizes of non-*Battus* visitors to assess pollen transfer despite extensive time in the field.

<h1>RESULTS

<h2>Floral visitation

We documented a total of 3091 floral visits over 55.5 h of observation, occurring between 0700 and 2030 hours from 23 April to 4 May 2022 (Figure 2A). *Battus philenor* was the most frequent visitor, contributing 2737 or 88.5% of all floral visits (Figure 2B). This observation was consistent across the study sites, with *B. philenor* representing the majority of observed floral visits at every population included in our study.

The remaining visits were made by a variety of lepidopterans, including skipper species (Family Hesperiidae), *Hyles lineata*, *Phoebis sennae*, and *Junonia coenia* (Figure 2B; Appendix S2). These visitor types each represented <10% of total visits. Skippers were the second most frequent visitor, making 7.4% of floral visits. *Hyles lineata* made 3.4% of visits, *P. sennae* made 0.6% of visits, and *J. coenia* was seen visiting a single flower. Notably, 95 of the 104 *H. lineata* visits observed were made by one individual during a single foraging bout.

Compared to other visitors, *B. philenor* tended to make longer bouts within study patches (Figure 2C). On average, *B. philenor* foraging bouts included visits to 9.23 individual plants while skipper foraging bouts included 3.81 plants. In addition to visiting more unique plants, *B. philenor* also tended to visit more flowers in succession on the same plant (Figure 2D; a typical observed foraging bout is shown in a video in Appendix S3). Successive visits to flowers on the same plant by *B. philenor* ranged as high as 10 flowers (average = 1.88). Successive visits to flowers on the same plant by skipper species ranged as high as 5 flowers (average = 1.32). Other floral visitors, including *H. lineata*, *P. sennae*, and *J. coenia*, were too infrequent to summarize patterns.

<h2>Timing of pollination

Pollinator exclusion had a significant effect on fruit set (Figure 3; Appendix S4). A higher proportion of open flowers set fruit in the diurnal pollination treatment than in the nocturnal pollination treatment (day 5: $z = -5.391$, $P < 0.001$; day 8: $z = -3.435$, $P < 0.01$). Our model predicts that the proportion of open flowers that set fruit was 0.45 in the diurnal pollination group (95% confidence interval [CI]: 0.32–0.59) and 0.06 in the nocturnal pollination group (95% CI: 0.03–0.12). This pattern was similar on day 8, when the proportion of open flowers that set fruit was 0.87 for the diurnal pollination group (95% CI: 0.71–0.95) and 0.37 for the nocturnal pollination group (95% CI: 0.20–0.59).

We detected no significant difference between total pollination and diurnal pollination (day 5: $z = -0.395$, $P = 0.978$; day 8: $z = -0.578$, $P = 0.9373$). Simultaneously, we detected no significant difference between full exclusion and nocturnal pollination at day 5 ($z = 1.694$, $P = 0.316$), although a significant difference emerged by day 8 ($z = 3.927$, $P < 0.001$).

Fruit set was high in the total pollination treatment: the proportion of open flowers that set fruit was 0.41 and 0.82 at days 5 and 8, respectively, confirming our model prediction (95% CI: 0.29–0.55 and 0.65–0.92, respectively). Rates of autonomous selfing were low throughout the course of the experiment. At days 5 and 8, the proportion of open flowers that set fruit was 0.02 under full exclusion/no pollination (95% CI: 0.01–0.06 and 0–0.08, respectively).

<h2>Pollen pickup

We detected *Phlox* pollen on proboscises of all insect visitors captured while foraging on *P. drummondii* flowers (Figure 4A). Among *B. philenor* individuals, *Phlox* pollen loads ranged from 81 to 3266 grains (mean = 1297.9, $n = 24$). *Phlox* pollen loads on both skipper species and *P. sennae* individuals tended to be lower than those carried by *B. philenor*. We observed pollen loads ranging from 40 to 728 grains on skipper species ($n = 4$), and pollen loads of 129 and 267 grains on two *P. sennae* individuals. The single *H. lineata* individual carried 2727 pollen grains.

<h2>Pollen deposition

The number of pollen grains on *P. drummondii* stigmas following a single visit by *B. philenor* was more than five times higher than for unvisited flowers (Figure 4B; $F = 24.34$, df = 46, $P < 0.001$). Stigmas from unvisited flowers had 85.65 pollen grains on average (95% CI: 0–205.48). After a single *B. philenor* visit, stigmas had 480.54 pollen grains on average (95% CI: 379.27–581.81).

