

Pollen Wars: Explosive Pollination Removes Pollen Deposited from Previously Visited Flowers

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ABSTRACT: Pollen grains from different plants potentially compete for ovule access because flowers produce many more pollen grains than ovules. Pollen competition could occur on pollinators, where there is finite space for pollen placement. Here, we explore the explosive pollen deposition in *Hypenia macrantha* (Lamiaceae, a perennial flowering plant native to South America that is frequently visited by hummingbirds) and determine whether it can improve male performance by reducing pollen loads deposited by previously visited flowers. Through the simulation of floral visits utilizing a hummingbird skull, we showed that explosive pollen deposition by untriggered flowers dislodges almost twice as many pollen grains as already-triggered flowers. In addition, pollen removal increases with the amount of deposited pollen by the floral explosion, suggesting that the precision or the explosive force of pollen deposition plays a pivotal role in this pollen removal process. These results suggest that explosive pollen placement, a mechanism that has evolved in many unrelated angiosperm clades, may confer a pre-pollination male competition advantage to plants.

Keywords: selection sexual, male-male competition, pollen placement, flower evolution, quantum dots.

Introduction

When female animals have multiple mates (polyandry), sperm from rival males compete for access to a limited number of eggs (Birkhead and Hunter 1990; Parker 1970). In some polyandrous animals, the first male that females copulate with may have a time advantage, and his sperm may fertilize more eggs than subsequent males (Eady 1995). This time advantage can be accentuated by using mating plugs, which prevent females from immediately mating with rival males (Wedell et al. 2010; Stockley et al. 2020). However, the females of some species can store sperm for later use, and many sperm competition studies in animals have found that males who mate last sire more offspring than the males who mated earlier (Parker 1970; Zeh and Zeh 1994; Parker and Pizzari 2010). In fact, for most insects and birds, sperm from the last mated male fertilizes the majority of eggs (Gwynne 1984; Birkhead and Hunter 1990). This can occur through two main mechanisms. In the first (stratification), sperm from the first mated male is pushed to the back of the female's sperm store by the sperm of the last mated male, giving the last mated male a reproductive advantage (Michiels and Dhondt 1988; Córdoba-Aguilar et al. 2003). In the second mechanism (sperm removal), animals

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remove sperm from the reproductive tracts of females, often by using structures on their penis that scoop or brush (Waage 1979; Córdoba-Aguilar et al. 2003).

Like animals, plants produce large numbers of male gametes that compete for access to a small number of ovules (Cruden 2000; Harder and Johnson 2023). However, unlike animals, mating is seldom direct and is usually mediated by pollinators (Murphy 1998; Delph and Ashman 2006; Ollerton et al. 2011; Tong et al. 2023). Furthermore, pollen grains (i.e., the male gamete carriers) are relatively short-lived and are not stored by plants after mating has taken place. Consequently, unlike animals, flowers of one plant are unable to manipulate pollen grains once they arrive on the stigmas of another plant, and it is unsurprising that pollen competition mechanisms like stratification and pollen removal have been poorly explored in plants (Stephenson and Bertin 1983; Moore and Pannell 2011). Instead, most pollen competition in plants has focused on competition in a postpollination context by studying differences in the germination and growth rates of pollen tubes down the styles of flowers (Minnaar et al. 2019). However, Minnaar et al. (2019) suggested that the bodies of pollinators may represent an additional arena in which pollen competition may occur, especially if space for pollen attachment is limited. They hypothesized that because flowers interact with and can potentially manipulate the pollinators' pollen loads, pollen competition on pollinators may facilitate the evolution of plant strategies that are similar to strategies of sperm manipulation in animals (also see Anderson and Minnaar 2020).

