Competition for pollen deposition space on pollinators generates last-male advantage

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Abstract

Many plants have precise pollen placement strategies so that large amounts of pollen can be found over very small and discrete areas located on pollinators. This may lead to male-male competition if pre-existing pollen (1) is smothered or displaced by pollen from subsequent male flowers or (2) prevents subsequent pollen from attaching to pollinators. We investigated these alternative hypotheses using caged sunbirds (*Cinnyris chalybeus*) and sunbird-pollinated flowers (*Tritoniopsis antholyza*). We labelled pollen from two different flowers with quantum dots so that their pollen grains could be distinguished. We offered these two male-phase flowers in succession to sunbirds before they were allowed to visit a female-phase flower. In a separate trial, we offered sunbirds a quantum-dot-labelled flower followed by a flower without reproductive structures. This trial established whether discernable amounts of pollen were being lost during the trials due to a “time effect” (over time, pollen falls off, or is groomed from the pollinator). We found that pollen from the second male flower was better represented on the stigmas of the subsequently visited female flowers and that this advantage was not due to a time effect (i.e. less time for the pollen from the last male to fall off the pollinator). Instead, it suggests that pollen from earlier-visited flowers is smothered or displaced by subsequently visited flowers. Because the last male visited may have a reproductive advantage (similar to last-male sperm precedence in animals), plants are likely to evolve strategies to both capitalize on this advantage but also to combat it.

Keywords: male fitness; pollen movement; pollen precedence; pollen smothering; sexual selection
Resumo (in Portuguese)

Muitas plantas possuem estratégias precisas de deposição de grãos de pólen que, como consequência, geram pequenas áreas cobertas com grandes quantidades de pólen no corpo dos polinizadores. Se (1) grãos de pólen pré-existentes no polinizador são cobertos ou empurrados pelas novas flores visitadas, ou (2) se estes grãos de pólen acabam por impedir que mais grãos de pólen sejam depositados no corpo dos polinizadores, espera-se que haja competição entre a função masculina dos indivíduos. Nós investigamos estas hipóteses alternativas através de experimentos conduzidos com pássaros da família Nectariniidae (*Cinnyris chalybeus*) que atuam como polinizadores das flores de *Tritoniopsis antholyza*. Para realizar esse experimento, nós utilizamos nanopartículas fluorescentes que, aderidas aos grãos de pólen, permitem distinguí-los através da emissão de diferentes cores. Nós oferecemos sequencialmente duas flores em fase masculina contendo grãos de pólen marcados com diferentes cores para os pássaros polinizadores; em seguida oferecemos uma flor na fase feminina. Adicionalmente, nós oferecemos uma flor em fase masculina com o grãos de pólen marcados, e em seguida oferecemos uma flor sem estruturas reprodutivas, de maneira que pudemos comparar o efeito de perca de pólen no corpo do polinizador devido ao tempo com a ausência de aderência de grão de pólen devido a pólen pré-existente. Através de amostragem dos estigmas das flores femininas, pudemos verificar a quantidade de grão de pólen que havia sido exportada e comparar se havia vantagem em ser o primeiro ou o último macho a ser visitado. Nós encontramos que pólen da segunda flor em fase masculina apresentam maior probabilidade de serem encontrados no estigma da flor masculina e essa vantagem não era por conta do tempo. Nossos resultados indicam que através de sobreposição ou de deslocamento, a última flor visitada é capaz de alterar o sucesso da primeira em atingir o estigma da próxima flor visitada. Tal reprodução diferenciada leva a uma vantagem competitiva da última flor em fase masculina visitada, similar a precedência espermática em animais.
**Introduction**

Pollen movement plays a key role in plant reproduction and flower evolution (Moreira-Hernández & Muchhala, 2019; Opedal et al., 2023). Given that most plants are hermaphroditic, pollen movement affects individual fitness via both the female pathway (seeds produced by pollen receipt on stigma) and the male pathway (seeds sired due to pollen export to other plants’ stigma; Alexander & Tinkle, 1981; Morgan, 1994). Male contributions to total fitness are usually constrained by mating opportunities, while female reproductive success is typically constrained by resource access (Bateman, 1948). As flowers typically produce many more pollen grains than ovules, the male fitness pathway can potentially make higher contributions to the total fitness of an individual (Cruden, 1977; Gong & Huang, 2014). The imbalance between potential male and potential female contributions to total fitness can lead to higher variability in male (Minnaar et al., 2019) than in female reproductive success (Tonnabel et al., 2019), and sets the stage for sexual selection in plants (Janzen, 1977; Willson, 1979).

Sexual selection in plants is most likely to occur through male competition to fertilize the available pool of ovules (Moore & Pannell, 2011). Indeed, Paterno et al., (2020) found evidence suggesting that traits involved in increasing pollen export (e.g. attractive traits) have evolved mainly through the male fitness pathway (Stanton et al., 1986). Despite the potential importance of the male fitness pathway in flower evolution, the mechanisms of competition between male gametes and how they promote siring success remains a challenge, since tracking the siring success of pollen grains is especially difficult. Additionally, plants potentially have several mates, which increases the challenge to understand the contribution of intrasexual competition to floral trait evolution (Christopher et al., 2019; Karron et al., 2006). This is made even more difficult by the fact that for most angiosperms, gametes are carried by another organism (Ollerton et al., 2011; Tong et al., 2023), adding the bodies of pollinators as another
potential arena for male-male competition to occur (Stanton, 1994; Stephenson & Bertin, 1983). The current work contributes to the understanding of how sequential fates of pollen deposition might facilitate intraspecific competitiveness (i.e. evolution in order to enhance the likelihood of succeed on the competitive process of achieving a stigma) between mates on pollinator bodies (Thomson, 2014).

