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As prey and pollinators, insects increase reproduction and allow for outcrossing in the carnivorous plant *Dionaea muscipula*

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Abstract

Premise: Understanding the factors that limit reproductive success is a key component of plant biology. Carnivorous plants rely on insects as both nutrient sources and pollinators, providing a unique system for studying the effects of both resource and pollen limitation on plant reproduction.

Methods: We conducted a field experiment using wild-growing *Dionaea muscipula* J. Ellis (Droseraceae) in which we manipulated prey and pollen in a factorial design and measured flower production, number of fruits, and number of seeds. Because understanding reproduction requires knowledge of a plant species' reproductive and pollination biology, we also examined the pollination system, per-visit pollinator effectiveness, and pollen-ovule (P/O) ratio of *D. muscipula*.

Results: Plants that received supplemental prey produced more flowers than control plants. They also had a higher overall fitness estimate (number of flowers × fruit set (total fruits/total flowers) × seeds per fruit), although this benefit was significant only when prey supplementation occurred in the previous growing season. Neither pollen supplementation nor the interaction between pollen and prey supplementation significantly affected overall plant fitness.

Conclusions: This study reinforces the reliance of *D. muscipula* on adequate prey capture for flower, fruit, and seed production and a mobile pollen vector for reproduction, indicating the importance of considering insects as part of an effective conservation management plan for this species.

KEYWORDS

carnivorous plants, *Dionaea muscipula*, Droseraceae, plant-insect interactions, pollination, pollination ecology, reproductive biology, resource limitation

An essential component of plant ecology is understanding the factors that limit plant reproduction. In particular, many studies have examined how reproduction can be limited by both pollen and resource availability (e.g., Haig and Westoby, 1988; Zimmerman and Aide, 1989; Burd, 2008; Cunningham et al., 2020). These two factors are not mutually exclusive (Campbell and Halama, 1993), and resource limitation can also indirectly influence pollen receipt via increased flower or reward production (Zimmerman and Pyke, 1988; Campbell and Halama, 1993; Carroll et al., 2001). Despite an increasingly nuanced understanding of how

resource and pollen limitation affect plant reproduction, these factors have infrequently been studied simultaneously in rare plants (Mattila and Kuitunen, 2003; Shi et al. 2010). This is concerning in instances where reduced reproduction can have repercussions for population viability (Wilcock and Neiland, 2002; Law et al., 2010). The goal of this study was to understand the degree to which reproduction in a rare carnivorous plant is limited by both pollen and resources.

Carnivorous plants get most of their mineral nutrients—chiefly nitrogen (N) and phosphorous (P)—from animal prey (Ellison and Gotelli, 2001; Ellison, 2006). Low prey

capture limits reproductive success in several carnivorous plant taxa, including *Pinguicula* L. (Lentibulareaceae) (Thorén and Karlsson, 1998; Alcalá and Domínguez, 2005) and *Sarracenia* L. (Sarraceniaceae) (Ne'eman et al., 2006). To our knowledge, only one study has investigated the simultaneous influence of pollen and resource limitation on a carnivorous plant (Ne'eman et al., 2006), finding that resource limitation from photosynthates and prey-derived nitrogen had a greater effect on reproductive output compared to pollen limitation in *S. purpurea* L. The overall importance of pollen limitation to carnivorous plant reproduction remains poorly known.

Since many carnivorous plants depend on insects both as pollinators and prey (Juniper et al., 1989), they also run the risk of capturing their own pollinators, a hypothetical dilemma known as pollinator-prey conflict (PPC) (Jürgens et al., 2012; El-Sayed et al., 2016). Evidence for PPC is scant (Cross et al., 2018) and has been shown in just a handful of instances (Zamora, 1999; Murza et al., 2006). In the Venus Flytrap, *Dionaea muscipula* J. Ellis (Droseraceae), there is minimal overlap between pollinator and prey guilds, likely due to spatial separation between the flowers and traps (Youngsteadt et al., 2018). However, it remains unclear whether this spatial separation was the result of selection to minimize PPC, or due to independent selective pressures on the flowers and on the traps. Studies on the closely related genus *Drosera* suggest that tall flowering scapes may largely be an adaptation to attract pollinators, rather than an adaption to minimize pollinator by-catch (Anderson and Midgley, 2001; Anderson, 2010; El-Sayed et al., 2016). To tease apart the relative selective pressures driven by pollinators and prey on traits in carnivorous plants, it is essential to understand whether and how these guilds limit plant reproductive success.

In this study, we investigated the simultaneous influence of pollen and prey limitation on the reproduction of *D. muscipula*, a rare carnivorous plant. We used a two-way factorial experiment in which we manipulated pollination and prey supplementation to ask: To what degree is reproduction limited by pollen, prey, and their interaction in *D. muscipula*? In other words, is an insect equally useful as pollinator or prey? We predicted that a multiplicative estimate of fitness (number of flowers × fruit set (total fruits/total flowers) × seeds per fruit) would be highest in plants provided both supplemental pollen and prey. We also asked: What is the pollination system and per-visit efficiency of pollinator taxa to *D. muscipula*? Determining the pollination biology of *D. muscipula* allows us to better understand factors that limit its reproduction.