Limited attachment space may be especially prevalent in plant species that utilize a very small part of their pollinators' bodies for pollen placement and receipt. Small deposition and receipt sites may be prevalent in many plants because it is advantageous to place pollen in "safe sites" (Koch et al. 2017), where the chance of being groomed by pollen-consuming vectors is lower (Macior 1967; Thorp 2000; Tong and Huang 2018). Furthermore, small deposition and receipt sites may also increase the accuracy of pollen transfer to stigmas or even reduce the chances of improper pollen transfer (Muchhala and Potts 2007; Armbruster et al. 2009; Moreira-Hernández and Muchhala 2019; Newman and Anderson 2020). Plants could potentially compete for the small deposition sites by depositing large pollen loads that smother rival pollen from previous visits (Minnaar et al. 2019; Santana et al. 2024). This could occur in a manner similar to sperm stratification if pollen from rival flowers is deposited in layers, where the last layers prevent earlier layers from reaching the stigmas of subsequently visited flowers (Price and Waser 1982; Lertzman and Gass 1983). Moir and Anderson (2023) found evidence for such layering, and Santana et al. (2024) found that pollen from the last flower visited was better represented on stigmas

than pollen from the first flower visited, suggestive of a pollen placement effect similar to sperm stratification. However, Moir and Anderson (2023) also found evidence suggesting a "saturation effect," where pollen already on the body of the pollinator may prevent the deposition of new pollen (pollen preclusion), potentially acting in a way similar to the copulation plugs found in animals. Similarly, two other studies also found that pollinaria on insect pollinators may reduce the chances of new pollinaria from attaching (Cocucci et al. 2014; Duffy and Johnson 2014; Harder et al. 2021). It is possible that placing large pollen loads onto pollinators may enhance the pollen-smothering effect; however, large loads may not adhere to pollinators if pollen preclusion occurs (Moir and Anderson 2023). Furthermore, large pollen loads may also increase the chances of pollinator grooming or increase the chances that pollen falls off while pollinators are in flight (Harder and Thomson 1989; Harder 1990; Harder and Wilson 1997). One way that plants may respond to this is through pollen dosing, which limits the amount of pollen deposited with each visit (Thomson et al. 2000; Castellanos et al. 2006). However, Minnaar et al. (2019) suggested that, similar to sperm removal in animals, plants may also respond by removing preexisting pollen before depositing their own. They suggested that the brushlike hairs surrounding the pollen dispenser of some flowers (e.g., *Lobelia* sp., Campanulaceae) may function to clean rival pollen from pollinators before they deposit their own pollen. However, pollen removal in plants is not yet supported by any empirical evidence. In this article, we examine an interesting pollen deposition strategy in plants (explosive pollination) and provide the first empirical evidence supporting competitive pollen removal in plants.

Plants from many different families have evolved convergent mechanisms to ballistically release their pollen, such as Fabaceae (Raju and Rao 2006; Alemán et al. 2014), Lamiaceae (Harley 1971; Aluri 1990), Marantaceae (Davis 1987), Loranthaceae (Feehan 1985), and Hydrocharitaceae (Zhang 2020). In these plants, all or most of a flower's pollen is fired at the pollinator when it visits a flower for the first time. Usually, this is done by keeping floral parts under tension (e.g., Brantjes and de Vos 1981). The pollinator then releases this tension by triggering a trip mechanism upon visitation, and the release of tension rapidly catapults the pollen onto the pollinator. Explosive pollination has been recorded for plants pollinated by bees (Raju and Karyamsetty 2008; Córdoba and Cocucci 2011; Alemán et al. 2014), flies (Raju and Karyamsetty 2008), and birds (Davis 1987). It has also evolved in aquatic plants, possibly to improve the probabilities of pollen reaching the water surface (Zhang et al. 2020). In insect-pollinated plants, Li et al. (2022) were able to disable the explosive mechanism and found that explosive pollination reduced pollinator foraging times and increased pollen export. Often, the rates of