<h2>Pollination efficiency

Battus philenor visitation had a significant effect on fruit set among greenhouse-grown *P. drummondii* plants (Figure 4C; Appendix S5). Flowers visited by *B. philenor* set a higher proportion of fruit than unpollinated flowers, regardless of visit order. We detected no significant difference in fruit set between flowers visited first, second, or third. However, mean values of fruit set declined slightly across visits. Our model predicts that the proportion of first-visit flowers setting fruit was 0.64 (95% CI: 0.34–0.84), compared to 0.58 of second-visit flowers (95% CI: 0.29–0.63) and 0.31 of third-visit flowers (95% CI: 0.10–0.63). This pattern suggests that the availability of outcross pollen may decline as pollinators make successive visits on flowers of the same self-incompatible plant, yet geitonogamous visits can still cause fruit set. Autonomous selfing was low, with the proportion of unpollinated flowers setting fruit at just 0.01 (95% CI: 0.0–0.08), as predicted by our model. Following manual pollination with outcross pollen, the proportion of flowers setting fruit was 0.95 (95% CI: 0.84–0.99).

<h1>DISCUSSION

Battus philenor is the primary pollinator of the Texas wildflower *P. drummondii*. Our experiments detailed each step of the pollination process and revealed that *B. philenor* is an overwhelmingly common visitor and an effective pollinator (Figures 2B and 4). We found that most pollination occurred diurnally (0700–1900 hours; Figure 3). During this window, *B.*

philenor was by far the most frequent visitor across all study sites, despite the presence of a diversity of lepidopteran species in the community (Figure 2B; Appendix S2). Our results further demonstrate that *B. philenor* visitation results in pollen transfer and affects fruit set, which provides evidence for its important role in pollination of *P. drummondii* (Figure 4; Appendix S5). Butterfly-pollinated systems have received relatively little attention compared to other pollination vectors, particularly in the Northern Hemisphere (Weiss, 2001; Mertens et al., 2021). Our study adds to evidence of the importance and effectiveness of butterflies as pollinators, which has been questioned historically (Levin and Berube, 1972; Wiklund et al., 1979; Jennersten, 1984).

Our results are consistent with lepidopteran syndrome predictions (Grant and Grant, 1965; Faegri and Van Der Pijl, 1979). A foundational assumption of the pollination syndrome concept is that floral trait variation is shaped through natural selection by the most effective pollinator(s) (Stebbins, 1970; Spears, 1983; Waser et al., 1996; Mayfield et al., 2001; but see Aigner, 2001). *Phlox drummondii* floral traits, including a narrow corolla tube and a small amount of nectar, suggest specialization toward pollination by lepidopterans (Figure 1; Grant and Grant, 1965; Faegri and Van Der Pijl, 1979). Our empirical pollination data support this prediction by demonstrating that lepidopteran species visit flowers, while other potential visitors (e.g., bees) do not.

A single butterfly species represents ~90% of the floral visits, which suggests surprisingly high specialization within the pollinator assemblage (Figure 2A). *Phlox drummondii* has floral traits consistent with a generalized lepidopteran syndrome, which led us to expect a more even distribution of visitors (Figure 1; Grant and Grant, 1965; Faegri and Van Der Pijl, 1979). Only with direct observation were we able to uncover the degree of specialization in this system. In previously characterized specialized systems, there are particular traits enforcing specialization—for example, a correlation between corolla tube length and the proboscis length of the primary pollinator (Alexandersson and Johnson, 2002). However, *P. drummondii* corolla tube length does not appear to closely match one specific visitor type. In fact, the proboscis lengths of the four visitor types (*B. philenor*, *H. lineata*, skipper species, and *P. sennae*) suggest that individuals of each can access nectar and sexual floral organs during visitation (Appendix S6). *Battus philenor* visitation is extremely common and significantly affects reproductive output, yet there is not apparent phenotypic specialization toward *B. philenor* over other lepidopteran visitors. More in-depth experimentation is needed to identify potential variation in traits that are cryptic to human observers but that may be important for pollinator specificity (e.g., chemical composition of scent or nectar; Adler, 2000; Brodmann et al., 2008; Shuttleworth and Johnson, 2009).

