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RESEARCH ARTICLE

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

Pamela C. Santana[1,2,3] | Jake Mulvaney[2] | Erika M. Santana[3] | Monika Moir[4] | Bruce Anderson[2]

[1]Biodiversity and Evolution Unit, Department of Biology, Lund University, Lund, Sweden

[2]Department of Botany and Zoology, Stellenbosch University, Stellenbosch, South Africa

[3]Departamento de Ecologia, Instituto de Biociências, Universidade de São Paulo, São Paulo, Brazil

[4]Centre for Epidemic Response and Innovation, School for Data Science and Computational Thinking, Stellenbosch University, Stellenbosch, South Africa

Correspondence
Pamela C. Santana
Email: pamela.santana@biol.lu.se

Bruce Anderson
Email: banderso.bruce@gmail.com

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Abstract

1. Many plants have precise pollen placement strategies, ensuring that large amounts of pollen are deposited on small and discrete areas of pollinators' bodies. 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.
2. We investigated these alternative hypotheses using caged sunbirds (*Cinnyris chalybeus*) and sunbird-pollinated flowers (*Tritoniopsis antholyza*). We labelled pollen from two different male-phase flowers with quantum dots, enabling us to distinguish their pollen grains. 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 male-phase flower followed by a flower without reproductive structures. The last trial established whether pollen loss over time (time effect) influenced our results, such as pollen falling off or being groomed from the pollinator.
3. We found that pollen from the second male-phase flower was better represented on the stigmas of the subsequently visited female-phase flowers. This advantage was not attributable to a time effect (i.e. less time for the last male's pollen to fall off the pollinator). Instead, our results suggest 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 exploit or mitigate this effect.
4. *Synthesis*: Our study demonstrates that pollen grains compete for space on pollinators' bodies. By uncovering how interference competition affects male reproductive success, our findings underscore the importance of pollen-related traits in sexual selection and open new avenues for investigating floral evolution.

KEYWORDS
male fitness, pollen movement, pollen precedence, pollen smothering, sexual selection

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1 | 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) and the male pathway (seeds sired) (Alexander & Tinkle, 1981; Morgan, 1994). Female contributions to total fitness are usually constrained by resource access (Bachelier, 2022; Clutton-Brock, 2009), while male contributions are typically constrained by mating opportunities (access to mates and their ovules—Janicke, 2024; Marshall & Folsom, 1991). As flowers typically produce many more pollen grains than ovules (Cruden, 1977; Gong & Huang, 2014), the male fitness pathway can potentially lead to higher variability in the total fitness of an individual (Schärer et al., 2012). The imbalance between potential male and potential female contributions to total fitness can lead to higher variability in male (Minnaar, Anderson, et al., 2019) than in female reproductive success (Tonnabel et al., 2019) and sets the stage for sexual selection in plants (Bateman, 1948; Janzen, 1977; Moore & Pannell, 2011; Willson, 1979). Indeed, Paterno et al. (2020) found evidence suggesting that traits involved in increasing pollen export (e.g., attractive traits) may have evolved mainly through the male fitness pathway (Stanton et al., 1986).

Despite the potential importance of the male fitness pathway in flower evolution (Mulcahy & Mulcahy, 1987; Paterno et al., 2020), 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 (Minnaar, Anderson, et al., 2019). Additionally, plants potentially have several mates, making it more challenging 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). This paper contributes to our understanding of how competition for pollen deposition space on pollinator bodies may shape floral evolution (Thomson, 2014).

The potential for males to compete for pollen deposition space may be facilitated 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 and stamp), which are likely to generate distinct pollen landscapes (Duffy & Johnson, 2014; Minnaar, Anderson, et al., 2019). Pollinator bodies may represent arenas for male–male competition when males compete for placement sites on pollinators in order to maximize pollen export and access to the ovules of subsequently visited female-phase flowers on other plants (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 competition even before pollen has been deposited on another stigma (Minnaar & Anderson, 2021). Muchhala and 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), 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, Anderson, et al., 2019).