pollen release can be explained in terms of dosing, where plants with high visitation rates are expected to apply their pollen onto pollinators in small doses, while plants with low visitation rates may be expected to apply larger pollen loads (Burd 1994; Thomson et al. 2000; Ashman et al. 2004). However, the evolution of explosive pollination may have very different driving mechanisms (as described above), and some species with explosive pollination have very high visitation rates (Aluri 1990), supporting the idea that explosive pollination may be explained by the existence of other pollen deposition strategies (Minnaar et al. 2019). For example, explosive pollination may fire pollen grains deep into the hairs of the pollinator's body, making them harder to groom, making them attach better, or allowing them to

travel greater distances. Explosive pollination may also keep pollen grains safe or hidden within the flower where they cannot be collected by pollen thieves. This pollen is released, all in one go, only when the legitimate pollinators trip the flower. Finally, pollen fired ballistically at pollinators may forcibly remove rival pollen grains from the bodies of pollinators. In this article, we test whether explosive pollination removes pollen grains from the bills of hummingbirds in *Hypenia macrantha*.

Hypenia macrantha (A.St.-Hil. ex Benth.) Harley (Lamiaceae) flowers are red and tubular and visited by hummingbirds (Carstensen et al. 2016; Matias et al. 2016; fig. 1). Short- and long-billed hummingbirds of the Cerrado have been demonstrated to have very overlapping feeding niches,