The potential contribution of infrequent non-*Battus* visitors may become more important if the pollinator assemblage varies across time or geography. As a result, pollinator-mediated selection on floral traits may also vary, offering a possible explanation for the apparent lack of phenotypic trait specialization on *B. philenor*. However, previous work in this system and community science data suggest that the pollinator assemblage has remained constant in recent years. In a fixed-array experiment conducted in 2012, *B. philenor* represented 81% of all floral visits, similar to the 88.5% observed in the present study (Hopkins and Rausher, 2014). A survey of community science data suggests that *B. philenor* abundance has remained consistent across the past 5 yrs (Appendix S7). Furthermore, community science data collected across the past 8 yrs demonstrate that variation in *B. philenor* abundance was correlated with variation in abundance of other pollinator types (Appendix S8; via GBIF.org;

https://doi.org/10.15468/dl.4ypz8w [accessed 6 December 2022]). In terms of potential geographic variation, *B. philenor* was the most frequent visitor at all study sites throughout daylight hours of observation. Collectively, these findings suggest that our data likely represent the dominant pollinator environment in most places and in most years.

The overall proportion of visits by *B. philenor* suggests a high degree of specialization in floral visitation, and the patterns of foraging, pollen transfer, and fruit set suggest that this visitor is also an effective pollinator. Although *B. philenor* make many floral visits in total, consecutive visits to flowers on the same self-incompatible plant could have diminishing returns and not affect overall reproductive output. However, our data do not support this scenario. In natural foraging conditions, we observed *B. philenor* individuals making successive visits to flowers of the same plant, with an average of 1.88 flowers per plant (Figure 2D). In our controlled foraging experiment, we found that fruit set is statistically indistinguishable between the first three visited flowers on a single plant, although point estimates of fruit set decrease, which suggests some self-pollen interference (Figure 4C; Appendix S5). Because *P. drummondii* is largely self-incompatible, it is likely that fruit set at the third visit represents carryover of outcross pollen across successive visits to flowers on the same plant. Our results demonstrate that *B. philenor* has high pollination success, and that visits to multiple flowers of the same plant will result in continued transfer of outcross pollen, positively affecting a plant's reproductive output.

A limitation of our study is the absence of pollen transfer data for non-*Battus* visitors. Rare pollinators may also be important in shaping floral trait variation (Pauw et al., 2020). The extremely low visitation frequency of these other visitor types prevented capture of adequate sample sizes to assess pollen transfer. Pollination efficiency data for each visitor would offer a more complete picture of pollinator specialization in *P. drummondii*. Although sample sizes are low, we observed pollen loads on all species captured while foraging within the range of loads carried by *B. philenor*, which suggests that visitation may affect reproductive output (Figure 4A). Yet the high efficiency of the most frequent pollinator leaves little room for these infrequent visitors to have a disproportionate impact on plant fitness. Our results are suggestive of ecological specialization toward *B. philenor* but do not rule out some contribution to reproductive output by infrequent lepidopteran visitors.

Importantly, *B. philenor* is a generalist. While we noted *B. philenor* visiting a variety of plant species in the area, we observed only *Phlox* pollen on individuals captured while foraging on *P. drummondii*. Previous work demonstrated high constancy in *B. philenor* foraging behavior (Hopkins and Rausher, 2012; Briggs et al., 2018). Foraging pattern may explain the lack of non-*Phlox* pollen observed if individuals tend to visit many *P. drummondii* flowers in succession (high constancy). It is further possible that *B. philenor* prefers *P. drummondii* where their ranges overlap, given that both learning and floral preference have been shown in this species (Weiss and Papaj, 2003). However, *B. philenor* is geographically widespread and visits a diversity of plants, which suggests some asymmetry in specialization between this species pair, based on our visitation data (Fordyce and Nice, 2003; Vázquez and Aizen, 2004).

Battus philenor represents an overwhelming proportion of floral visits, but low levels of visitation by other Lepidoptera may offer some redundancy in pollination services if these visitors are able to affect pollination. *Phlox drummondii* occurs throughout regions in Texas that have experienced significant anthropogenic change in land-use, pollution, and weather patterns. Lepidopterans are vulnerable to changing climate, and species loss is accelerating (Forister et al., 2021). While *B. philenor* is currently widespread, it is unclear how potential future population reduction might affect pollination of *P. drummondii* and its long-term persistence (Appendix S7;