MATERIALS AND METHODS

Study system and site

Dionaea muscipula is a carnivorous perennial endemic to pocosin and longleaf pine savanna ecosystems in North and South Carolina, USA. It is the only terrestrial carnivorous plant with a snap-trap mechanism for capturing prey (Cameron et al., 2002). Each plant has approximately 4 to 12 trapping leaves arranged in a rosette around a short stem (Bailey and McPherson, 2012). The entire prey digestion process takes up to 12 days. After several bouts of prey capture, the trap will no longer close and will remain purely photosynthetic until senescence (Jaffe, 1973). Ground-crawling arthropods (mainly spiders, ants, and beetles) comprise the bulk of captured prey (Hutchens and Luken, 2009; Youngsteadt et al., 2018).

Dionaea muscipula reach reproductive maturity three years after germination (Smith, 1929). Reproductive stems in *D. muscipula* become visible in early April (Smith, 1929; Roberts and Oosting, 1958), but it is unclear when the floral primordia begin to develop in this species. Flowering occurs in late May to mid-June. Reproductive individuals typically produce a single inflorescence, comprising 8 to 12 flowers arranged in an umbelliform cyme. The inflorescence is elevated above the trapping leaves by a 15- to 30-cm scape. The white flowers are perfect and protandrous, with anthers dehiscing about 24 hrs before the stigma flares (Smith, 1929; Williams and Scholl, 2021). Youngsteadt et al. (2018) found that *D. muscipula* flowers are visited by myriad insect taxa, with sweat bees and beetles identified as the likely primary pollinators. There is minimal overlap between the guild of insects visiting the flowers and those that are caught in the traps (Youngsteadt et al., 2018). Though originally reported to be self-incompatible (Roberts and Oosting, 1958), Juniper et al. (1989) later indicated that *D. muscipula* may be self-compatible, which has also been reported by hobbyist and commercial growers of the plant (ex. www.FlytrapCare.com; Anonymous, 2022). The lifespan of *D. muscipula* in the wild is unknown but is presumably long-lived, as individuals in the horticultural trade can live for up to twenty years (Bailey and McPherson, 2012).

We studied a large population of *D. muscipula* (>2000 plants) at a managed area in Pender County, North Carolina, USA (Hamon et al., 2021). Due to the poaching risk for this plant, specific site locations within the large population have been omitted. Instead, we assign arbitrary ID numbers to sites (Appendix S1, Table S1). Prey and pollen supplementation trials and the pollination system study occurred within the same site. Pollen-ovule counts were estimated using plants from three sites which were separated from one another by 0.70 to 5.8 km. The year 2019 was unusually dry for the southeastern USA, with the driest September on record for the region (NOAA, 2020). Consequently, many *D. muscipula* individuals entered dormancy in May and June 2019 (Hamon et al., 2021).

Prey and pollen supplementation

Prey and pollen supplementation to wild plants was conducted as a two-way factorial design with two trials at a single site, i.e., one starting summer 2019 and a second starting in spring 2020, with both culminating in 2020

reproduction. On 09-August-2019, we haphazardly selected 80 plants of a similar size and assigned them to a prey supplementation treatment (hand-fed or control) and a pollen supplementation treatment (hand-pollinated or control) (N = 20 plants per treatment combination). Plants were labeled using metal tags staked into the nearby soil. The site had been burned in May 2019, and plants with the persistent remains of burnt flowering stems were preferentially chosen. However, since flowering stems can sometimes persist from the previous year, it is not certain which plants in this trial had flowered earlier that year.

Prey supplementation

In the trial that began in 2019 (hereafter referred to as 2019 plants), we hand-fed all plants in the prey supplementation treatment on five dates (09-August, 23-August, 15-September, 29-September, and 25-October). These dates were selected because the site had been burned in May, and by late summer plants had a trap size that allowed for hand feeding with crickets. By November, daytime temperatures were too low to consistently support arthropod prey activity and trapping ability (Bailey and McPherson, 2012). Prey supplementation dates were at least 14 days apart to allow for complete digestion. To supplement prey, we used frozen house crickets (*Achetus domesticus* L. (Gryllidae)) in two sizes labeled by the distributor: 0.635 cm or 0.476 cm in length (Premium Crickets, Winder, Georgia, USA). We chose crickets because Orthopterans are among the natural prey items for *D. muscipula* (Hutchens and Luken, 2009; Youngsteadt et al., 2018). Upon receiving each shipment of crickets, we immediately froze the insects for at least 24 h. In 2019, we fed plants with either 0.635 cm or 0.476 cm crickets, using smaller crickets only for traps that were too small to effectively digest large crickets. To hand-feed plants, we placed a thawed cricket in each open trap using a pair of forceps and stimulated the trigger hairs on the trap surface. Following trap closure, we gently squeezed the trap over a duration of roughly 1 minute to continue stimulating the trigger hairs, mimicking struggling prey. During each hand-feeding session, we recorded the number of naturally closed traps at the time of feeding, open traps, and developing traps on plants in both supplemented and non-supplemented treatments. We also recorded the number of 0.635 cm or 0.476 cm crickets that were fed to each plant. Plants in the non-supplemented treatment were also handled (but not triggered) to mimic the handling of leaves in the supplemented treatment. The day following feeding events, we confirmed that a subsample of hand-fed traps proceeded to digestion, indicated by tightly appressed margins.