Pollen preclusion may occur when pre-existing pollen loads prevent or preclude the deposition of new pollen grains onto pollinators (Figure 1; Cocucci et al., 2014; Moir & 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 1; Minnaar, Anderson, et al., 2019). There are a few examples of pollen smothering and preclusion from 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, only one study has documented pollen preclusion in plants with granular pollen: Moir and Anderson (2023) established the first empirical evidence for pollen layering when non-grooming flies visited *Moraea lurida* (Iridaceae—Moir et al., 2022). They found that earlier-visited plants appeared to deposit more pollen on pollinators' bodies than subsequently visited plants, suggestive of pollen preclusion. However, the effects of pollen preclusion and smothering or displacement 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 deposition onto stigmas.

Our study sought to investigate male–male competition in *Tritoniopsis antholyza* **(Poir.)** Goldblatt (Iridaceae) plants when they are visited by sunbird pollinators (*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

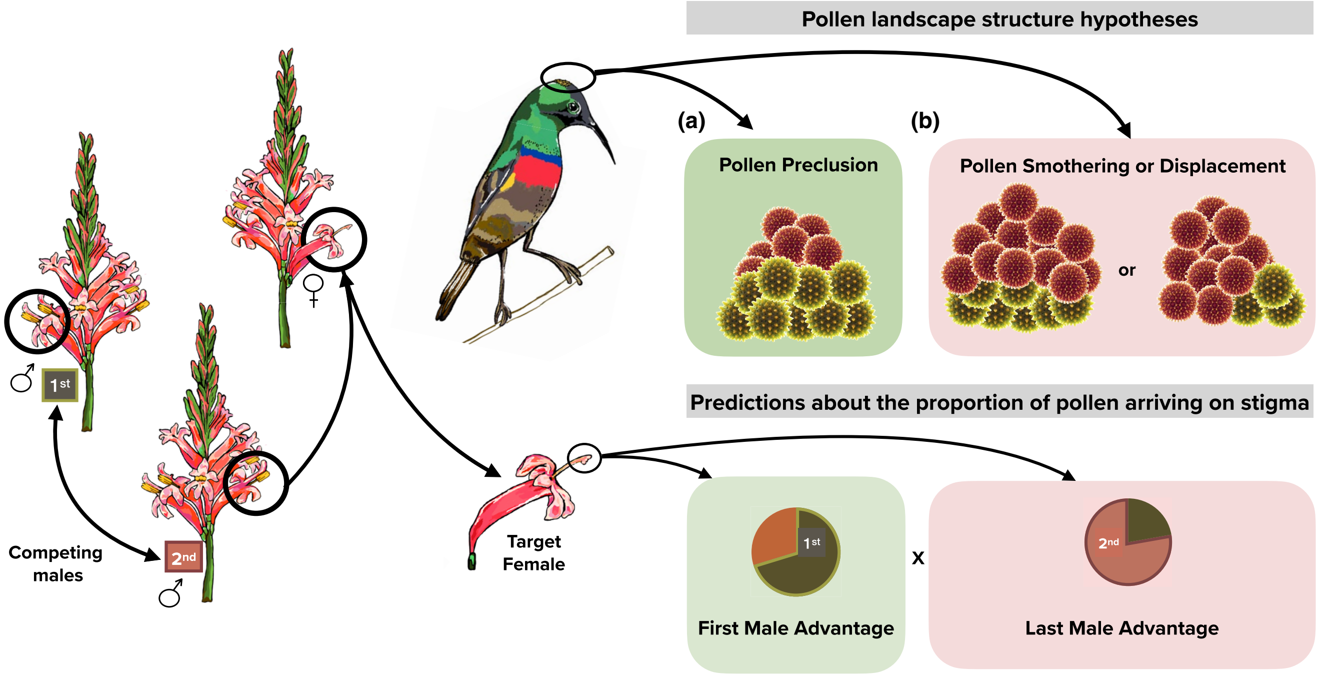

FIGURE 1 Two alternative male–male competition hypotheses and predicted pollen landscapes. When pollen from successively visited flowers (starting sequentially with male-phase flowers on plant 1, then 2) is deposited on the pollinator's body, it can result in different kinds of pollen landscapes which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