Steiner and Whitehead, 1996; Potts et al., 2010; Brosi and Briggs, 2013; González-Varo et al., 2013). One possible source of redundancy in pollination services could come from both diurnal and nocturnal visitation (Blüthgen and Klein, 2011; Funamoto and Sugiura, 2021; Alison et al., 2022). In our temporal exclusion experiment, we found that although nocturnal pollination is much less frequent than diurnal, we observed some fruit set among flowers exposed to nocturnal-only pollination, likely due to visitation by the hawk moth *H. lineata* (Figures 2B and 3; Appendix S4). A survey of community science data suggests that across the past 5 yr, *H. lineata* abundance was highest in 2022 (Appendix S7; via GBIF.org; https://doi.org/10.15468/dl.4ypz8w). As a result, the present study may represent a year with above-average nocturnal pollinator activity. Notably, there was no significant difference in the proportion of fruits between diurnal-only pollination and total pollination (diurnal + nocturnal pollination), despite some fruit set in the nocturnal-only treatment (Figure 3; Appendix S4). A possible explanation for this pattern is that because flowers open in the morning, flowers pollinated during the day may not be receptive to pollination during the night. However, if no visits occur during the day, nocturnal visitors can elicit some fruit set. If daytime pollinator loss occurs, our results suggest that nocturnal hawk moths could become a more important participant in the pollination process (Walton et al., 2020). Future studies of the temporal dynamics involved in pollen-pistil interactions could clarify redundancy in the pollination network and how these relationships may be affected by changing climate.

Plant-pollinator interactions exist along a continuum of specialization (Armbruster, 2017). Centuries of pollination studies have contributed examples ranging from highly restricted systems that rely on a single pollinating species to broad generalists that receive visitation from many pollinator types (Waser et al., 1996; Martén-Rodríguez et al., 2009; Mitchell et al., 2009). *Phlox drummondii* occupies a position along this continuum toward specialization. In the present study, we observed that *P. drummondii* is visited by a handful of Lepidoptera while effectively filtering out other potential visitors. Specialization on Lepidoptera is consistent with pollination syndrome predictions based on floral trait variation (Grant and Grant, 1965; Faegri and Van Der Pijl, 1979). Within this lepidopteran visitor assemblage, we observed a strong bias in visitation rates toward a single generalist butterfly species, *B. philenor*, which we also found to be an effective pollinator. While these results are suggestive of ecological specialization toward a single butterfly species, they do not rule out a potentially important role for less frequent visitors, particularly if the pollinator assemblage changes over time. Identifying these pollination dynamics in *P. drummondii* required direct observation and could not be deduced from floral trait variation. Most flowering plants do not have obvious specialized floral trait adaptations to a single pollinating species; our findings suggest that this does not imply absence of strong bias in visitation rate toward a particular pollinating species (Waser et al., 1996; Ollerton et al., 2007; Muchhala et al., 2009).

<h1>CONCLUSIONS

Based on floral morphology, we hypothesized that *P. drummondii* is pollinated by a variety of lepidopteran species. We tested this prediction by directly observing each step of the pollination process in the Texas wildflower. While the results are consistent with our prediction, they further reveal details of *P. drummondii*'s pollination biology that carry ecological and evolutionary implications. We find that a single butterfly species, *B. philenor*, is responsible for the vast majority of visitation. Further, we find that *B. philenor* visitation results in pollen transfer and affects reproductive output. A limitation of our study is the lack of pollen transfer data for other insect visitors, which we were unable to collect due to their extremely low visitation frequencies. Our results are consistent with ecological specialization toward a dominant butterfly species but do not rule out a potential contribution of infrequent visitors to pollination. If this redundancy in pollination services exists, *P. drummondii* may be relatively robust to the effects of changing climate and the rapid loss of butterfly species in the Northern Hemisphere. This study demonstrates how empirical observations of each step of the pollination process can offer a more complete picture of a species' pollination biology.

<h1>ACKNOWLEDGMENTS

The authors thank L. Gilbert, T. Juenger, R. Mecredy, and the Brackenridge Field Laboratory for graciously hosting experiments. Our statistical analyses were greatly improved by feedback from A. Berardi, and we thank her for her thoughtful guidance. Comments from two anonymous reviewers improved our manuscript immensely and we appreciate their contributions. Research was supported by funding from National Science Foundation grant DEB1844906 to R.H.

<h1>AUTHOR CONTRIBUTIONS

G.A.B. and R.H. conceptualized and designed the study. G.A.B. led and organized data collection to which all authors contributed. G.B. and O.B.-M. analyzed the data and generated figures. G.A.B. and R.H. wrote the manuscript. All authors edited the manuscript.

<h1>DATA AVAILABILITY STATEMENT

Data available from the Dryad digital repository: doi.org/10.5061/dryad.1c59zw3zj

<h1>SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Appendix S1. Map of central Texas with the approximate range boundary of *P. drummondii* shown in green. The three focal populations included in our study are marked as black dots (901, BFL, 765). Other populations included in our study with fewer observation hours are indicated as open dots (902, 758, 807, 794). GPS coordinates for each population are reported.