For the trial that started in 2020, on 22-May we selected an additional 80 plants of similar size (hereafter "2020 plants"). All selected plants had reproductive stems with buds and had not begun flowering. Plants in this trial in the prey supplementation treatment were fed on two dates, 22-May and 07-June, using similar methods as in 2019. Both 2019 plants and 2020 plants bloomed in 2020, such that 2019 plants received supplemental prey the season before flowering, whereas the 2020 plants received supplemental prey after reproduction was already initiated. Thus, these two trials were intended to serve as comparison of how the timing of resource acquisition affects the degree of prey limitation on plant reproduction. While it would have been ideal to have a similar number of feeding events in the 2019 and 2020 trials, feeding supplementation in 2020 was delayed due to the COVID-19 pandemic. Therefore, these trials necessarily constitute a preliminary, qualitative exploration of the role of timing on reproductive output.

Pollen supplementation

Plants in the 2019 trial that successfully flowered in 2020, as well as all plants in the 2020 trial, were hand-pollinated over three or four dates, respectively, between 28 May and 3 June 2020. Across these dates, 1 to 4 flowers with receptive stigmas from plants in the hand-pollination treatment were pollinated to saturation by rubbing the stigmas with dehiscing anthers collected from one or more individuals at least 5 m away. Each plant in the 2019 trial was pollinated on two dates due to overcast conditions during the first hand pollination bout, compared to one date per plant in the 2020 trial. Simultaneously, we haphazardly selected 1 to 4 flowers with receptive stigmas on plants in the control pollination treatments to serve as open-pollinated controls. Experimental flowers (including both hand-pollinated and control flowers) were marked using a piece of green embroidery thread tied around the pedicel and were left for additional open pollination. In total, each flowering plant had 1 to 4 flowers marked for fruit collection and seed counts, representing 1 to 2 days of hand-pollination bouts for each plant. We did not observe a significant effect of the number of experimental flowers on our fitness estimates (Appendix S2). At the time of the first hand-pollination bout for each plant, we recorded the number of open, closed, and developing traps, as well as the number of buds, flowers, and fruits on pollen-supplemented and open-pollinated controls. We also recorded the flowering scape height by measuring the distance in cm from the ground to the bottom of the lowest pedicel.

We assessed fruit and seed production at least 14 days after hand-pollination. To determine the number of fruits, we counted the number of fruits (including experimental fruits) that successfully produced seeds. For the 1 to 4 focal fruits per plant, seeds were counted and weighed to the nearest 0.001 mg in the lab. We calculated the average seed weight per fruit by dividing the total seed weight by the number of seeds in each fruit. Seeds were then returned to the site to approximate natural seed rain.

Statistical analyses

Statistical analyses were performed using R version 4.1.1 (R Core Team, 2021). To determine whether prey

supplementation in 2019 plants affected the probability of producing leaves (i.e., not dead or dormant) or flowers in 2020, we conducted a Pearson's chi-squared test comparing the proportion of growing and flowering individuals between prey-supplemented and control treatments. To examine the effects of pollen and prey supplementation on reproductive success, we calculated a multiplicative fitness estimate for each plant by multiplying the flower number × fruit set (total fruits/total flowers) × average seeds per fruit. We then fit a linear model in which fitness estimate was the response, and pollen supplementation, prey supplementation, and their interactions were the explanatory variables. We tested the outputs of the model using an ANOVA. To compare number of seeds per fruit and average seed weight per fruit between treatments, we fit a linear mixed effects model wherein pollen supplementation treatment, prey supplementation treatment, and their interaction were the explanatory variables, and plant identity was included as a random variable. Linear mixed effects models were fitted using the package 'lme4' (version 1.1.27.1; Bates et al., 2015) and summarized using the 'Anova' function within the package 'car', using a Type II test (Fox and Weisberg, 2011). For post-hoc comparison between treatments, we conducted a Tukey test using the package 'emmeans' (version 1.7.2, Lenth, 2022). We also conducted a t-test to compare number of flowers and number of fruits between prey supplementation treatments, as well as to compare scape height and number of traps at the time of hand-pollination. Plants that did not flower or had missing inflorescences were excluded from analyses of inflorescence traits. The 2019 and 2020 trials were analyzed separately.

Plant reproductive biology

To test whether *D. muscipula* is self-compatible, we conducted hand-pollinations over six days in late May and early June 2020. At a single site, we selected 30 blocks of four plants, in which each block had inflorescences with a similar number of total flowers (N = 120 plants total, wherein each individual had a single experimental flower). We selected one flower from each of the four plants to be haphazardly assigned to one of four pollination treatments: (1) self-pollinated with autogamous pollen; (2) self-pollinated with geitonogamous pollen; (3) pollinated with outcrossed pollen from an individual at least 5 m away; and (4) unpollinated control. The focal flower was identified with a piece of green thread tied around the pedicel. Each inflorescence was then enclosed in a drawstring organza bag (pollinator exclusion bag hereafter) before anther dehiscence and about 24 hr before the stigma became receptive. The day after inflorescence bagging, we conducted hand pollinations by rubbing dehiscing anthers from the relevant treatment against the receptive stigma of the focal flower using forceps. Pollinator exclusion bags were removed

following anthesis of the experimental flower, approx. 2 to 4 days after hand-pollination, when the petals on the focal flower had started to whither.

