Received: 13 October 2022 | Accepted: 18 February 2023

DOI: 10.1002/ajb2.16184

American Journal of Botany

SPECIAL ISSUE ARTICLE

Pollen layering and male–male competition: Quantum dots demonstrate that pollen grains compete for space on pollinators

Monika Moir | **Bruce Anderson**

Department of Botany and Zoology, Stellenbosch University, Stellenbosch, South Africa

Correspondence
Bruce Anderson, Department of Botany and Zoology, Stellenbosch University, Stellenbosch, South Africa.
Email: banderso.bruce@gmail.com

This article is part of joint special issues of the *American Journal of Botany* and *Applications in Plant Sciences*: "Pollen as the Link Between Phenotype and Fitness."

Abstract

Premise: Almost nothing is known about what happens to pollen grains once they attach to pollinators, although some have postulated that pollen from different donors may form complex, two- or three-dimensional landscapes (e.g., layers or mosaics) that can facilitate male–male competition. For example, pollen that is already on pollinators may preclude the deposition of subsequent pollen grains.

Methods: Using quantum dots to mark the pollen of individual flowers, we explored the possibilities of layering and preclusion in a fly-pollinated iris, *Moraea lurida*.

Results and Conclusions: The proportion of labeled pollen from the last flower visited diminished in sequential pollen samples taken from the top to the bottom of the pollen load, representing the first empirical evidence for pollen layering. However, the consequences in terms of pollen preclusion were equivocal: Although the pre-existing pollen load size was not a good predictor of new pollen receipt, labeled pollen loads from the last flower visited were significantly smaller than pollen loads from the previous flower visited. Thus, pollen from the previous flower may preclude pollen placement from a subsequently visited flower, and pollen from different flowers may compete for space on pollinators.

KEYWORDS

male fitness, *Moraea lurida*, pollen competition, pollen fates, pollen layering, pollen movement, pollen preclusion, pollen tracking, quantum dots, sexual selection

Pollen that is discretely rubbed or stamped from successive flowers onto a pollinator may form a three-dimensional landscape like a "pollen layer cake" (Armbruster et al., 2009; Minnaar et al., 2019a). The structures of these landscapes can theoretically affect the distance and timing of a flower's pollen dispersal (Harder and Wilson, 1998), with potentially far-reaching effects on floral evolution and ecology. For example, layering may increase gene flow if pollen is not removed immediately from pollinators by stigmas, but is instead buried underneath subsequently deposited pollen, only to resurface much later (Lertzman and Gass, 1983; Morris et al., 1995; Marcelo et al. 2022 [preprint]). Pollen landscapes may also generate variability in the ability of pollen grains to adhere to or leave the bodies of pollinators. For instance, pollen may build up on pollinators, saturating the available attachment space so that additional pollen loads adhere poorly. This lack of space is one potential reason why pollen export may decrease with the amount of pollen removed by each pollinator and why flowers often dispense many small pollen loads instead of a few large pollen loads (Harder and Thomson, 1989). Inflorescence architecture, pollen-dispensing strategies, and structures that brush existing pollen from pollinators may all be shaped in part by pollen landscapes and the competition for space by pollen grains from rival pollen donors (Minnaar et al., 2019a; Anderson and Minnaar, 2020). Despite the potentially important ecological and evolutionary consequences of pollen landscapes, their existence in flowers with granular pollen has not been verified by empirical studies. Nor is there empirical evidence in plants that granular pollen grains compete for space on pollinators.

There is, however, evidence to suggest that pollen packaged in pollinaria may generate structured pollen landscapes on pollinators, which may result in competition between individuals (Cocucci et al., 2014; Duffy and Johnson, 2014; Harder et al., 2021). For example, Harder et al. (2021) demonstrated that the presence of pre-existing pollinaria on bee pollinators reduced the export of pollen from subsequently visited flowers by 27%. Cocucci et al. (2014) also found evidence that the pollinaria of some milkweed species have horns that prevent or preclude the attachment of pollinaria from subsequently visited flowers.

Among plants with granular pollen, evidence for pollen landscapes is far less convincing, perhaps because prior difficulties in marking pollen made it difficult to gather data on pollen landscapes (Anderson and Minnaar, 2020). However, recent studies (e.g., Minnaar et al., 2019b; Minnaar and Anderson, 2019, 2021) have used fluorescent nanoparticles to mark and distinguish pollen grains from different flowers of the same species, potentially facilitating the study of pollen landscapes and intraspecific pollen competition on pollinators. One study demonstrated the presence of two-dimensional, granular pollen mosaics on the wings and bodies of bees visiting enantiostylous flowers (Minnaar and Anderson, 2021). Here, the proportions of pollen from left- and right-handed morphs varied across the pollinator bodies. If pollen landscapes on pollinators can be broadly structured by a left or right morph, perhaps they can also be structured at the level of individual pollen donors.

Similarly, there is also very little evidence for intraspecific pollen competition on the bodies of pollinators in plants with granular pollen. However, Muchhala and Thomson (2012) demonstrated that different species of plants compete for available pollen space on pollinators, raising the possibility that the same may be true for rival pollen donors of the same species. Minnaar et al. (2019a) interpreted the brush-like structures on lobelias as an evolutionary consequence of intraspecific pollen competition; an adaptation to displace pollen from rival pollen donors before depositing their own pollen onto pollinators. Such pollen-cleaning strategies may be beneficial if pollinators arrive at flowers carrying large, pre-existing loads that prevent the deposition of new pollen. We refer to this as “pollen preclusion”. Large pollen loads may enhance the possibilities of pollen preclusion, especially if pollen residence on a pollinator requires a firm substrate, with feathers, fur, or hairs aiding the entrapment of pollen (Muchhala and Thomson, 2010; Anderson and Minnaar, 2020). Pollen preclusion is most likely in pollination systems where pollinators seldom groom pollen from their bodies (e.g., birds, beetles or flies) or in plants that place pollen on pollen-safe sites, which are parts of the pollinator that are hard to groom (Koch et al., 2017). It is also likely to be most beneficial in plants that produce few flowers because it would be counter-productive to preclude pollen from other flowers on the same plant (Minnaar and Anderson, 2021).