Appendix S2. Total pollinator visits across time are plotted in the upper left. Each panel indicates visits across time for a particular pollinator species. A smoothed line was fitted to each histogram to show general trends in visitation across the day using gam smoothing in ggplot2. Vertical dashed gray line at 19:00 indicates the beginning of nocturnal pollinator exclusion/end of diurnal pollinator exclusion in our temporal pollinator exclusion experiment.

Appendix S3. *Battus philenor* individual forages on *P. drummondii* flowers at focal population 765 (30.1049 N, −97.207428 W) on 30 April 2022. Video by Grace A. Burgin.

Appendix S4. Results from post hoc pairwise comparisons testing whether temporal pollinator exclusion groups differ in the proportion of open flowers setting fruit at days 5 and 8 using generalized mixed-effects models with binomial errors. Bolded values indicate significant differences in the proportion of fruits set per flower between exclusion treatments.

Appendix S5. Results from post hoc pairwise comparisons testing whether flowers under different pollination treatments differ in the proportion of flowers that set fruit using a generalized mixed effect model with binomial errors. Specifically, we compare flowers with outcross pollen applied in a controlled cross, flowers visited in succession by *B. philenor*, and unvisited flowers. Bolded values indicate significant differences in the proportion of fruits set per flower between pollination treatments.

Appendix S6. Distributions of proboscis length (mm) among captured individuals of the four visitor types (*B. philenor*, *H. lineata*, skipper species, and *P. sennae*) summarized as boxplots. The minimum and maximum corolla tube lengths are indicated as horizontal gray lines and labeled. Individuals of all four species fall within the range of corolla tube length in *P. drummondii*.

Appendix S7. (A) Total *B. philenor* (in green) and *H. lineata* (in blue) observations from 2018 to 2022. While the number of *B. philenor* observations remains relatively constant across time, the number of *H. lineata* observations varies considerably. In 2022, which corresponds to our study period, *H. lineata* observations were particularly high. (B) We implemented a binomial model using the R package lme4, comparing observations of *B. philenor* and other floral visitors collectively. Model predictions are plotted as points. 95% CIs are plotted as bars. Community science data used in these analyses was downloaded from the Global Biodiversity Information Facility (via GBIF.org; https://doi.org/10.15468/dl.4ypz8w [accessed on 6 December 2022]). We filtered GBIF data to include occurrences only if they fall within the range of observation sites included in our study and during *P. drummondii*'s flowering period (March-July).

Appendix S8. Sightings of *B. philenor* correlate with sightings of other lepidopteran species observed visiting *P. drummondii* flowers in our study. Community science data used in these analyses was downloaded from the Global Biodiversity Information Facility (via GBIF.org; https://doi.org/10.15468/dl.4ypz8w [accessed 6 December 2022]). We filtered GBIF data to include occurrences only if they fall within the range of observation sites included in our study and during *P. drummondii*'s flowering period (March–July). We removed larval observations for this analysis to retain only individuals that could potentially serve as pollinators.

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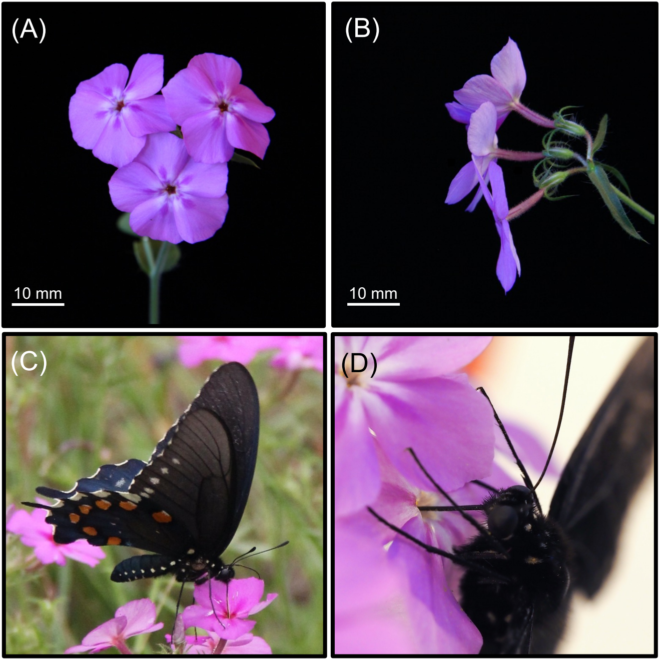
Figure 1. *Phlox drummondii* flowers display a general lepidopteran floral syndrome. (**A**) Flowers are radially symmetric, showy, and borne in clusters. Star-shaped center patterning is common. (**B**) Nectar is produced at the bottom of a narrow corolla tube of medium length. Sexual organs (pistil and anthers) are fully inserted within the corolla tube. (**C**) *Battus philenor* female visiting a *P. drummondii* flower in a natural population. (**D**) During visitation, a *B. philenor* individual inserts its proboscis into the corolla tube. Photos by Grace A. Burgin (A, B), Austin G. Garner (C), and Robin Hopkins (D).