We collected fruits 18 to 21 days after hand pollination. Seeds were then counted and weighed in the lab and returned to their sites of origin. To compare number of seeds per fruit and average seed weight among treatments, we fit a linear mixed effects model in which number of seeds per fruit or average seed weight was the response variable, treatment was the explanatory variable, and block was included as a random effect.

Pollen-ovule ratio

In May 2018, we selected one flower from 28 to 30 inflorescences from each of three sites. We counted the anthers in each flower and then collected all anthers per flower prior to dehiscence (N = 88 flowers total). Anthers were stored in open vials in a desiccator for two weeks after collection, then in closed vials in a desiccator for 4 to 7 mo until they could be counted. In the lab, we used a pestle to lightly grind the anthers until all the anthers were powdery in appearance. We then added 750 µL of 70% ethanol to each vial. Pollen solutions were sonicated for five minutes, followed by five seconds of vortexing. To count pollen grains, we removed 10 µL of the pollen suspension and placed it under the cover slip of one half of a hemocytometer, which was then viewed under a dissecting microscope at 20× magnification. We counted 12 to 16 subsamples from each sample. Samples were vortexed for an additional five seconds between each count (adapted from Kearns and Inouye, 1993). To estimate the total number of pollen grains per flower, we calculated the average number of pollen tetrads counted across the subsamples per flower. We then extrapolated the total number of pollen tetrads in the 750 µL solution by multiplying the average value by 75. Like other members of Droseraceae, the pollen grains of *D. muscipula* are combined into permanent tetrads (Halbritter et al., 2012). Therefore, to obtain the total number of pollen grains per flower, we multiplied the total number of tetrads per flower by four (Murza and Davis, 2003). We henceforth use the term tetrad only to refer to the aggregated unit of four grains (compared to Youngsteadt et al., 2018 and Hamon et al., 2019, where the term "grain" refers to tetrads).

From each of the same sites where we collected anthers, we also collected the ovaries of 30 to 32 flowers (N = 93 ovaries total). Ovaries were stored in 70% ethanol. Five weeks after collection, we added one-part glacial acetic acid to the samples for every three parts 70% ethanol. Ovules were held in this mixture at room temperature for 6 to 16 d, rinsed in distilled water, and covered with 80% lactic acid for 19 to 22 h. The ovaries were again rinsed in distilled water and returned to 70% ethanol solution. We dissected the cleared ovules out of the ovaries and counted them under a dissecting scope at 10× magnification. This ovary clearing protocol was adapted from the methods outlined by Kearns and Inouye (1993).

We tested for differences among the three study sites in number of anthers, ovules, and estimated pollen tetrads per flower using a one-way ANOVA, followed by pairwise comparisons between sites using a Tukey test. We then calculated the average pollen-ovule (P/O) ratio per site according to Cruden (1977):

$$\frac{P}{O} \; ratio = \frac{pollen \; count \; per \; flower}{number \; of \; ovules \; per \; flower}$$

Per-visit pollinator effectiveness

We observed pollinators over the course of two flowering seasons: 2018 and 2020. In 2018, we conducted pollinator observations between 14-May and 16-June for a total of 20.2 person-hours. In 2020, we conducted pollinator observations on eight dates between 31-May and 11-June for a total of 12.6 person-hours. To compare single-visit pollen deposition to the stigma by different floral visitors, we placed pollinator exclusion bags over groups of inflorescences still in bud. During each observation session, the observer removed the exclusion bags from up to 11 inflorescences and watched all flowers that had undergone anthesis while in the exclusion bags. We observed from a distance of at least 0.2 m. Floral visitors to these flowers were identified in the field to family level; only honey bees (*Apis mellifera* L. (Apidae)) were identified to species in the field. Visitors were also allowed to visit as many flowers as were within the field of view before we attempted capture, regardless of whether the flowers had been bagged or not. We then collected all visited experimental stigmas into individual vials. From the bagged inflorescences, we collected five unvisited stigmas in 2018 and ten stigmas from unvisited flowers in 2020 to serve as unvisited controls. Stigmas were stored in a cooler with ice until they could be returned to the lab. Stigmas were subsequently stored at –30°C until analysis.

To count pollen receipt to stigmas, we stained the pollen on the stigmas using basic fuchsin dye (Kearns and Inouye, 1993) and counted the total number of conspecific pollen grains (i.e., number of tetrads multiplied by four) deposited on the stigma using a compound microscope at 200×. To compare pollen deposition per visit between taxa, we fit a linear mixed effects model wherein number of pollen grains per stigma was the response variable and visitor taxon (to family) was included as the explanatory variable. Individual insect ID number was included in the model as a random effect. We observed many visits from *A. mellifera*. Therefore, to compare per-visit effectiveness of native and non-native visitors, we treated *A. mellifera* separately from other members of the family Apidae. Visits from individuals that could not be positively identified to family were excluded from analysis. This model was visualized using the package 'sjPlot' (version 2.8.10; Lüdecke, 2021).