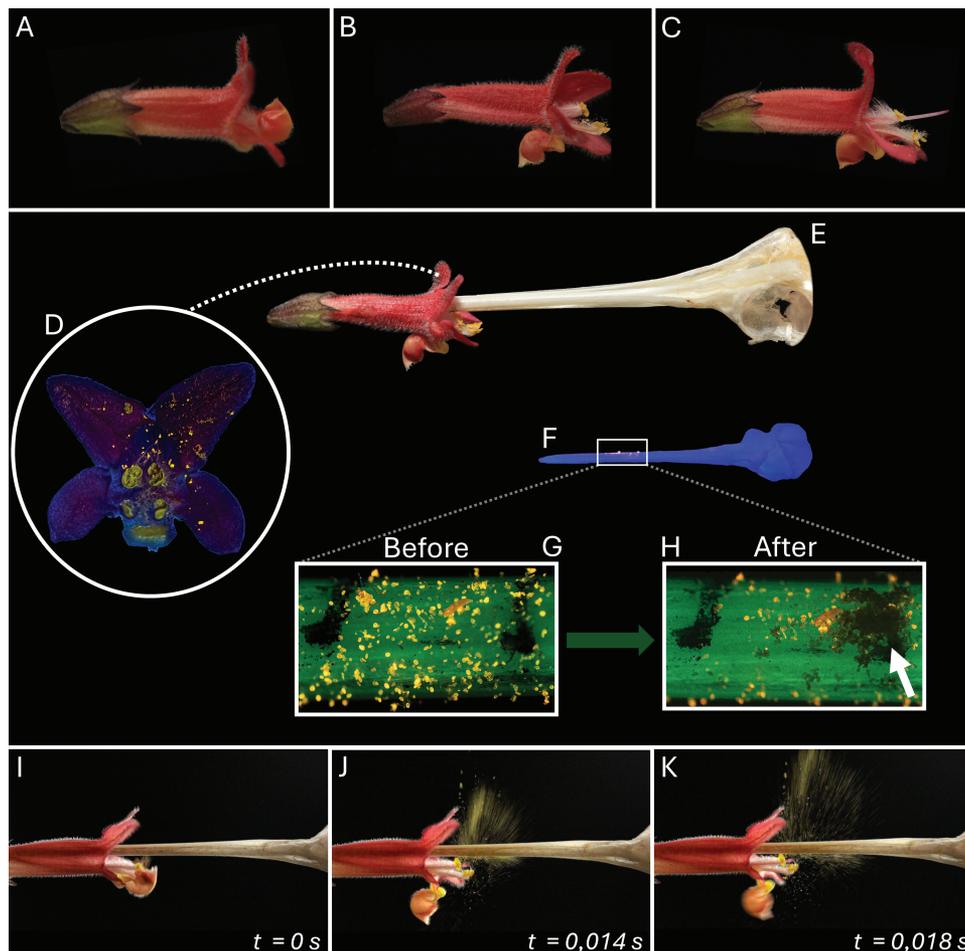


Figure 1: *Hypenia macrantha* flowers in the male phase before (A) and after (B) being triggered and in the female phase (C) evidenced by the elongated style. In field conditions, flowers are triggered when visited by hummingbirds. During the explosion, the specialized petal called carina flips back and catapults the pollen load onto the hummingbird's bill. We simulated the visits in the lab using a hummingbird skull (E) and triggered or untriggered flowers. We manually placed labeled pollen on the bill and after the explosion, counted the labeled pollen on floral structures (D) and on the hummingbird's bill (F). Specifically, we counted the number of labeled pollen grains before (G) and after (H) simulated visits in triggered and untriggered flowers. We also counted the number of unlabeled pollen grains (arrow) on the bill after the floral explosion. Three time frames (I–K) show the explosive pollen release of *H. macrantha*.

and most flowers are visited by multiple hummingbird species with varying bill morphology (Matias et al. 2016). Indeed, the corollas of *H. macrantha* (mean \pm SD: 13.36 \pm 1.2 mm; $n = 14$) are considerably shorter than the bills of most hummingbirds of the Cerrado and are unlikely to exclude many species from visiting. This may also explain the prevalence of both short-billed (e.g., *Amazilia versicolor*) and long-billed (e.g., *Eupetomena macroura*) hummingbird visitors on the flowers (B. Anderson, personal observation). In unvisited flowers, the anthers of *H. macrantha* are not visible and are covered by a keel-like petal lobe called a carina (Harley 1971; fig. 1A). Triggering by the pollinator causes the carina to flip back and bend the anther filaments backward. The carina then releases the anthers, allowing them to swing upward and catapult their pollen onto the bill of the pollinator (fig. 1B, 1I–1K; video S1, available online; Brantjes and de Vos 1981). Later, the anthers, which are on the ends of unusually hairy anther filaments, bend downward, and the style elongates past the anthers as the flower enters its female phase (fig. 1C). This explosive pollination mechanism is thought to have evolved at least twice within the Lamiaceae (Brantjes and de Vos 1981) and is also found in several bee-pollinated species with very high visitation rates (Aluri 1990; Amorim et al. 2021). Here, using a recently developed technique to label individual pollen grains, we experimentally test two ideas: (1) explosive pollination removes pollen deposited from previously visited flowers and (2) the amount of pollen removed by the explosion is positively related to the strength or accuracy of the explosion and is thus proportional to the amount of new pollen deposited. A demonstration of effective pollen removal by floral explosion would provide the first evidence that male-male competition may have contributed to the evolution of this trait.

Methods

Experiment

The experiment was conducted at the Laboratório de Morfologia, Microscopia e Imagem at Universidade Federal de Uberlândia, Uberlândia, Brazil, in March 2023. Cut stems from a flowering population of *Hypenia macrantha* were placed in plastic jars filled with water and promptly transported approximately 30 km (around 20 min) by car to the laboratory. Transportation along the road would have caused minimal damage owing to shaking. Upon arrival, all open flowers were removed to ensure that only newly developed flowers were used for the experiment on the next day, after floral anthesis. Before proceeding with the experiments, we visually checked whether the chosen flowers were properly turgid and in good condition for the experiments.