Figure 2. Summary of pollinator observations. (**A**) Total visits observed to *Phlox drummondii* flowers by time of day. All species observed visiting flowers are included. (**B**) Relative proportion of floral visits made by *Battus philenor* vs. other visitor types throughout the day. Proportion of visits made by *B. philenor* is shown in green, while all other visitor types are grouped and shown in gray. All but one of the floral visits observed during 2000–2100 hours were made by the hawk moth *Hyles lineata*. (**C**) Average numbers of plants visited during a foraging bout by *B. philenor* (green), skipper species (purple), and other visitors (gray), plotted as points, with standard error plotted as bars. (**D**) Average numbers of flowers visited per plant by *B. philenor* (green), skipper species (purple), and other visitors (gray), plotted as points.

Figure 3. Model predictions for the proportion of open flowers that set fruit in each of the pollinator exclusion treatments, plotted as points, with 95% CIs plotted as bars. Data collected on

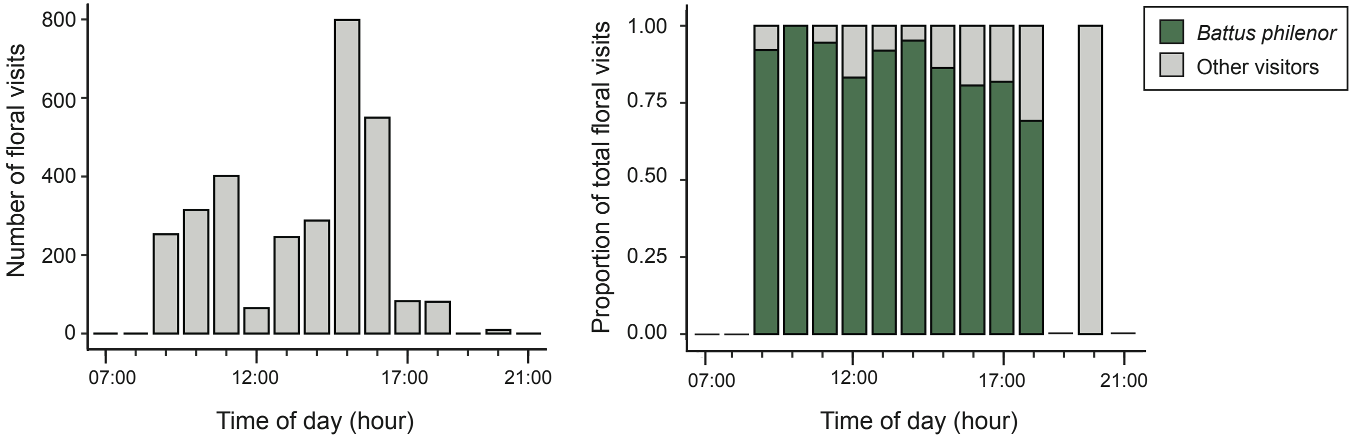
days 5 and 8 after beginning exclusion were analyzed and plotted separately. Lowercase letters indicate significant differences in the proportion of open flowers that set fruit across treatment groups. Fruits measured on each plant in the experiment are listed in the table. Full model results are reported in Appendix S2.