RESULTS

Pollen and prey supplementation

Out of the 80 plants that were tagged in the 2019 trial, 68 produced leaves (i.e., were not dead or dormant) during the 2020 flowering season and 59 produced flowers. There was no significant effect of prey supplementation on whether the plant produced leaves ($X^2 = 1.57$, $P = 0.21$) or flowers ($X^2 = 1.60$, $P = 0.21$) in 2020. Prey-supplemented 2019 plants produced an average estimate of 76% more seeds per individual compared to controls ($F_{1,49} = 6.74$, $P = 0.01$, Figure 1A). Among the 2020 plants, prey-supplemented plants produced an average estimate of 25% more seeds per individual compared to controls, but this trend was not statistically significant ($F_{1,75} = 3.76$, $P = 0.06$, Figure 1B). We observed no significant effect of pollination treatment alone on fitness estimate (2019 plants: $F_{1,49} < 0.01$, $P = 0.98$; 2020 plants: $F_{1,75} < 0.01$, $P = 0.98$), nor an interaction between pollination and prey treatment (2019 plants: $F_{1,49} = 0.44$, $P = 0.51$; 2020 plants: $F_{1,75} = 0.45$, $P = 0.51$).

When examining the effect of prey supplementation on individual estimates of reproductive success, prey treatment had a significant effect on flower production in both 2019 ($t_{40} = -3.98$, $P = 0.0003$; Appendix S3, Figure S1a) and 2020 ($t_{67} = -2.37$, $P = 0.02$; Appendix S3, Figure S1b), with fed plants producing an average of 6.2 and 1.9 more flowers per individual, respectively, than control plants. Prey-supplemented plants also had a significantly higher number of fruits compared to control
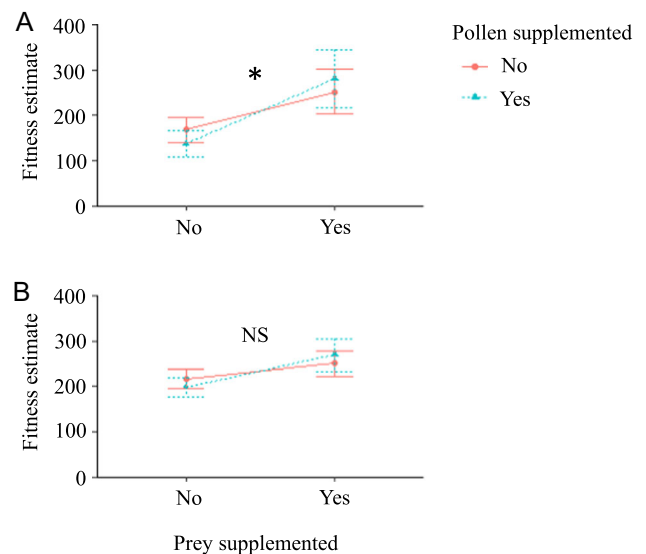

FIGURE 1 Venus flytrap (*Dionaea muscipula*) fitness estimates (flower number × fruit set (total fruits/total flowers) × average seeds per fruit) in plants that received supplemental prey or not, and supplemental pollen or not. Plants received supplemental prey in either 2019 (A) or 2020 (B); all pollen supplementation was in 2020. Prey supplementation in 2019 increased plant fitness in 2020; these plants produced more flowers and seeds per individual compared to controls. Pollen supplementation did not increase plant fitness. Bars are mean estimate ± SE.

plants (2019: $t_{41} = -3.02$, $P = 0.004$, Appendix S3, Figure S1c; 2020: $t_{66} = -2.07$, $P = 0.04$, Appendix S3, Figure S1d), with plants fed in 2019 and 2020 producing an average of 3.7 and 1.4 more fruits per individual, respectively. However, we found no significant effect of the prey treatment, pollination treatment, or their interaction on number of seeds per fruit in either trial (2019: $F_{1,51} \leq 2.83$, $P \geq 0.10$, Appendix S3, Figure S1e; 2020: $F_{1,76} \leq 61$, $P \geq 0.44$, Appendix S3, Figure S1a). We also observed no significant effect of the prey treatment, pollination treatment, or their interaction on average seed weight (2019: $F_{1,46} \leq 0.32$, $P \geq 0.58$; 2020: $F_{1,75} \leq 0.94$, $P \geq 0.14$).

When examining plant traits, we observed no significant effect of prey supplementation on scape height for the 2019 or 2020 trial (2019: $t_{55} = -0.14$, $P = 0.89$, Appendix S3, Figure S2a; 2020: $t_{76} = -1.12$, $P = 0.26$, Appendix S3, Figure S2b). Similarly, we did not observe a significant effect of prey treatment on number of traps produced in 2020 (2019 plants: $t_{72} = -1.44$, $P = 0.15$, Appendix S3, Figure S2c; 2020 plants: $t_{73} = -1.60$, $P = 0.11$, Appendix S3, Figure S2d).