pollen smothering/displacement by tracking pollen deposition success of different flowers after their pollen was labelled with different colour quantum dots (Minnaar & Anderson, 2019). Our experiment did not attempt to distinguish between the two different mechanisms (pollen smothering vs. pollen displacement) which may give rise to a last-male advantage.

2 | MATERIALS AND METHODS

2.1 | 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 (CapeNature permit number CN41-28-16214 and SAFRING ringer number 1622). Our focal flowering species was *Tritoniopsis antholyza*, 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).

Sunbird-pollinated flowers in the Cape Floral Region of South Africa typically conform 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 (*Cinnyris chalybeus*) and orange-breasted sunbirds (*Anthobaphes violacea*), with a butterfly (*Aeropetes tulbaghia*) playing the role of a secondary pollinator or nectar thief (Newman et al., 2014; B. Anderson, *personal observation*). 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; B. Anderson, *personal observation*).

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

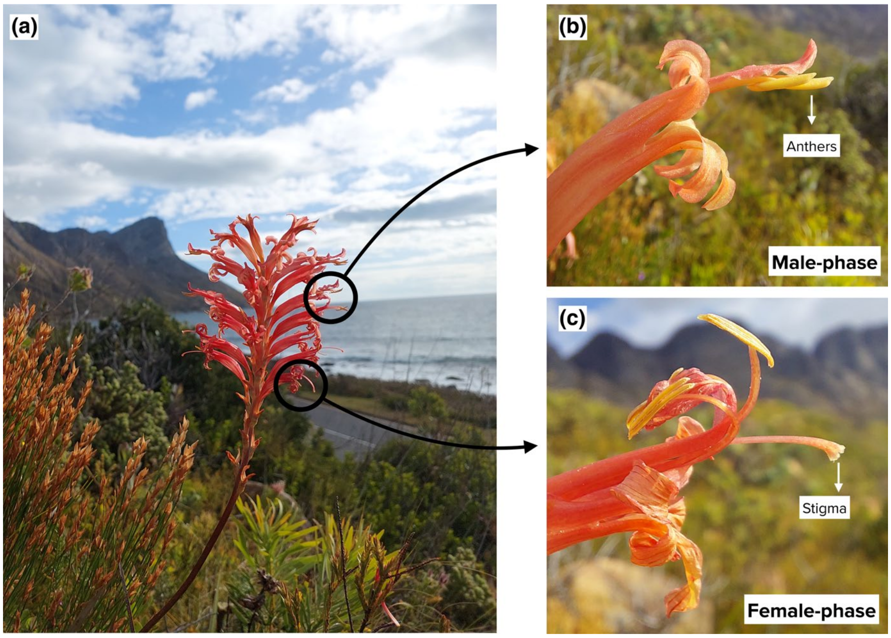

FIGURE 2 *Tritoniopsis antholyza* (Iridaceae) inflorescence showing: (a) flowers spirally arranged, and maturing so that, (b) male-phase flowers are on the top; and (c) female-phase flowers are at the bottom.

open nets monitored every 20 min. All captured birds were identified and banded, with by-catch species released immediately thereafter (Permits granted by Northern Cape Province and Cape Nature for bird banding—permit no. CN41-28-16214—all birds were banded using materials supplied by SAFRING/University of Cape Town under SAFRING licence no. 1622; Stellenbosch University granted ethics approval for this research). *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 (80/100 × 60 × 60 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 h 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.