Male competitiveness may be generated by floral strategies that affect pollen distributions on pollinator bodies (Castellanos et al., 2006; Harder & Johnson, 2008; Harder & Thomson, 1989; Harder & Wilson, 1994, 1998). Plants display different strategies of placing pollen on pollinators (e.g. diffuse, stamp, etc), which are likely to generate distinct pollen landscapes (Minnaar, Anderson, et al., 2019a). Pollinator bodies may represent arenas for male-male competition, where males compete for placement sites on the pollinator that maximize pollen export and access to interindividual ovules of subsequently visited female-phase flowers (Anderson & Minnaar, 2020). Limited space on pollinator bodies may facilitate three-dimensional, layered pollen landscapes (Armbruster et al., 2009; Moir & Anderson, 2023), which could result in male-male gamete competition even before pollen has been deposited on another stigma (Minnaar & Anderson, 2021). Muchhala & Thomson, (2012) demonstrated that plants of different species compete for space on pollinator bodies and that different species can displace or smother granular pollen from previous visits. Within-species (i.e. intraspecific) competition between pollen grains may be equally or even more intense because there is likely to be more overlap of pollen placement sites between plants of the same species than there would be between plants of different species (Simón-Porcar et al., 2024). While some studies have theorized how such pollen landscapes may affect gene flow (Harder & Wilson, 1998; Marcelo et al., 2022), recent empirical evidence suggests that successive pollen layering promotes interference competition between individual plants (Moir & Anderson, 2023). This
interference may occur through smothering, displacement, or preclusion of pollen grains from other individuals (Minnaar et al., 2019).

Pollen preclusion may occur when pre-existing pollen loads prevent or preclude the deposition of new pollen grains onto pollinators (Figure 1a, Moir and Anderson, 2023). In contrast, pollen smothering or displacement may occur when flowers are able to cover or displace pre-existing pollen on pollinators from previous floral visits, so that their own pollen has a higher probability of reaching the stigma of subsequently visited flowers (Figure 1b, Minnaar et al., 2019). There are a few examples of pollen smothering and preclusion in the Asclepiadaceae and Orchidaceae families, where plants package their pollen in pollinaria (Cocucci et al., 2014; Duffy & Johnson, 2014; Harder et al., 2021). Cocucci et al., (2014) found evidence for both smothering and preclusion in milkweeds: some species possess pollinaria with horns that prevent the attachment of pollinaria from subsequently visited plants; while other species possess pollinaria which attach to and smother pollinaria previously placed on pollinators (Cocucci et al., 2014). However, to the best of our knowledge, only one study has documented pollen preclusion in plants with granular pollen: Moir and Anderson (2023) established the first evidence for pollen layering in flies visiting Moraea lurida (Iridaceae - Moir et al., 2022) and found that earlier-visited plants appeared to deposit more pollen on pollinators than subsequently visited plants, suggestive of pollen preclusion. However, the effects of smothering and pollen preclusion in plants with granular pollen have not been investigated beyond the stages of pollen deposition onto pollinators and it is unclear how this translates to pollen export onto stigmas.

Our study sought to investigate pollen competition by Tritoniopsis antholyza (Poir.) Goldblatt (Iridaceae) plants when they are visited by sunbird pollinators (i.e. Cinnyris chalybeus). We tested two alternative hypotheses (Figure 1): a) pollen preclusion – where pollen from the first-visited male is expected to have a higher probability of being deposited
on the stigma of a subsequently visited female flower; b) pollen smothering or displacement – where pollen from the last male visited has a higher probability of deposition on the stigma of the next female-phase flower. We found evidence supporting pollen smothering/displacement by tracking pollen deposition success of different flowers after their pollen was labelled with different color Quantum dots (Minnaar & Anderson, 2019). Our experiment did not attempt to distinguish between the two different mechanisms (pollen smothering versus pollen displacement) which may give rise to a last male advantage.

Figure 1: Two alternative male-male competition hypotheses and predicted pollen landscapes. When pollen from successively visited flowers (starting sequentially with flowers on plant 1, then 2 and lastly 3) are deposited on the pollinator body, it can result in different kinds of pollen landscapes which affect male success differently: a) first male advantage resulting from pollen preclusion – where pollen from the first flower precludes pollen placement by subsequently visited flowers. The higher pollen loads of the first-visited should result in better pollen export than later-visited flowers; b) last male advantage resulting from pollen smothering or displacement – where pollen from the last flower smothers or displaces previously deposited pollen. This should result in better pollen export for the last flowers visited by the pollinator.