Figure 4. (**A**) Distribution of *Phlox* pollen grain number on proboscises of floral visitors. *Battus philenor* individuals are shown in green and summarized as a box plot. Individual pollen loads quantified from non-*B. philenor* proboscises are indicated as diamonds and colored by visitor type. Data represent pollen loads after at least one floral visit. (**B**) Number of *Phlox* pollen grains on stigmas after no pollinator visitation or after a single visit by *B. philenor* as predicted by the model, plotted as points, with 95% CIs plotted as bars. Lowercase letters indicate significant differences. (**C**) Model predictions of the proportion of flowers that set fruit after manual application of outcross pollen (controlled cross), autonomous selfing, and successive visits by *B. philenor* to flowers of the same plant in visit order (first flower, second flower, third flower). Model predictions are shown as points and 95% CIs are plotted as bars. Lowercase letters indicate significant differences in proportion of flowers that set fruit across treatment groups. Full model results are reported in Appendix S4. (**D**) Images depicting the process of pollination from pollen pickup to fruit set in *P. drummondii*. An individual *B.philenor* has a visible pollen load on its proboscis after foraging on *P. drummondii* flowers. Then, after a single visit by *B. philenor*, a *P. drummondii* stigma is coated in pollen. The same stigma is shown after a few days when it has developed into fruit. Photos by Robin Hopkins (left) and Grace A. Burgin (right two).


(A)
10 mm
(B)
10 mm
(C)
(D)

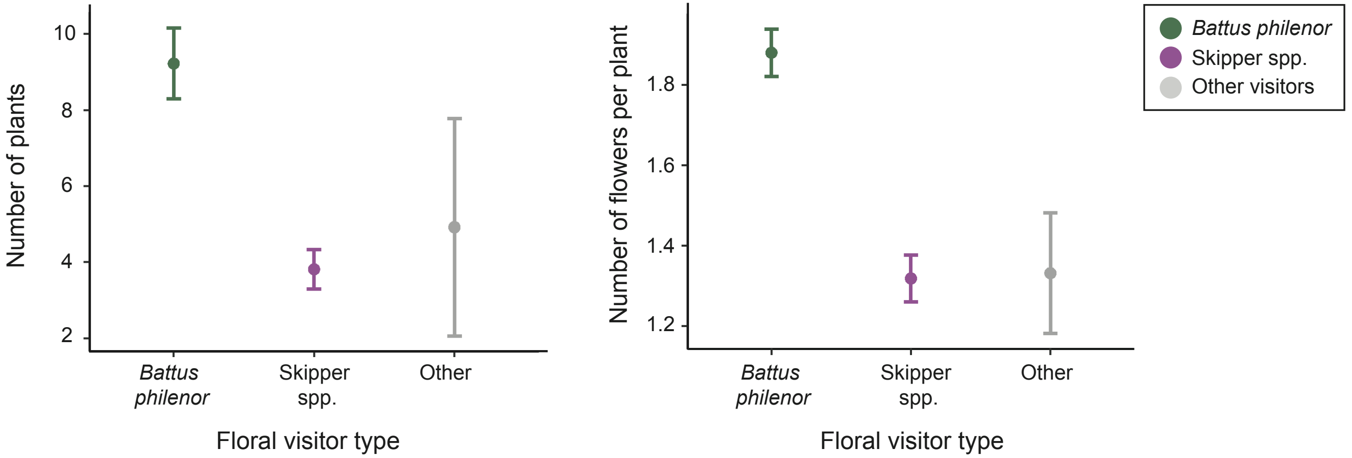
(A) Total floral visits made by all species

(B) Proportion of total floral visits at each hour made by *Battus philenor* vs. other floral visitors