2.2 | 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 & 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 colours 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 laboratory 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; and (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 placed onto a slide and stored in the freezer for later examination under a M125 Stereo dissecting microscope (Leica, Germany). We examined the stigma using a Qdot excitation box to compare the pollen grain quantities from first versus second male-phase flowers (Minnaar & Anderson, 2019).

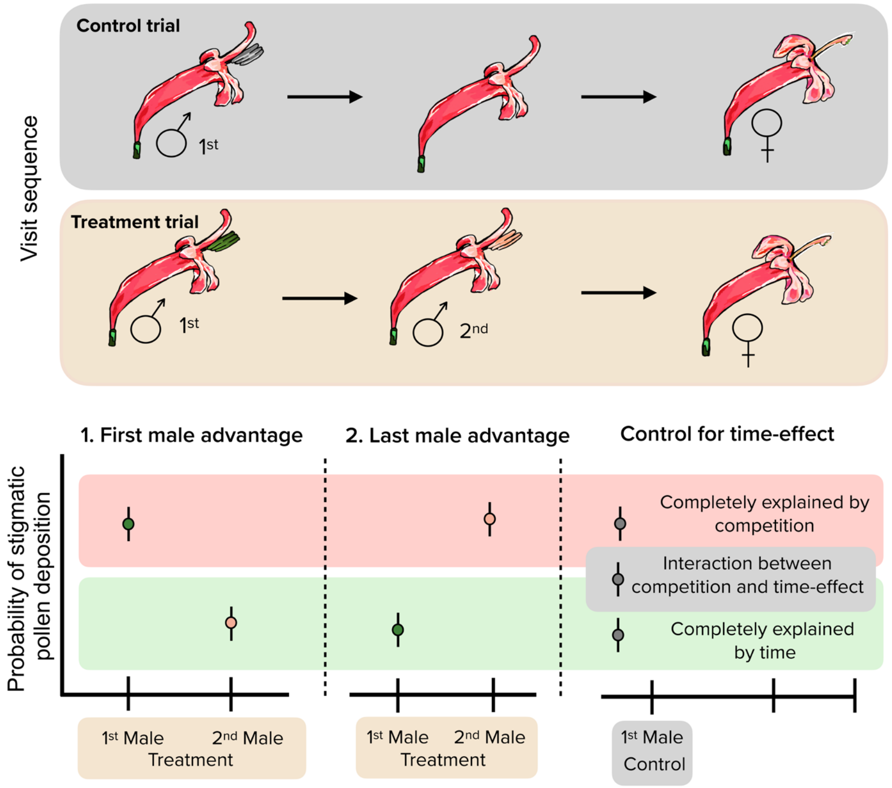

FIGURE 3 Experimental setup and hypothetical differences in stigmatic pollen representation to distinguish between time effects, last and first male advantage. Control trials consisted of probes to a Qdot-labelled male-phase flower, followed by a flower without reproductive structures, followed by a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

From this, we were able to determine whether the first or the second male-phase flower was more successful at exporting pollen to the stigma of the third female-phase flower. However, an advantage to the second male-phase could occur just because pollen grains from the second male-phase have less time to fall from the pollinator (time effect). To identify a possible 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-min 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 1-h apart to minimize cross-contamination of pollen grains. Across paired trials, Qdot colours were also randomized for the first and second male-phase flowers to remove any potential effects of Qdot colour 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).

2.3 | 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

TABLE 1 Replication statement: (i) inferences were made at the scale of individuals, as we investigated intrasexual 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.

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

Note: We included birds as a random factor in the models and used the same bird for pairwise control and treatment trials.

preclusion), we expected the first male-phase flower to have greater pollen deposition success than the second male-phase flower. In this first scenario, we predict a positive relationship between sequence position and pollen grains deposited. 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 male-phase flower has greater pollen deposition success than the first male-phase flower. In this second scenario, we predict a negative relationship between sequence position and pollen grains exported.