Materials and methods

Study area and focal species

We conducted this study in the Fynbos biome from October to December (2021) on two private properties (with landowner permission) in Betty’s Bay and in Stellenbosch (Western Cape, South Africa), complying with national regulations (see Acknowledgements;
CapeNature permit number CN41-28-16214 and SAFRING ringer number 1622). Our focal flowering species was *Tritoniopsis antholyza* (Iridaceae), a fynbos-endemic, summer-flowering plant. *T. antholyza* presents hermaphroditic, protandrous flowers, spirally arranged along the inflorescence which matures sequentially from the bottom upwards (Manning & Goldblatt, 2005) (Figure 2a). Flowers first open in male-phase, with three backward-reflexing stamens emerging on the second day (Figure S1). At this time, anthers are positioned beneath the upper tepal (Figure 2b). The style then gradually elongates, and the anthers reflex backwards as the flower transitions into the female-phase (Figure 2c).

\[\text{Figure 2: } \text{Tritoniopsis antholyza inflorescence, showing: a) flowers spirally arranged, and maturing so that, b) male-phase flowers are on the top; and c) female-phase flowers are at the bottom.}\]

Sunbird pollinated flowers in the Cape Floral Region of South Africa typically confirm to one of two guilds: short tubed flowers pollinated by two functionally analogous short billed sunbird species; or long tubed flowers pollinated by long billed malachite sunbirds (Geerts & Pauw, 2009). *Tritoniopsis antholyza* tube lengths fall within the short tubed guild and are typically visited by southern double collared and orange breasted sunbirds (Newman et al.,
2014; B.A. Pers. Obs). Typically, *T. antholyza* deposits pollen on the probing sunbird’s forehead/crown (Manning & Goldblatt, 2005), allowing pollen accumulation on an area not readily groomed while the birds feed. These birds usually visit several flowers on the inflorescence and probe them directly from the front while perching below them on the stem (Goldblatt et al., 1999; Goldblatt & Manning, 2006; Manning & Goldblatt, 2005; Newman et al., 2014; BA Pers. Obs).

We selected the southern double collared sunbird, *Cinnyris chalybeus* (family Nectariniidae) as the focal pollinator due to its abundance at the sites where *T. antholyza* was collected. We captured *C. chalybeus* using mist-nets (16 × 16 mm mesh) during early mornings and late afternoons under non-inclement weather, with open nets monitored every 20 minutes. All captured birds were identified and banded, with by-catch species released immediately thereafter. *Cinnyris chalybeus* individuals which did not possess brood patches (indicative of breeding), which were not in moult (which would interfere with pollen deposition), and which were not fledglings (still in need of parental care) were kept captive for experiments. These individuals were placed into separate birdcages (80/100x60x60 cm), with no more than two birds retained concurrently. To minimize physiological stress, the cages were blanketed, placed in warm, sheltered environments, and oriented to prevent visual distraction between sunbirds. Cages were also provisioned perching branches, water baths and 20% w/w sucrose solutions with Ensure® nutritional supplements (Abbot Laboratories, South Africa) to promote avian health (Fleming et al., 2004; Lerch-Henning & Nicolson, 2013). The sucrose solutions were placed in Eppendorf tubes modified to secure a *T. antholyza* flower through a hole in the lid, such that each sunbird could only access the solution by probing the flower corolla; these solutions were replenished throughout the day (Figure S2). An acclimation period of 3-8 hours was employed for each sunbird before commencing experimental trials. Trials lasted 2-4 days,
after which sunbirds were released on site of capture, with the contingency of premature release if notable stress was apparent after acclimation.

*Male-phase flower presentation experiments*

To investigate the potential for male-male interference competition on pollinator bodies, we conducted 43 pairwise trials (treatment and control), in which a sunbird was allowed to visit two sequences of three flowers (detailed below). We used Quantum dots (Qdots) to label the pollen grains in all three newly dehisced anthers of male-phase flowers (*see Minnaar and Anderson, 2019* for detailed quantum dot protocols), enabling us to distinguish pollen from different flowers. Approximately 55 µl of Qdot solution was required to visibly saturate all the anthers from a single flower. We used three colors of Qdot solution (green – 523 nm, yellow 590 nm, red – 628 nm) to label the flowers and differentiate the pollen grains from different flowers. All flowers were collected in bud and allowed to open under lab conditions before being used in the experiment. For the male-phase flowers, only those with all their anthers dehisced were used in the experiments. We removed the anthers from the female-phase flowers, ensuring that there could be no interference from male reproductive structures at this stage of the experiment.

Each experimental trial comprised the following steps (illustrated in Figure 3): (1) place an initial Qdot-labelled male-phase flower within an Eppendorf containing sucrose solution, and present it to the sunbird; (2) allow the sunbird to probe the first male-phase flower three times; (3) replace the first male-phase flower with second Qdot-labelled ‘rival’ male-phase flower (*i.e.* distinct individual); (4) allow the sunbird to probe the second male-phase flower three times; (5) replace the second male-phase flower with a female-phase flower; (6) allow the sunbird to probe the female-phase flower three times; (7) end the trial by collecting the female-phase flower and replacing it with a non-trial flower (described below) to allow
continued sunbird feeding. After these steps, the stigma from the female-phase flower was examined under a M125 Stereo dissecting microscope (Leica, Germany) using a Qdot excitation box to compare the pollen grain quantities from first versus second male-phase flowers (Minnaar & Anderson, 2019).