Plant reproductive biology and pollen-ovule ratio

There was a significant effect of pollination treatment on number of seeds per fruit ($F_{3,87} = 57.37$, $P < 0.001$, Figure 2A).
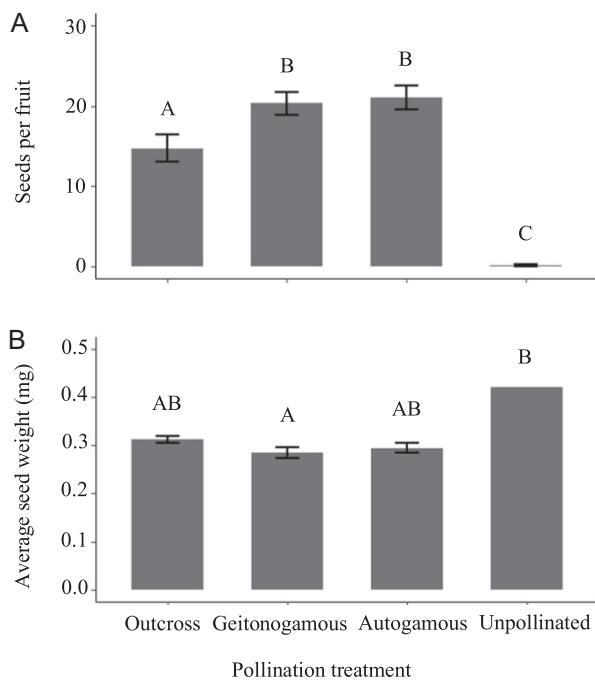
FIGURE 2   Seed set per fruit (A) and mean seed weight (mg) (B) by hand-pollination treatment. Bars are means ± SE. Different uppercase letters above the bars indicate significant differences in response variables from a Tukey HSD test at $P < 0.05$. Average seed weight for unpollinated flowers is based on a single fruit that produced four seeds. Otherwise, unpollinated flowers did not set seed.

Both outcrossed ($t_{87} = -3.52$, $P = 0.0038$) and geitonogamously pollinated flowers ($t_{87} = 11.56$, $P < 0.0001$) had a higher number of seeds per fruit than unpollinated flowers. Only one out of thirty unpollinated control flowers set seed, likely due to a leaky pollinator exclusion bag. This single unpollinated flower produced four seeds, compared to 21.1 seeds, on average, in outcrossed fruits and 20.3 seeds in geitonogamously pollinated fruits. In addition, outcrossed ($t_{87} = -3.52$, $P = 0.0038$) and geitonogamously ($t_{87} = -3.09$, $P = 0.01$) pollinated flowers had higher numbers of seeds per fruit compared to autogamously pollinated flowers, producing an average of 32.5% and 28.5% more seeds per fruit, respectively. However, there was no difference in number of seeds per fruit between outcrossed and geitonogamously pollinated flowers ($t_{87} = -0.42$, $P = 0.97$).

There was also an effect of pollination treatment on average seed weight ($F_{3,64} = 3.38$, $P = 0.023$, Figure 2bB). However, this significant result was driven by a single control flower that set seed, which produced unusually heavy seeds (29.4% heavier than seeds in all other treatments, on average). When we excluded the control treatment from analysis, there was no significant effect of pollination treatment on average seed weight ($F_{2,56} = 2.12$, $P = 0.13$).

Across all three sites where we collected anthers and ovaries in 2018, we observed an average of 13.96 ± 0.29 anthers per flower, 26.40 ± 0.79 ovules per ovary, and 8776.72 ± 460.23 pollen grains per flower (Appendix S1, Table S2). There was a significant effect of site on number of anthers, ovules, and pollen grains per flower, with S14 displaying an average of 11% fewer anthers, 30% fewer ovules, and 48% fewer pollen grains per flower compared to S3 and S10 (anthers: $F_{2,87} = 3.88$, $P = 0.02$; ovules: $F_{2,90} = 21.43$, $P < 0.0001$; pollen grains: $F_{2,85} = 17.21$, $P < 0.0001$; Appendix S1, Table S2). For each of sites S3, S10, and S14, we estimated a P/O ratio of 350.8, 355.3, and 267.3 pollen grains per ovule, respectively, with an average P/O ratio of 332.5 (Appendix S1, Table S2).

Per-visit pollinator effectiveness

In 2018 and 2020, we identified 33 flower visitors to family representing 98 flower visits (Appendix S3, Table S1). Out of the flower visitors identified to family, we further identified those that we could to genus (number of individuals in parentheses): *Apis mellifera* (5); *Bombus* spp. (Apidae) (2); *Augochlorella spp.* (Halictidae) (1); and *Junonia coenia* Hübner (Nymphalidae) (1). Out of 70 flower visits where we recorded the reward being sought, 100% (70) were observed foraging for nectar, while 2.85% (2) were observed foraging for both pollen and nectar. Excluding unvisited control flowers, we counted a mean of 149.24 ± 15.12 grains per stigma. There was a significant effect of visitor taxon on number of pollen grains per stigma ($F_{7,18} = 2.89$, $P = 0.03$, Appendix S3, Figure S3), with flowers visited by *A. mellifera* receiving an average of 198.64 ± 50.00 more grains than flowers visited by Halictid bees. However, unvisited control

stigmas had an average of 28.52 ± 7.44 grains, with no significant difference between control stigmas and stigmas of any taxon ($-0.95 < t < 0.19$, $P \geq 0.19$ in all cases, Appendix S3, Figure S3).