Our control allowed us to disentangle whether the 'last male advantage' pattern 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 could be better represented on stigmas than pollen from the first male-phase flower, because pollen from the first male-phase 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 or a competitive effect (Figure 3).

To distinguish between the effects outlined in Figure 3, we counted the number of pollen grains deposited by each of the male-phase flowers (i.e., different Qdots colours) onto the stigma of the female-phase flower. In 12 of the 43 replicates (~27%), there were no pollen grains deposited on the stigma. Therefore, we first looked at the probability of pollen deposition on stigma by analysing the data as presence (1) or absence (0) of pollen grains for each male-phase flowers (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 from the treatment and the control. We set up models to compare the effect of the sequence position with different random factor combinations: (a) the identity of the bird or (b) the identity of the bird nested within the experiment trial. We compared these models to a null model using the Akaike information criterion (AIC) and selected the model that best-fit our data. Models with ΔAIC lower than 2 were considered equally plausible (Table 2i).

We also modelled 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, then was it transferred from bird to stigma (yes or no) and how many. 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 (i.e., composed by a zero portion and a truncated count portion—Poisson or negative binomial) and zero-inflated (i.e., 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 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* (v1.10.2 Lenth, R. 2024) and *phia* (v0.3-1 De Rosario-Martínez, H. 2024) packages, by computing contrasts of estimated marginal means 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 (CIs).

All analyses were done in R (R version 4.4.2, Core Team, 2024), using the main packages *lme4* for linear models (Bates et al., 2015), *glmmTMB* (Brooks et al., 2017), *DHARMa* (Hartig, 2022), *bbmle* (Bolker, R Development Core Team, 2023), *performance* (Lüdecke, 2021), *ggeffects* (Lüdecke, 2018), *MASS* (Venables & Ripley, 2002), *pscl* (Jackman, 2024) and *AER* (Kleiber & Zeileis, 2022). The complete list of packages, together with the code and data is available at GitHub (SantanaPC).

TABLE 2 Model selection results for (i) the probability of pollen deposition on the stigma and (ii) the amount of deposited pollen grains according to the presentation sequence in treatment and control trials.

Models	Probability distribution	AIC	dAIC	df	Weight
(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|sequence	**Binomial|negative binomial**	**429.26**	**0.85**	**7**	**0.24**
(zero-inflated) ~ sequence|sequence	**Binomial|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|sequence	Binomial|Poisson	606.94	178.54	6	0
(zero-inflated) ~ sequence|sequence	Binomial|Poisson	606.94	178.54	6	0
~sequence	Poisson	915.36	486.96	3	0

Note: 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. For all analyses, male sequence affected the response variable (probability of deposition on the stigma and number of pollen grains deposited on the stigma). For the probability of stigmatic deposition, the equally plausible models contained different random factor structures. For the number of pollen grains being deposited on the stigma, both hurdle, zero-inflated or only negative binomial probability distribution models were equally plausible. Therefore, we used the simplest model to predict our response variable as this was also our best-fit variable.

3 | RESULTS

Pollen transfer was highly variable (varying from 0 to 150 pollen grains) and was often ineffective. When we found pollen on stigmas, the mean number of pollen grains was low (Treatment mean: 2.2; Control mean: 2.1 grains), while the standard deviation was higher (Treatment standard deviation: 5.84; Control standard deviation: 4.21 grains).

Two pollen transfer models were equally plausible (Table 2i) and both included the visit sequence as a predictor, suggesting that visit sequence is the most important predictor of variation in pollen export success. Here, the last male consistently had a higher probability of pollen deposition success (conditional R^2: 0.275; marginal R^2: 0.128, Figure 4i; Table 2i). In particular, the control (mean response = 0.44|95% 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/displacement effect. Furthermore, because the second male export success was similar to control male, the success of the second male could not have been the result of a time effect (see possible outcomes and interpretations in Figure 3).