From this, we were able to determine whether the first male or the second male flower was more successful at exporting pollen to the stigma of the third flower. However, an advantage to the second male could occur just because pollen grains from the second male have less time to fall off from the pollinator. To control for the time-effect, we paired each experimental trial with a control trial which allowed us to distinguish between a possible time-effect and a competition-effect (Figure 3). The control trial modified steps 3-4, so that the second male phase flower was substituted for a flower without reproductive structures (Figure 3). Pairwise experimental and control trials for each bird were conducted consecutively (randomly ordered) with 30-minute intervals between them. The non-trial flowers that were introduced after each set of pairwise trails had their reproductive structures removed and replaced with clear adhesive attached to the upper tepal to remove pollen residue off the sunbird’s head. We spaced paired trials at least one-hour apart to minimize cross-contamination of pollen grains. Across paired trials, Qdot colors were also randomized for the first and second male-phase flowers to remove any potential effects of Qdot color variation. We performed the experiments during the day, presenting on average three pairwise treatments per day per bird.

In total, we conducted 86 experimental trials (43 controls and 43 treatments), using eight birds.

Table 1 - Replication Statement: i) inferences were made at the scale of individuals, as we investigated intra-sexual competition occurring on one sunbird pollinated plant species. ii) our treatment was applied at the experimental unit scale; iii) we did 43 pairwise experiment trials (86 in total), using three flowers for each (258 in total), and with 8 birds, in total. We included birds as a random factor in the models and used the same bird for pairwise control and treatment trials.
Scale of inference | Scale at which the factor of interest is applied | number of replicates at the specific scale
---|---|---
Individual | Experimental trial | 86 experimental trials (43 controls paired with 43 treatments)
Individual | Plants | 258 flowers (as proxy of individual plants) used in total (3 for each experimental trial)
Individual | Birds | 8 birds

Figure 3: Experimental setup and hypothetical differences in stigmatic pollen representation to distinguish between time effects, last and first male advantage. Control trials consisted of probes to a Qdot labeled male-phase flower, followed by a flower without reproductive structures, followed by a female-phase flower. Experimental treatment trials consisted of successive visits to two male-phase flowers (labelled with different color Qdots), followed by a female-phase flower. When interpreting the results, we first need to determine whether the first male has the advantage (panel 1) or whether the second male has the advantage (panel 2). If pollen from the first male is better represented on the stigma, it suggests a first male advantage resulting from pollen preclusion, where pollen from the first male prevents pollen from the second male from adhering to the pollinator. Such a pattern cannot be explained by a time effect. If pollen from the second male is better represented on the stigma, it suggests a second male advantage. Such an advantage could result because the second male gains a competitive edge from smothering or displacing pollen from the first male, but it could also arise because there is less time for pollen from the second male to fall off the pollinator. If the first control male is as successful as the second male, there is no detectable effect of time, suggesting that the second male advantage can completely be explained by competition. But if the first control is less successful than the second male, and equally successful to the first male of the treatment trial, it suggests that the time effect on its own may explain the success of the second male. Between these two points is a zone where a combination of both time and competition may play a role in giving the second male an advantage.

Data analyses
We tested alternative hypotheses by comparing patterns of stigmatic pollen deposition from our control trials versus treatment trials. For our first hypothesis (i.e., first male advantage, resulting from pollen preclusion), we expected the first male flower to have greater pollen deposition success than the second male flower. In this first scenario, we predict a positive relationship between sequence position and pollen grains exported. Since there are no other plausible explanations for this pattern, the success of the control male is not important when interpreting this difference. For our second hypothesis (i.e., last male advantage), the second flower has greater pollen deposition success than the first flower. In this second scenario, we predict a negative relationship between sequence position and pollen grains exported.

Our control allowed us to disentangle whether any of the patterns described above could have been due to differences in the amount of time pollen from the first versus the second male had spent on the pollinator. Pollen from the second male-phase flower would be better represented on stigmas than pollen from the first male-phase flower, because pollen from the first flower would have had more time to fall off the bird. By adding a control trial, we were able to distinguish whether the second male advantage was the result of a time effect and a competitive effect (Figure 3). Therefore, besides our control does not withdraw time effect from the probability of a pollinator carrying a pollen grain, it establishes a standard of comparison from what would be expected due to time effect.

To distinguish between the effects outlined in figure 3, we counted the number of pollen grains deposited by each of the male-phase flowers onto the stigma of the female-phase flower as they differed in color. In twelve of the 43 replicates (~27%), there were no pollen grains deposited on the stigma. Therefore, we first looked at the probability of pollen placement on the stigma by analyzing the data as presence or absence of pollen grains for each male (a binomial perspective). We used generalized linear mixed-effects binomial models with a logit
link function and Laplace maximum likelihood approximation of theta, in which the success
of deposition was the response variable (i.e., 0 for no deposition; 1 for success deposition),
dependent on sequence position and each treatment (1st or 2nd male Treatment or 1st male
Control). This structure allowed us to make all comparisons between males, even between male
treatment and male control. We set up models to compared the effect of the sequence position
with different random factor combinations: a) the identity of the bird or b) the identity of the
bird nesting the experiment trial. We compare these models with a null model using the Akaike
Information Criterion (AIC) and select the model that best fit our data. We considered equally
plausible models the ones with a ΔAIC lower than 2 (Table 2i).