DISCUSSION

Pollination and nutrient resources, individually and in combination, can affect plant reproductive success (Campbell and Halama, 1993; Mattila and Kuitunen, 2003). For carnivorous plants, their interactions with insects can mediate both pollination and resource acquisition, such that plants risk trapping their pollinators, and may face reproductive tradeoffs between attracting pollinators and attracting prey. *Dionaea muscipula* rarely traps its pollinators (Youngsteadt et al., 2018), and our results confirm its reliance on insect pollinators for reproduction. Nevertheless, our experimental results also indicate that trapping pollinators would not likely reduce plant fitness. Pollinator-prey separation in this species may have arisen incidentally from separate selection pressures on flowers and traps.

Pollen and prey supplementation

We found no effect of pollen supplementation on number of seeds per fruit or on the whole-plant fitness estimate. This result differs from other pollen supplementation studies we conducted in 2017 and 2020, wherein pollen supplementation increased number of seeds per fruit by approximately 8% and 27%, respectively (Hamon et al., 2019; Hamon, 2022). Variation in pollen limitation within species is not unusual (Burd, 1994; Ashman et al., 2004; Burd et al., 2009), and can vary across years and populations, depending on environmental factors, presence of co-flowering species, fluctuations in pollinator abundance, plant population size, and other factors (Knight et al., 2005). For example, significant interannual variation in pollen limitation was observed in the rare plant *Polemonium caeruleum* L. (Polemoniaceae), possibly in part due to variation in pollinator visitation frequency and temperature (Ryniewicz et al., 2021). Our results compared to prior studies suggest that there is also interannual or geographic variation in pollen limitation in *D. muscipula*.

Though prey supplementation also did not boost number of seeds per fruit, we did observe that plants fed in 2019 or 2020 produced more flowers and fruits in 2020, which boosted whole-plant reproduction. This was somewhat surprising in the case of the 2020 plants, given that the budding stalks in this trial were relatively well-developed at the time of hand feeding, in addition to the fact that plants were only supplemented with prey two times, rather than four times as in 2019 plants. Therefore, *D. muscipula* may be able to quickly mobilize nutrients into more buds. Other studies have found that current season prey acquisition can boost investment in flowering, a relatively cheap investment compared to fruits and seeds (Hanslin and Karlsson, 1996;

Krowiak et al., 2017). For example, Krowiak et al. (2017) found that individuals of *Pinguicula vulgaris* L. (Lentulariaceae) that were given supplemental prey had a flowering probability roughly four times greater than plants with natural prey capture. Flowering is likely a major sink of prey-derived nitrogen in *D. muscipula*, with upwards of 76% of nitrogen in floral tissues sourced from current-season insect prey (Schulze et al., 2001).

Three caveats are important to consider in the interpretation of our results. First, our experiments assessed reproduction in a single year (2020), and do not account for the effect of supplemental prey or pollen on survival, growth, and reproduction in subsequent years. Reproduction in a given year may impose a cost or change in resource allocation in subsequent years (Thorén et al., 1996; Thorén and Karlsson, 1998). Flowering was also measured approximately one year after the most recent burn. Since burns can boost flowering rate (Roberts and Oosting, 1958; Luken, 2007), this study represents a 'best-case scenario' in terms of time since last burn for reproduction, and it remains unclear to what degree prey and pollen limit reproduction in plots with a less-recent burn history. In addition, the previous flowering and fruiting success of our experimental plants was not known. This is relevant because previous season fruit production may have influenced success at the time of this study (Zimmerman and Aide, 1989). Second, our experiments were designed to assess if excess pollen or prey would benefit plant reproduction. We do not know how a reduction in pollination or prey capture would affect plant reproduction. Reducing pollen and prey is more challenging to experimentally impose but would be relevant to consider for *D. muscipula* in future research given its rarity and the potential for habitat degradation to impose negative effects on important ecological processes that affect plant reproduction. Third, the fitness effect of prey supplementation was smaller for 2020 plants than 2019 plants, but given that these plants were given differing amounts of supplemental prey, it remains unclear to what degree this was due to the timing of supplementation or the quantity of prey. It is worth noting that in both trials, plants with more traps necessarily received more crickets, as is biologically relevant in a field setting. In a post-hoc analysis, the plant fitness estimate indeed scaled with the amount of prey, such that plants given more crickets had a higher fitness estimate (Appendix S4). Therefore, results should be interpreted in light of the fact that, while supplemental prey confer a clear benefit to reproduction, plant size was confounded with prey weight. However, this subtlety does not affect the conclusions of our factorial experiment where prey supplementation was analyzed as a categorical variable (fed vs. control).