To investigate whether the explosive pollination mechanism removes previously deposited pollen grains from the hummingbird bill, we simulated floral visits to *H. macrantha* flowers by utilizing the skull and bill (length = 31 mm) of a dead long-billed hummingbird (*Helioaster squamosus*), which is commonly found in the area and frequents the open habitats in which *H. macrantha* is found (fig. 1E). Bill length of this species was recorded as 31.6 mm by Matias et al. (2016). We inserted the bill of the bird into recently opened, untriggered flowers to determine the approximate region of pollen placement on the hummingbird's bill. This region (12–17 mm from the tip) was marked with pencil. Most (75%) of the hummingbirds that could possibly occur at the study site have bills exceeding 17 mm in length (Matias et al. 2016), suggesting that these plants often place their pollen on the bills of hummingbird visitors. Unfortunately, we were unable to label pollen grains inside untriggered flowers without triggering them. Consequently, we collected pollen from *H. macrantha* flowers by triggering many flowers inside a clean Eppendorf centrifuge tube (1.5 mL). This pollen was then labeled using quantum dots (Minnaar and Anderson 2019). Pollen labeled with quantum dots fluoresces under ultraviolet (UV) light, which allowed us to distinguish labeled grains from unlabeled grains. Quantum dots also do not change the adhesive properties of pollen (Minnaar and Anderson 2019). The labeled pollen was manually applied in a thin layer within the penciled region on the bird bill using a small brush (fig. 1F) so that the total number of labeled grains could be quantified using a stereomicroscope coupled with a UV excitation chamber (fig. 1G; Minnaar and Anderson 2019). Subsequently, the hummingbird bill with labeled pollen grains was inserted into the floral tubes of either a triggered flower ($n = 16$) or an untriggered flower ($n = 14$). After the simulated visits, we counted the remaining labeled pollen grains as well as the total number of unlabeled pollen grains that had been placed on the marked region of the hummingbird bill (fig. 1H). Pollen grains were cleaned off of bills between trials using a swab embedded in 70% ethanol, and new labeled grains were applied at the start of each trial.

Statistical Analyses

We compared the number of labeled pollen grains on the hummingbird bill before and after insertions into triggered versus untriggered flowers using a generalized linear mixed model. In this case, our response variable was the total number of labeled pollen grains, while the predictors were the time when pollen was counted (before or after the flower visit), the phase of the visited flower (triggered or untriggered), and the interaction between these two variables. Flower identity was used as a random factor,

enabling pairwise comparisons of labeled pollen loads before versus after simulated visits to the flower. For this analysis, we assumed a Poisson distribution of the response variable with log transformation. A significant interaction term would indicate a difference in the pollen removal rate (number of grains before vs. after visitation) by triggered or untriggered flowers.

We also fitted a generalized linear model using a beta distribution with a logit link to test the relation between the proportion of labeled pollen grains removed and the number of pollen grains deposited by untriggered flowers ($n = 14$) on the hummingbird bill. Data from triggered flowers were not used in this analysis, as we specifically wanted to determine whether the number of pollen grains deposited was proportional to the number removed. Here, we ln transformed the predictor to achieve better residual dispersion.

All the statistical analyses were run in R environment version 4.3.1 (R Development Core Team 2023) using default packages and the following: glmmTMB (Brooks et al. 2017), emmeans (Russell 2022), and car (Fox and Weisberg 2019). Residual dispersion around the fitted models was checked using the DHARMA package (Hartig 2022).

Results

Explosive pollination removed pollen previously placed on the hummingbird's bill (see video S1; fig. 2). Although experimental visits to both triggered and untriggered flowers decreased the number of labeled pollen grains on the bill of the bird, the decrease was greater after experimental visits to untriggered flowers than to triggered flowers (fig. 2; table S1; tables S1–S3 are available online; χ^2 interaction = 15.76, $df = 1$, $P < .001$). This suggests that the explosive mechanism removed additional pollen. On the basis of the estimated marginal means, untriggered flowers removed 1.93 times more pollen grains than previously triggered flowers (table S2). Furthermore, the proportion of removed pollen grains is positively related to the number of pollen grains placed by the explosive mechanism (fig. 3; table S3; estimate = 0.35, $z = 2.94$, $P < .01$).