We also modeled the total amount of pollen grains deposited on the stigma by each
male. In our experiment, the number of pollen grains deposited on the stigma was dependent
on two steps: 1) pollen was transferred from anthers to bird (yes or no), 2) if pollen was
transferred to the bird, was it then transferred from bird to stigma (yes or no). The two steps
may generate a combined probability distribution, where there is a zero-response probability
(pollen deposited or not) and a count portion probability (the magnitude of the variable -
number of pollen grains transferred from anthers to the bird and number of pollen grains
deposited to the stigma). Even though we could not count how many pollen grains were
deposited on the bird, our result is a consequence of both processes. Therefore, to investigate
the differences in the number of pollen grains deposited by each male, we compared models
using negative binomial, hurdle (composed by a zero portion and a truncated count portion -
poisson or negative binomial) and zero-inflated (composed by a zero portion and an
untruncated portion - poisson and negative binomial) probability distribution (Table 2ii).
Hurdle and zero-inflated types of models split the response variable into two latent variables
and account for more than one process generating the failure of pollen export to the stigma.
Similarly to the previous analysis, we included the sequence of deposition as the predictor
variable and tested different combinations of random factor (Table 2ii) against the null model.

Again, we selected the model that best fitted our data based on Akaike Information Criterion (AIC) and considered equally plausible models which had a ΔAIC lower than 2 (Table 2ii).

After selecting the model that best fitted the data, we compared the pairwise factor combinations using Marginal Means through *emmeans* (v1.5.5-1 Lenth, R.V. 2021) and *phia* (Rosario-Martínez, H. 2015 v0.2.1) packages, by computing contrasts of Estimated Marginal Means (EMMs) between the levels of fixed factors. The confidence level adjustment was conducted with the Tukey method for comparing a family of three estimates and we back-transformed from the log scale to obtain the estimates. We present the estimates for 95% confidence intervals.

All analyses were done in R (R version 4.4, Core Team, 2022), using the main packages *lme4* for linear models (Bates et al., 2015), *glmmTMB* (Brooks et al, 2017), *DHARMa* (Hartig, 2016), *bbmle* (Bolker, R Development Core Team, 2023), *performance* (Lüdecke, 2021), *ggeffects* (Lüdecke, 2018), *MASS* (Venables and Ripley, 2015), *pscl* (Jackman, 2015), *AER* (Kleiber and Zeileis, 2022). The complete list of packages, together with the code and data will be available at Github upon acceptance.

**Results**

Pollen transfer was highly variable (varying from 0 to 150 pollen grains). Pollen transfer was often ineffective and when we found pollen on stigmas, the mean number of pollen grains was low (Treatment mean: 0.70, Control mean: 3.7 grains), while the variance was high (Treatment variance: 3.31, Control variance: 61.04 grains).
Figure 4: Probability of stigmatic pollen deposition and number of pollen grains on stigma for both control and treatment trials. Letters show statistically significant differences among treatments according to Tukey method for pairwise comparisons of interval confidence on each graph. i) Probability of stigmatic pollen deposition by different male-phase flowers on the stigma of the female-phase flower, depending on sequence position in control and treatment experimental trials. Points represent trials with pollen deposited on the stigma (1) or not (0). ii) Number of pollen grains deposited by different male-phase flowers on the stigma of the female-phase flower in control and treatment experimental trials. Both panels show a similar pattern: the second male is more successful than the first male (second male advantage). This can be the result of a smothering/displacement effect or a time effect. In panel i, the time effect is undetectable as the first control male and second experimental male are equally successful. However, in panel ii, the success of the control male appears to be intermediate (between that of the first and second experimental males).

Two models were equally plausible for the probability of reaching a female flower (Table 2i). Both included the sequence of visiting as a predictor, with the difference that the best-fitted model does not include the trials as a random factor (condition $R^2$: 0.275; marginal $R^2$: 0.128). Therefore, we found a significant effect of the visiting sequence on the probability of depositing pollen onto the stigma, being the last flower the one in advantage (Figure 4i – I and Table 2). In particular, the control (mean response = 0.44 | 95% confidence interval (CI): 0.27-0.64) and the second male (mean response = 0.50 | 95% CI: 0.32-0.68) had triple the probability of depositing pollen, compared to the first male (mean response = 0.15 | 95% CI: 0.06-0.33). The higher pollen deposition by the second male is suggestive of a smothering effect and equal deposition to the control male suggests that this difference is not the result of a time-effect (see possible outcomes and interpretations in Figure 3).
When we looked at the number of pollen grains on the stigma, four models were equally plausible (Table 2ii). All of them included the sequence of visiting as a predictor, being the probability distribution of the response variable the main difference. Hurdle and zero-inflated models were equally plausible to negative binomial including or not the bird identification as a random factor. Our best-fitted model (Nagelkerke's $R^2$: 0.141) indicates that the second male (mean response = 3.73 | 95% confidence interval (CI): 1.96-7.07) and the control male (mean response = 2.12 | 95% confidence interval (CI): 1.08-4.14) also had equal success on the quantity of pollen grains deposited. However, we could not completely disregard the time effect, as the control male success was not significantly different from the success of the first male (mean response = 0.70 | 95% confidence interval (CI): 0.34-1.44) (Figure 4ii and Figure S3).