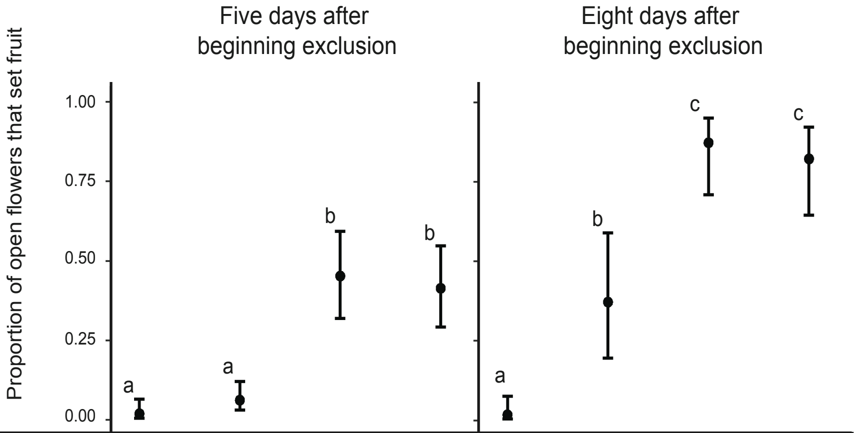


(C) Number of plants visited per foraging bout

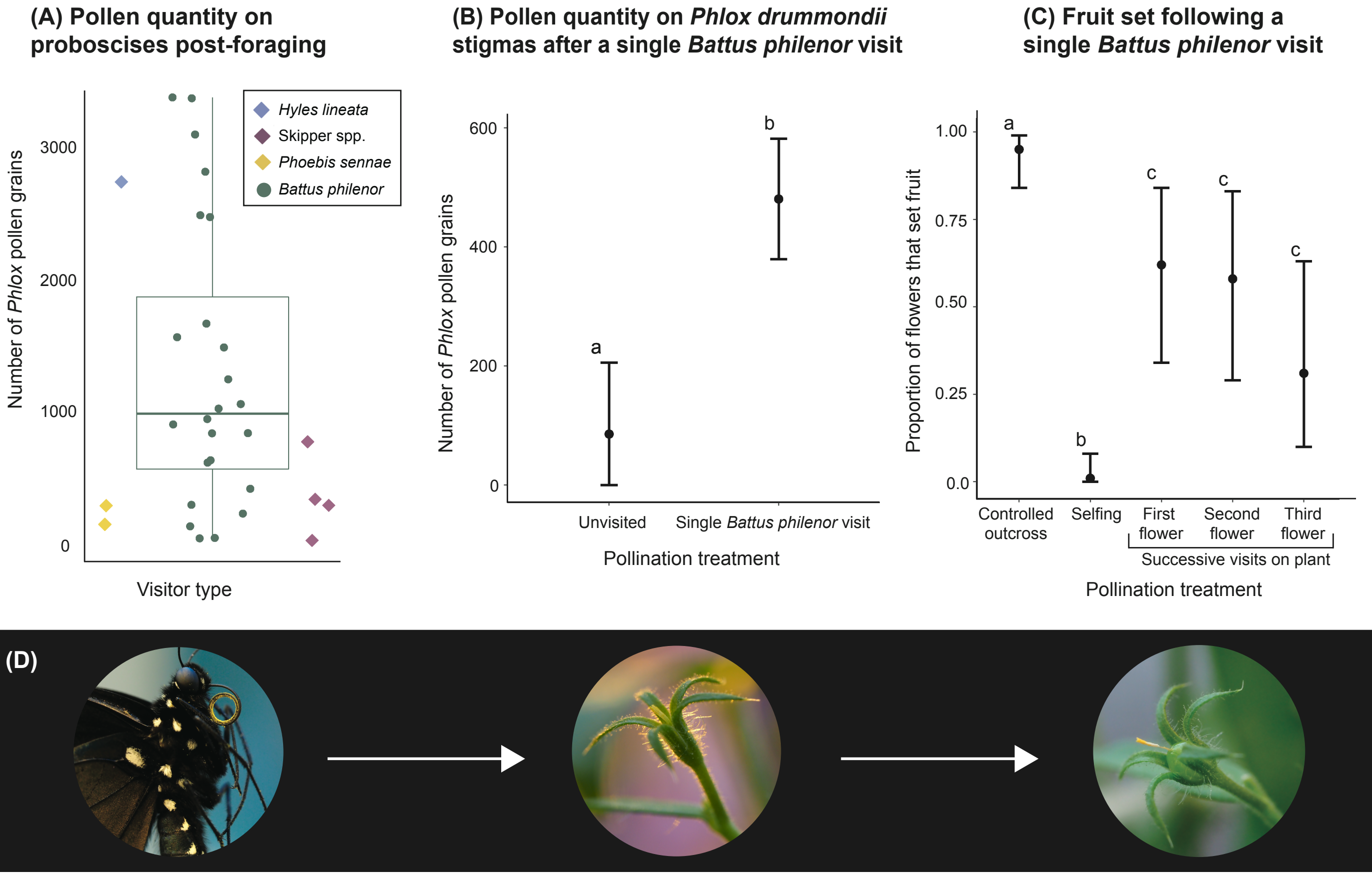
(D) Number of flowers visited per plant



Proportion of open flowers that set fruit in pollinator exclusion treatments



Pollination period	None	Nocturnal	Diurnal	Nocturnal + Diurnal	None	Nocturnal	Diurnal	Nocturnal + Diurnal
Pollinators excluded	Always	07:00-19:00	19:00-07:00	Never	Always	07:00-19:00	19:00-07:00	Never
Number of fruits	4	25	102	102	7	105	162	175
Number of calyxes	124	309	235	250	-	-	-	-
Number of plants	15	23	23	25	14	22	23	25



(A) Pollen quantity on proboscises post-foraging

(B) Pollen quantity on *Phlox drummondii* stigmas after a single *Battus philenor* visit

(C) Fruit set following a single *Battus philenor* visit

(D)