Discussion

Although experimental visits to triggered flowers remove pollen from the bill of the hummingbird, visits to explosive, untriggered flowers remove even more pollen. Pollen is launched at the bill with what appears to be a considerable force. In fact, slow-motion video footage reveals that pollen travels at approximately 2.62 m/s after it is released (see “Calculation of pollen velocity” in the supplemental

PDF). Furthermore, pollen clumps on the bill can clearly be seen being dislodged by the catapulted pollen (video S1). The difference in pollen removal between triggered flowers and untriggered flowers makes it clear that explosive pollination on its own accounts for significant amounts of pollen being removed from the pollen load residing on the bird's bill. This is also supported by the observation that the more explosively deposited pollen within the focal area of the bill, the more preexisting pollen was removed from that area, suggesting that explosion accuracy may also have a role to play in the removal of preexisting pollen. Slow-motion video footage also suggests that it is primarily the force of the pollen hitting the pollen loads and bill that removes the residing pollen rather than the rapidly moving carina or the movement of the hairs on the anther filaments. It is also not clear how much pollen is removed by these explosions, as our experiments placed only very small loads of pollen in a single layer. However, slow-motion video footage suggests that if pollen loads were higher and clumped (as expected in nature), large quantities may be removed by explosive pollen.

Removal of preexisting pollen from pollinators is likely to homogenize the identity of pollen loads and increase the relative proportion of pollen from the last flower visited. This should give pollen from the last flower a siring advantage if the next flower visited is in the female phase. Consequently, visits to multiple male flowers on the same plant could negatively affect male fitness by removing “own pollen” but simultaneously provide a positive effect of purifying the pollen loads so that most bill pollen is from the last plant visited. It is unclear at this stage what flower display sizes and ratios of male- to female-phase flowers would be optimal for these kinds of competitive strategies. *Hypenia macrantha* produces small floral displays. We typically found only about three or four untriggered flowers per plant (B. Anderson, personal observation), suggesting that in this case, explosions may remove only a little bit of “own pollen” from pollinators' pollen loads.

Explosive pollen removal may also result in intersexual conflict if the presence of male flowers on a plant reduces the chances of outcross pollen reaching the stigmas of female flowers on the same plant. This conflict is likely to increase with the size of the male display relative to the female display. However, some intersexual conflict is mitigated by the fact that there is no explosive pollen release when flowers are in the female phase. Unlike hermaphroditic plants, the evolution of sperm removal strategies in animals is not constrained by this intersexual conflict because animals usually have separate sexes. However, intersexual conflict in plants could be mitigated if flowers are visited sequentially (females first and males last), as has been demonstrated for upright inflorescences (Harder et al. 2000; Zhu et al. 2020). This is unlikely the case in *H.*