Table 2 – Model selection results for i) the probability of reaching the stigma (female flower) and ii) the amount of deposited pollen grains according to the sequence it was presented on Treatment or Control. Equally plausible models ($\Delta$AIC < 2) are in bold. Our variable sequence is composed of three levels (Male 1 – treatment, Male 2 – treatment and Male 1 – control). This structure allowed us to make all comparisons between males.

<table>
<thead>
<tr>
<th>Models</th>
<th>Probability distribution</th>
<th>AIC</th>
<th>dAIC</th>
<th>df</th>
<th>weight</th>
</tr>
</thead>
</table>
| i) probability of reaching the stigma
| ~ sequence + (1|bird_id) | binomial | 166.52 | 0 | 4 | 0.66 |
| ~ sequence + (1|bird_id/exp_trial) | binomial | 167.98 | 1.45 | 5 | 0.32 |
| ~ NULL | binomial | 174.9 | 8.37 | 2 | 0.01 |
| ~ treatment + (1|bird_id) | binomial | 175.29 | 8.77 | 3 | 0.01 |
| ii) amount of pollen grains reaching the stigma
| ~ sequence | negative binomial | 428.4 | 0 | 4 | 0.36 |
| (hurdle) ~ sequence | negative binomial | 429.26 | 0.85 | 7 | 0.24 |
| (zero-inflated) ~ sequence | negative binomial | 429.26 | 0.85 | 7 | 0.24 |
| ~ sequence + (1|bird_id) | negative binomial | 430.4 | 2 | 5 | 0.13 |
| ~ NULL | negative binomial | 434.52 | 6.12 | 2 | 0.02 |
| ~ 1 + (1|bird_id) | negative binomial | 434.58 | 6.17 | 3 | 0.02 |
| (hurdle) ~ sequence | negative binomial | 606.94 | 178.54 | 6 | 0 |
| (zero-inflated) ~ sequence | negative binomial | 606.94 | 178.54 | 6 | 0 |
| ~ sequence | poisson | 915.36 | 486.96 | 3 | 0 |
Discussion

Our study shows that male reproductive success is influenced by interference competition for space on the pollinator’s body, before they even reach the stigma of a receptive flower. This conclusion relies on the fact that the second male-phase flower had a greater probability of depositing pollen onto the stigma of a female-phase flower and deposited more pollen onto the stigma than the first male-phase flower visited. Because the success of the first control male is similar to the success of the second male, the second male’s success cannot be attributed completely to a time effect. Overall, our results suggest a last male advantage, which is most likely the consequence of smothering or displacement, where pre-existing pollen is smothered or displaced by the last flower (Figures 3 and 4). In addition to the competitive advantage of the second male, we also found some evidence for a time effect on the numbers of pollen grains deposited. This time effect, was not strong enough to be detected when analyzing the probability of pollen deposition on stigmas. Here, the advantage of the second male could be attributed almost completely to its competitive edge and ability to smother or displace pollen loads on pollinators. This is the first study to demonstrate a last male advantage in plants with granular pollen. In multi-flowered plants, we similarly expect the pollen deposited by the last plant visited to have a reproductive advantage over the pollen deposited by previously visited plants.

Plant traits mediating male-male competition

Competition between rival pollen grains is facilitated by the build-up of structured pollen landscapes resulted after pollen layering from sequentially visited plants, as demonstrated by Moir and Anderson (2023). Consequently, traits that facilitates or breaks down the formation of these layers could potentially be selected through male-male competition. For example, any floral traits that enhance pollen deposition by removing rival pollen from pollinators may be selected (Minnaar, de Jager, et al., 2019). In fact, whole-genome
sequences from natural populations have captured molecular signatures consistent with sexual selection on genes involved in pollen competition (Gutiérrez-Valencia et al., 2022). Such genes could be associated with pollen exine structures or their chemical properties that promote the adhesion of pollen grains to one another. Lin et al (2013) showed that a combination of pollen surface morphology (size and shape of echinate or reticulate features) and pollenkitt volume provides pollen grains with remarkable adhesion to surfaces. They found that the adhesive capacity was higher for plants that depend on insect-pollination than for wind-pollinated flowers (Lin et al., 2013). It is conceivable that these adhesive properties may extend beyond just attaching to pollinators but may also include attachment to other similar pollen grains (Lin et al., 2013). Thus, the evolution of adhesive pollen grains may be tightly linked to smothering strategies, as was found for hook-like structures found on the pollinaria of some milkweeds (Cocucci et al., 2014). Variability in heritable pollen traits (e.g. tapetal secretions and deposits or the determination of exine pattern) sets the condition for evolution through selection to happen; (see Kumar & Nair, 1986 for details). Our study provides evidence of interindividual variation on male success before the encounter with the female, setting the stage for sexual selection through male-male competition in acquiring mates while in pollinator body (Ganeshiah & Shaanker, 2001; Stephenson & Bertin, 1983). Consequently, we envisage that pollen traits connected to layering and adhesion may be targeted by sexual selection.