Plant reproductive biology and pollen-ovule ratio

Plants that can self-pollinate are predicted to have less pollen limitation (Larson and Barrett, 2000) and reduced

PPC (Jürgens et al., 2012). We observed no significant difference in seed set between outcrossed and geitonogamously pollinated fruits, confirming that *D. muscipula* is self-compatible. However, only one out of thirty unpollinated flowers set seed. We suspect this one expanded fruit in the control ('unpollinated') treatment was due to leakiness in the pollinator-exclusion bag and is not a result of apomixis or facultative self-pollination. Though we did not emasculate unpollinated flowers, anthers flex downwards and away from the stigma during anthesis, minimizing the possibility of self-pollination within a flower in the absence of a visitor (Williams and Scholl, 2021). Therefore, it is most likely that *D. muscipula* relies on the presence of a mobile pollen vector for reproduction.

The P/O ratio provides additional clues about the reliance of *D. muscipula* on pollinators. Plants that can self-pollinate are generally predicted to have low P/O ratios (Cruden, 1977). Across all three sites, we observed an average P/O ratio of 332.5. In comparison, *Aldrovanda vesiculosa* L. (Droseraceae)—the closest relative to *D. muscipula*—has a P/O ratio of 28.5 (Okada 2008). Lower P/O ratios have also been observed in other members of Droseraceae, including 9.1 in *Drosera anglica* Huds., 18.7 in *D. linearis* Goldie, 9.0 in *D. rotundifolia* L. (Murza and Davis, 2003), and 23.9 in *D. tracyi* (Diels) Macfarl. (Wilson, 1995), a pattern which is supported by the prevalence of autogamy and cleistogamy in these taxa. If P/O ratios are indeed an overall indicator of the likelihood that a given pollen grain will help fertilize an ovule, the relatively high P/O ratio exhibited by *D. muscipula* relative to other Droseraceae supports outcrossing as a more predominant reproductive strategy for this species. Variation in P/O ratios observed between sites may point to variation in local selective pressures imposed by differences in pollinator communities and the environment (Erbar and Langlotz, 2005). For example, at Site S14, absolute numbers of pollen and ovules, as well as the P/O ratio, were lower than at other sites. Several individuals at this site were growing on a slight slope directly adjacent to a road, and drier conditions in this habitat may have limited their capacity to produce ovules and pollen grains.

Per-visit pollinator effectiveness

When comparing single-visit pollen grain deposition, *A. mellifera* outperformed Halictid bees. Several apiaries are maintained at the managed area where we measured per-visit pollinator effectiveness, resulting in a high number of floral visits from this non-native pollinator. Despite this result, our observations of pollinator behavior may provide clues about the most effective native pollinators of *D. muscipula*. Halictid bees were observed foraging for nectar and pollen, but both of these resources are presented away from the stigma. Consequently, the small sweat bees were rarely observed touching the stigma. By comparison, larger bees, such as *A. mellifera* and *Bombus* spp., tended to touch the stigma while foraging. These observations point to

the possibility that larger visitors may play an outsize role in pollinating *D. muscipula*.

It is worth noting that, despite the prevalence of beetle visitors to flowers of *D. muscipula* in prior research (Youngsteadt et al., 2018; Hamon et al., 2019), we observed no beetle visits to our experimental flowers in 2018 or 2020. Beetles were observed visiting adjacent, non-experimental flowers, so it is unlikely that the presence of the observer prevented these visits. More observation is necessary to determine the per-visit effectiveness of beetle visitors to *D. muscipula* and other plant taxa with a generalist pollination system.

CONCLUSIONS

The increase in number of flowers and fruits in prey-supplemented plants reinforces the reliance of *D. muscipula* reproduction on insect prey. Moreover, we confirmed that *D. muscipula* requires pollinators for the movement of pollen within and among plants for successful reproduction. Nevertheless, plants in this study were not pollen limited; unlike prey supplementation, pollen supplementation did not increase plant fitness. It remains unclear whether *D. muscipula* exhibits adaptations to minimize PPC, but our results suggest that occasional pollinator capture may grant an outsize benefit to fitness due to a boost in flower numbers. To better understand how pollinator and prey access affect reproductive success in this species, future studies should focus on the multi-year effects of pollen and prey limitation on plant fitness and population demographics.

AUTHOR CONTRIBUTIONS
L.E.H., E.Y., C.E.S., and R.E.I. conceived and designed the experiments. L.E.H. performed the experiments, analyzed the data, and wrote the manuscript. All authors provided edits and feedback on the manuscript.

DATA AVAILABILITY STATEMENT
All data, as well as code used to analyze pollen and prey supplementation, self-compatibility, and per-visit pollinator

effectiveness are available in the Github repository: website https://github.com/lhamon31/Hamon.et.al.2023.PollenVPrey.

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SUPPORTING INFORMATION

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

Appendix S1. Summary of experiments and plant reproductive biology by site.

Appendix S2. Prey and pollen supplementation: influence of number of experimental fruits.

Appendix S3. Supplemental results for prey and pollen supplementation experiment and pollination efficiency.

Appendix S4. Prey and pollen supplementation: cricket weights.