For number of pollen grains on the stigma, four models were equally plausible (Table 2ii). All of them included the visit sequence as a predictor, suggesting the consistent importance of visit sequence in explaining variation in pollen grain export and deposition to the stigma. Additionally, this suggests that the probability distributions tested across different models did not significantly improve predictions of variation in pollen grain export to the stigma. Hurdle and zero-inflated models performed similarly to the negative binomial model, both with and without bird identification included as a random factor (Table 2ii). The best-fit of the four equally plausible models (Nagelkerke's R^2: 0.141) was also the simplest model and clearly shows that the second male (mean response = 3.73|95% CI: 1.96–7.07) and the control male (mean response = 2.12|95% CI: 1.08–4.14) had equal pollen export success. Success of the control male was not significantly different from the first male, suggesting that part of (but not all) of the second male's success was a result of time and that a large part was due to a smothering/displacement effect (mean response = 0.70|95% CI: 0.34–1.44) (Figure 4ii; Figure S3).

4 | DISCUSSION

This study shows that male reproductive success is influenced by interference competition for space on the pollinator's body: second male-phase flowers 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. 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

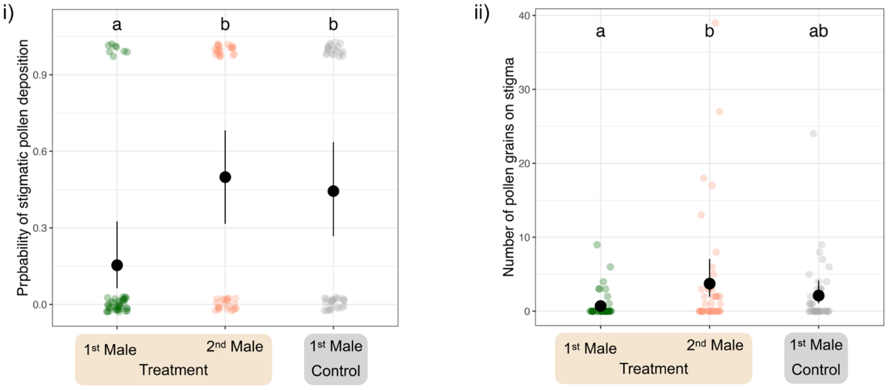

FIGURE 4 Probability of stigmatic pollen deposition and number of pollen grains on stigma for both control and treatment trials. Letters show statistically significant differences among treatments according to Tukey method for pairwise comparisons of interval confidence on each graph. (i) Probability 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).

strong enough to be detected when analysing 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.

4.1 | Plant traits mediating male–male competition

Competition between rival pollen grains is facilitated by the build-up of structured pollen landscapes resulting from pollen layering after sequential visits to different male-phase flowers (demonstrated by Moir & Anderson, 2023). Consequently, traits that facilitate or break down the formation of these layers could potentially be selected through male–male competition (Anderson et al., 2024; Minnaar, de Jager, et al., 2019). In fact, whole-genome sequences have captured molecular signatures consistent with sexual selection on genes involved in pollen trai (Gutiérrez-Valencia et al., 2022; Mulcahy & Mulcahy, 1987). Such genes could be associated with pollen exine structures or 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 (Pacini & Franchi, 2020). 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 pollinator attachment and may also include attachment to similar pollen grains. Thus, the evolution of pollen adhesiveness 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; Ganeshaiah & Shaanker, 2001; Stephenson & Bertin, 1983). Additionally, since male success may be influenced by traits that facilitate the building-up or breakdown of 'pure' pollen loads, pollen-cleaning strategies such as brushing or explosive pollination may be expected to evolve. For example, Anderson et al. (2024) found that some plants may use explosive pollen as projectiles to remove rival pollen from pollinators. 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 (Anderson et al., 2024).