We suggest that traits that facilitate the building-up of “pure” pollen loads, consisting mostly of the last plant visited, may enhance the male fitness of that plant. Pollen cleaning strategies such as brushing or explosive pollination could promote the build-up of more pure pollen loads on pollinators (Minnaar, Anderson, et al., 2019). Cleaning strategies may be even more effective if plants have multi-flowered displays so that each flower manipulates the pollen load until it consists mostly of pollen from that plant. One problem with this idea is that it potentially reduces female fitness if female phase flowers on the same plant receive mostly
geitonogamous pollen. However, certain inflorescence structures could minimize this problem. For instance, in plants like *T. antholyza*, and many other protandrous species, flowers are displayed on upright inflorescences with young male-phase flowers at the top and older, female-phase flowers below. Harder et al (2000) demonstrated that bee pollinators typically visit these inflorescences from the bottom upwards (i.e. first the female flowers and then the male flowers) and showed that this inflorescence design reduces geitonogamy. Reduced geitonogamy occurs because the male flowers on an inflorescence are only visited after the female flowers, thus reducing within-plant pollen movement. We suggest that the same inflorescence design may also improve male reproductive success if the stigmas of the female flowers “clean” rival pollen from the pollinators, desaturating their pollen loads and making space for male flowers to deposit their pollen as the pollinator moves up the inflorescence. This process might be particularly important because pollen load sizes are likely to be finite (Price & Waser, 1982) and the pollen loads of most non-grooming pollinators are probably close to saturation when they arrive at a flower. Because females – phase flowers evolved to promote more pollen pick up than they need just for ovule fertilization allowing the filter for quality of pollen grains (e.g. female choice) the inflorescence design consequently generates a “pollen cleaning” on the pollinator. Therefore, the pattern of flower maturation, in addition to decreasing geitonogamy, could play a role in increasing pollen export.

It is also known that younger flowers (i.e. the last flowers visited on such inflorescences) tend to have greater proportions of viable pollen than the older flowers located below in the plant (Pauldasan et al., 2023). Pollen stratification, as a pollinator moves up an inflorescence (from young to older flowers) may result in older male flowers depositing pollen which plays a predominantly smothering role while the more viable pollen of the younger flowers (last visited in an inflorescence) will be better positioned for mating (Anderson & Minnaar, 2020). Alternatively, other non-sexual floral structures may also play an important
role in generating pollen layering. In the case of *T. antholyza*, the upper petal may act by pressing the anther on the pollinator’s head, potentially facilitating the smothering or displacement. Curiously, when the flower changes to female-phase, the upper petal also reflexes backwards (Manning & Goldblatt, 2005) indicating some temporal correlation between both sex-phases and the upper petal (P.S., *Pers. Obs.* - Figure 1b-c, S1).

Pollen competition on pollinators may select on how pollen is presented by the anthers. Pollen presentation theory relies on the idea that there is a “carrying capacity” for the amount of pollen that a pollinator can carry (Price & Waser, 1982). If so, the deposition of large pollen loads onto pollen-saturated pollinators may result in pollen wastage and reductions in male fitness (Price & Waser, 1982). Here, individual pollen grains are likely to have greater siring success if they are deposited in small loads with a lower chance of exceeding the pollen carrying capacity of the pollinator. This is thought to have led to pollen dosing, a strategy where flowers deposit multiple small pollen loads onto pollinators when visitation rates are high. Under pollen presentation theory, the deposition of large pollen loads is thought to evolve when pollinator visitation rates are low, forcing plants into risking large pollen load deposition. However, it is possible that flowers may be able to desaturate pollen loads by removing pre-existing pollen from pollinators. This may allow for the deposition of large pollen loads after pre-exiting pollen is removed. Smothering and pollen removal may explain pollen deposition strategies which do not clearly fit the expectations of the current pollen presentation theory.

In this section, we have speculated on how pollen competition for limited space on pollinators may select on a diversity of plant traits including pollen grains, floral structures, pollen presentation strategies and even inflorescence architecture. In our experiment, we show a snapshot in the life of a pollen journey, but it is unclear how these experiments would have played out if pollen loads on the birds were much larger, or if pollen from the first male may subsequently resurface after the pollinator had visited more flowers than we provided in our
experiment. We hope that our results provides the stimulation for more studies on this interesting idea of research.

**Gamete competition in flowers and animals**

In animals, sperm competition can select on a similar array of animal traits, making this an interesting point of comparison. The last male advantage which we found in this study has for example, frequently been found in animals in which females sperm storage organs provide a siring advantage for the last-mated male (Birkhead & Hunter, 1990). Last-male sperm precedence thus arises in animals when females store sperm prior to fertilization, including allowing the stored sperm to be manipulated during subsequent copulation (Birkhead & Hunter, 1990). In animal pollinated plants, where a vector (*i.e.* pollinator) is needed to transport pollen, male gametes may accumulate on the pollinator’s body, where these pollen loads can be manipulated by subsequently visited flowers. This potentially gives rise to similar conditions to those promoting sperm competition in animals. For animals, sperm precedence competition occurs through displacement of sperm, mainly by *i)* sperm stratification or *ii)* sperm removal (Birkhead & Hunter, 1990). Sperm stratification occurs when the 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 (Austad & Howard, 1984; Birkhead & Hunter, 1990). This is similar to the pollen smothering hypothesis, except that the stratification process occurs on the body of the pollinator and not inside the female reproductive tract. In contrast, sperm removal occurs when males remove pre-existing sperm from the reproductive tracts of females, often by using structures on their penis that scoop or brush (Córdoba-Aguilar et al., 2003). For plants, it is also possible that something similar may occur on the bodies of pollinators, where pre-existing pollen is brushed or displaced by floral structures before pollen deposition occurs, also giving a siring advantage to the last-male visited. Pollen removal may be a result of stroking actions by anthers during pollen application or, alternatively, there may be specialized floral structures evolved for this
very purpose (as proposed for the brushes in *Lobelia* flowers – Minnaar et al 2019). It is also
possible that flowers with explosive pollination (*e.g. Thalia geniculate* – Santana et al., 2019)
may use flower triggering to displace pollen deposited by rival males from the pollinator’s
body. Our study shows that the last male has a siring advantage, but we were unable to
determine whether the advantage is due to pollen smothering or pollen displacement.