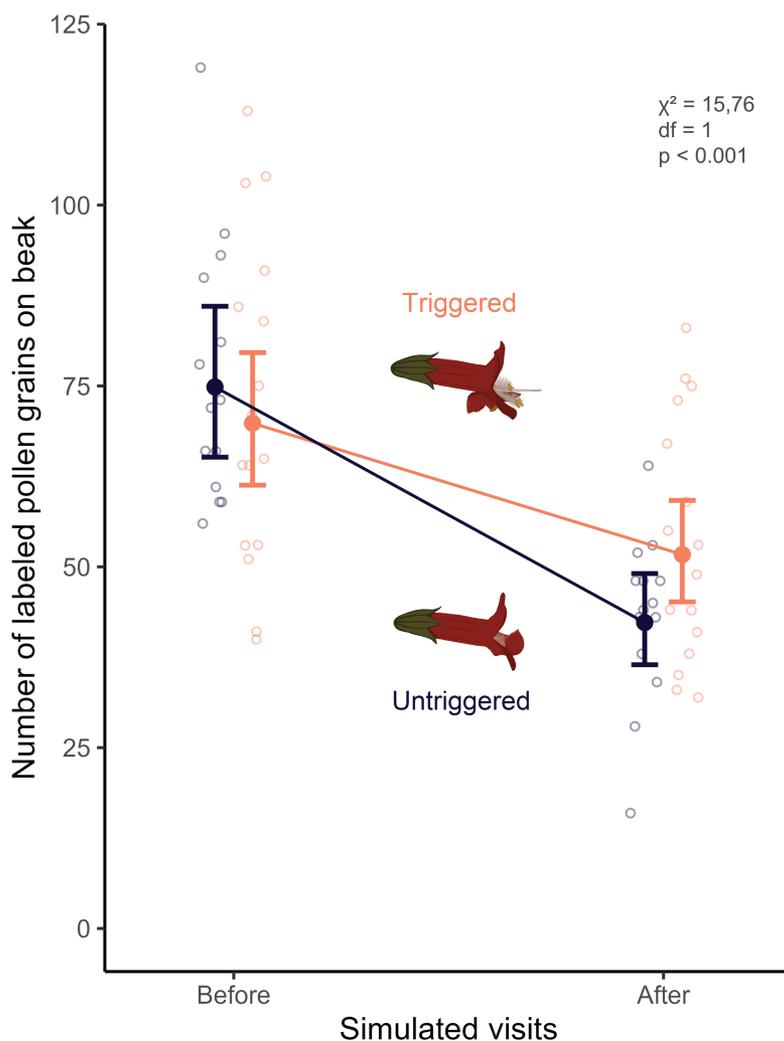


Figure 2: The floral explosive mechanism in *Hypania macrantha* reduces the number of previously placed pollen grains on the bill. In lab conditions, we simulated visits by a hummingbird's bill loaded with labeled pollen grains in triggered and untriggered flowers. We counted the number of labeled pollen grains before and after visits. In general, floral visits remove pollen grains previously placed on a hummingbird's bill, but the effect is greater when the flower was untriggered, evidencing that explosive pollination removes pollen deposited from previously visited flowers.

macrantha, which does not have a regular upright display and untriggered, male-phase flowers appear to be found interspersed throughout the display (B. Anderson, personal observation). That said, plants typically have many more female-phase flowers than male-phase flowers (B. Anderson, personal observation), and consequently, it is likely that a pollinator will visit a female flower before visiting a male flower. The magnitude of intersexual conflict is also likely to depend on how pollen-limited flowers are, how reliant they are on outcross pollen for seed set, and what the chances are of receiving outcross pollen in spite of pollen removal. We know nothing about self-compatibility or pollen limitation in *H. macrantha*, although other explosive flowers in this family appear to be extremely self-

compatible and set prodigious amounts of seed (Aluri 1990; Amorim et al. 2021). If plants are highly self-compatible, intersexual conflict can be minimized.

To put these pollen speeds into perspective, many animals are able to move much faster than the 2.62 m/s recorded here (Usain Bolt runs approximately four times faster at 10.44 m/s). However, these movements are still fast for plants and are orders of magnitude faster than the speed of a Venus flytrap closing (Forterre et al. 2005). While they rank among the fastest plant movements recorded (the second fastest are the pollen catapult mechanisms of bunchberry dogwoods, 7 m/s; Edwards et al. 2005), they are far slower than the pollen catapult mechanisms found in white mulberries, which can exceed 170 m/s (Taylor et al.