Pollen layering, and thus the likelihood of pollen competition on pollinators, may also select on how pollen is presented by the anthers. Pollen presentation theory suggests that a pollinator has a 'carrying capacity' for pollen, and exceeding this capacity can lead to pollen wastage, reducing male fitness (Price & Waser, 1982). To maximize siring success, flowers may deposit smaller pollen loads and/or extend the duration of pollen presentation. This concept is reflected in pollen dosing, where multiple small loads are transferred to pollinators. In plants, such as *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. We propose that this design may help male reproductive success by allowing female flowers to 'clean' pollen from pollinators, making room for subsequent pollen deposits. Female-phase flowers may also encourage excessive pollen pickup, facilitating selective filtering (i.e., female choice—Stephenson & Bertin, 1983; Waser & Price, 1993). Thus, the flower maturation pattern may reduce geitonogamy (Harder et al., 2000) and enhance 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 (Pacini et al., 1997; 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).

4.2 | 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. A last-male mating advantage has been found in animals were females store sperm (Birkhead & Hunter, 1990). This advantage arises because stored sperm can be manipulated during subsequent copulations (Birkhead & Hunter, 1990). While plants do not store sperm or pollen grains, pollen grains may accumulate on the pollinator's body where they 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 when sperm is displaced, 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 pollen smothering, except that the pollen stratification (i.e., layering) 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). Similarly, plants may remove or displace rival pollen grains from their pollinators by using brushing structures (as proposed for the brushes in *Lobelia* flowers—Minnaar, Anderson, et al., 2019) or by using explosive pollination (e.g., Santana et al., 2019; to dislodge pollen grains as demonstrated by Anderson et al., 2024). 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.

4.3 | 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., 2024; 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. 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). Muchhala and Thomson (2010) demonstrated differences in pollen loads associated with birds versus bats, which may be related to their body coverings (feathers vs. fur) or differences in grooming behaviour. Grooming behaviour by pollinators may remove pollen loads in a similar way to pollen-cleaning strategies (e.g brushing, explosive pollination) (Holmquist et al., 2012). However, it is unclear how grooming is likely to affect pollen competition: on the one hand, grooming could 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).

5 | CONCLUSION

This study shows that the sequence of flower visitation affects the probability and amount of pollen exported. Reproductive success can be increased when pollen from the last male-phase flower smothers or displaces pollen from previously 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 'pre-mating' pollen competition, 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.

AUTHOR CONTRIBUTIONS

Pamela C. Santana, Monika Moir and Bruce Anderson conceived the study; Pamela C. Santana and Bruce Anderson designed the experiments; Pamela C. Santana, Jake Mulvaney and Bruce Anderson collected the data; Pamela C. Santana performed the data analysis with conceptual contributions from Bruce Anderson. Pamela Santana, Erika M. Santana and Bruce Anderson interpreted the analysis; Pamela C. Santana led the writing of the study; all authors contributed to the drafts and gave final approval for publication.

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CONFLICT OF INTEREST STATEMENT

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.

DATA AVAILABILITY STATEMENT

All data used in analyses can be found at Zenodo (https://doi.org/10.5281/zenodo.14293009, Santana, 2024) and the code can be found at https://github.com/SantanaPC/Pollen_Competition.

ORCID

Pamela C. Santana https://orcid.org/0000-0002-7517-6671
Jake Mulvaney https://orcid.org/0000-0001-6877-5265
Erika M. Santana https://orcid.org/0000-0001-5883-4284
Monika Moir https://orcid.org/0000-0003-1095-1910
Bruce Anderson https://orcid.org/0000-0002-7264-4505

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SUPPORTING INFORMATION

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

Figure S1. Five stages of the *Tritoniopsis antholyza* flower, showing that it first opens in male-phase, from the left to the right.

Figure S2. Sunbird *Cinnyris chalybeus* probing the experimental flowers placed on the Eppendorf tubes: (a) sunbird probing a labelled 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 labelled 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.

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