**Possible effects of pollinator traits on male-male competition**

The evolution of smothering or displacement strategies may also be influenced by the
surface properties of pollinators and how quickly they saturate with pollen (Carneiro et al.,
2023; Castellanos et al., 2003; Pearson et al., 2023). Pollen-presentation theory recognizes that
a saturation effect may provide an advantage to plants that deposit small pollen doses (*i.e. the
deposition of large pollen doses onto already pollen-saturated pollinators may be wasteful*).
However, the surfaces of some pollinators (*e.g. feathers, fur or hairs*) may take longer to
saturate than smooth body surfaces (*e.g. some beetles and flies*). Muchhala & Thomson, (2010)
demonstrated differences in pollen loads associated with birds versus bats, which may be
related to their body coverings (*feathers versus fur*) or differences in grooming behavior.

Grooming behavior by pollinators may remove pollen loads in a similar way to pollen cleaning
strategies (*e.g. brushing, explosive pollination, stigmatic cleaning*) (Holmquist et al., 2012).
However, it is unclear how grooming is likely to affect the emergence of layering and how
different males are advantaged: on the one hand, grooming is likely to disrupt the layers which
promote smothering; on the other hand, sporadic grooming may also reduce pollen loads, so
that the pollen carrying thresholds are not reached, allowing large pollen loads to attach
(Marcelo et al., 2022).

**Conclusion**
This study shows that the sequence of flower visitation affects the probability of pollen reaching the next stigma. Pollen from the last male-phase flower visited can smother or displace the pollen from previously visited flowers, increasing the reproductive success of the most recently visited flowers. Consequently, male-male competition may promote the evolution of strategies that capitalize on this brief advantage (e.g., smothering) or strategies to combat smothering (e.g., less adhesive pollen grains). Little work has been done on pollen competition before reaching a female flower, and we hope that this paper provides a starting point for future work. We also hope that this paper stimulates discussion and debate about how pollen grains may compete with one another on pollinators, which will help us to think about the evolution of floral and pollen traits in a very different way. In particular, it may help us to understand the evolution of many pollen presentation strategies, pollen traits associated with adhesiveness and floral traits such as hairy anthers and ballistically fired pollen.

Acknowledgments

We thank Paulo Roberto Guimarães Jr, Ana Paula Aprígio Assis, Anselmo Nogueira, Pietro Murayama, Flávia Marquitti, Sam McCarren and members from SpACE group for feedback in earlier drafts. We also thank the anonymous referees for their helpful comments that improved the quality of the manuscript. We thank the Northern Cape Province and Cape Nature for permits for bird banding (permit no. CN41-28-16214). Birds were banded using materials supplied by SAFRING (University of Cape Town) under SAFRING license no. 1622. Ethics approval for this research was granted by Stellenbosch University, South Africa. This study counted on funding from the National Research Foundation (South Africa), Society for Study of Evolution (SSE) and European Society for Evolutionary Biology (ESEB). PCS is currently supported by a postdoctoral fellowship from Carl Tryggers Foundation and was supported by a doctoral fellowship from the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (Coordination for Improvement of Higher Education Personnel - CAPES | PrInt; process number: 88887.571648/2020-00) during the execution of this project.
Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Author Contributions

Study conception and design – PCS, MM, BA; acquisition of data – PCS, JM, BA; analysis and interpretation of data – PCS, EMS, BA; manuscript drafting and revision – PCS wrote the first draft and all authors contributed; scientific supervision – BA.

Data Availability Statement

All data used in analyses will be found at https://github.com/SantanaPC upon acceptance of the study.

References


Figure S1: Five stages of the *Tritoniopsis antholyza* flower, showing that it first opens in male-phase, from the left to the right. The first anther to open is the center one (left photo), and later the other two (side) anthers open. On the second day, all anthers are open, and the stamens starts to backward-reflex, positioning their anthers beneath the upper tepal. The style then gradually elongates as the flower transitions into the female-phase (Right photo).
Figure S2: Sunbird *Cinnyris chalybeus* probing the experimental flowers placed on the Eppendorf tubes: a) sunbird probing a labeled male-phase flower where is possible to see the anthers touching its forehead and potentially depositing pollen; b) sunbird probing the female-phase flower where is possible to see the stigma above its head and potentially receiving the labeled pollen.

Figure S3: Number of pollen grains deposited by different male-phase flowers on the stigma of the female-phase flower in control and treatment experimental trials as predicted by the best fitted model. The graph
shows that the second male is more successful than the first male (second-male advantage) which can be the result of a smothering/displacement effect or a time effect. The success of the control male appears to be intermediate (between that of the first and second experimental males), with the confidence intervals overlapping both with first and second male, potentially indicating also a time-effect.