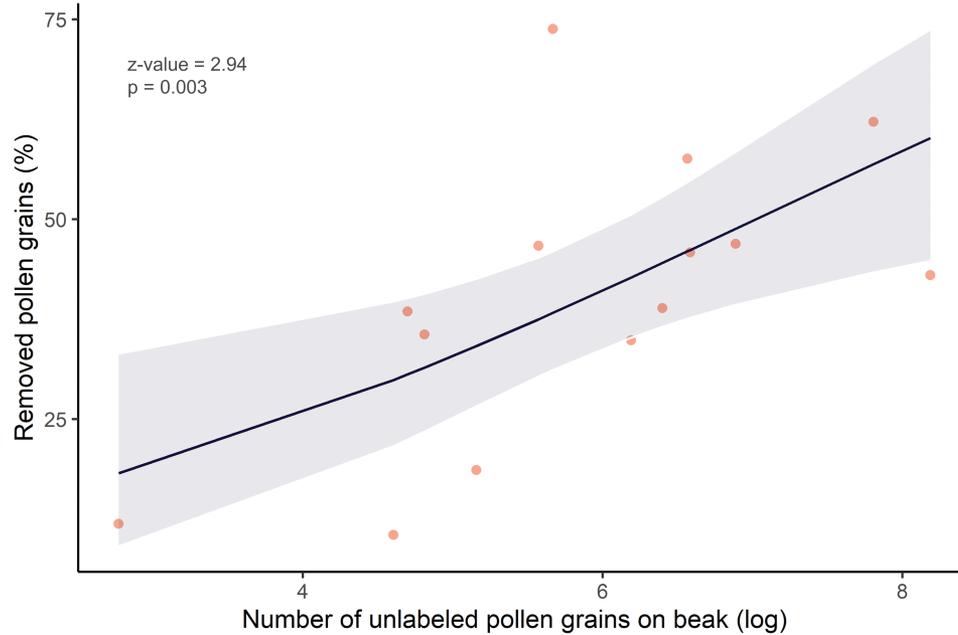


Figure 3: Pollen removal by *Hyponia macrantha* flowers is positively related to the number of pollen grains deposited by the explosion. In controlled experiments, we simulated hummingbird visits to untriggered flowers and counted the number of labeled pollen grains before and after visits and number of unlabeled pollen grains placed by the last visited flower. The proportion of removed pollen grains scales with the number of pollen grains deposited, evidencing the effect of pollen projectiles launched by the last visited. We ln transformed the predictor to achieve better residual dispersion. Points represent real values, while the line is estimated according to the predicted values. The shaded area represents the 95% confidence interval around the model.

2006). In fact, the catapults of white mulberries exhibit the fastest known movements in the plant or animal kingdom.

Conclusion

While there are potentially many competing hypotheses that could explain the evolution of explosive pollination (outlined in the introduction), this article explores a single hypothesis (pollen removal). Consequently, although we can say that explosive pollen removes existing pollen from the bird, we cannot say whether this was the primary selective pressure explaining its evolution (Gould and Lewontin 1979). Nevertheless, this is the first article to demonstrate proof of concept: that explosive pollination could be selected through pollen competition, in a way similar to the evolution of sperm removal in animals. This article suggests the possibility that explosive pollination can evolve solely as a pollen removal strategy; however, it could also evolve as a contributing selective pressure in conjunction with other selective pressures. Most importantly, these results force us to think a little differently about plants and recognize that they may be competing with one another in previously unimagined ways (Anderson and Minnaar 2020). However, it is also important to recognize that the

conditions under which pollen competition can drive pollen removal strategies in plants are likely to be more limited than in animals because of the complexities resulting from hermaphroditism and large display size (discussed above). We also show that it is possible to learn much about the mechanics of flowers through simulated approaches like this, as evidenced by Darwin, who predicted the existence of an extraordinary long-tongued hawkmoth after tinkering with the Madagascan star orchid using needles, bristles, and cylindrical rods as probes (Darwin 1862).

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Statement of Authorship

B.A. conceived the study, collected the data, designed the analysis, and wrote the manuscript. V.L.G.d.B. collected the data, designed and performed the analysis, and wrote the manuscript. C.A.A. collected the data and designed and performed the analysis. All authors collected the data; prepared figures, tables, and video; discussed the results; and gave input on the manuscript.

Data and Code Availability

Data and code used in this study are available at Zenodo (<https://doi.org/10.5281/zenodo.12789942>; Brito 2024).

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“Still another very common and at the same time a very curious plant is the *Fouquieria splendens*.... It grows all over the deserts of Arizona and among the rocks on the mountains.... The flowers are of a bright scarlet, and form racemes at the end of the branches.” From “Botanical Notes from Tucson” by Jos. F. James (*The American Naturalist*, 1881, 15:978–987).