In the present study, we looked for evidence of pollen layering and pollen preclusion in *Moraea lurida* (Iridaceae). *Moraea lurida* is pollinated by flies, which access nectar by passing through a narrow gap where they squeeze past a single anther (Moir et al., 2021). As the fly squeezes through this gap, the anther makes contact with the thorax of the fly, sometimes leaving a thick smear of pollen along the length of the insect's thorax (Figure 1) (Goldblatt et al., 2005; Moir et al., 2021). *Moraea lurida* may be a good candidate system to investigate layering and its effects because the plants typically display only one flower at a time, pollinators often appear to carry large, thick pollen loads placed precisely on their thorax (Goldblatt et al., 2005), and the fly pollinators do not appear to make any effort to groom these pollen loads from their bodies (B. Anderson, personal

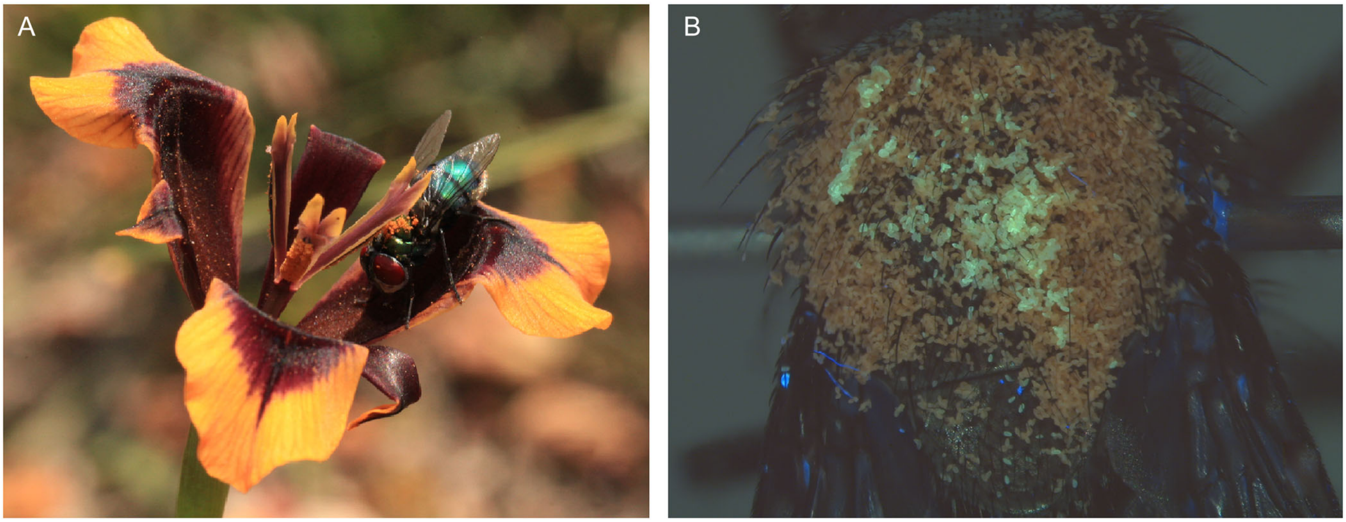

FIGURE 1 (A) A calliphorid fly visiting a *Moraea lurida* flower with an anther smearing a thick load of pollen on the fly's head and thorax. (B) Thorax of a fly with pre-existing (unlabeled) orange *Moraea lurida* pollen and quantum-dot-labeled pollen fluorescing yellow in a quantum-dot excitation box. Note that the yellow-labeled pollen appears to lie on top of the unlabeled pollen from previously visited flowers, representing the first photographic evidence of pollen layering. See Appendix S1 for further photographic evidence of layering.

observations). We hypothesized that if sequential samples of pollen are taken from the top to the bottom of a pollinator's pollen load, proportions of pollen from the last flower visited may diminish with each subsequent sample, which could be taken as evidence for pollen layering. Pollen preclusion could be inferred if the number of pollen grains deposited onto a pollinator or the probability of pollen deposition is inversely proportional to the numbers of pollen grains already present on the pollinator. A decrease in pollen load size after each successively visited flower would also suggest that pollen from previously visited flowers precludes the placement of pollen from subsequently visited flowers.

MATERIALS AND METHODS

A flowering *M. lurida* population near Caledon in the Western Cape Province of South Africa (34.222°S, 19.406°E) was visited from 5 to 15 October 2020. Similar to flowers in the genus *Iris*, *Moraea* flowers consist of three units (meranthia), each comprising just a single stamen and stigma situated between a tepal (below) and an ornately expanded style branch (above) as can be seen in Figure 1A. In the morning, before pollinators started visiting, we collected several flowers with newly dehisced anthers. We used quantum dots (q-dots) to label and subsequently identify the pollen grains from individual plants (Minnaar and Anderson, 2019, 2021; Minnaar et al., 2019b; Kern et al., University of Minnesota, unpublished manuscript). The q-dots are fluorescent nanocrystals, which, dissolved in a volatile solution (in this case toluene), can be applied using a pipette to freshly dehisced anthers (Minnaar and Anderson, 2019). The solution quickly evaporates, leaving the q-dots attached to the pollen grains by ligands. The q-dots are commercially available in different colors, that fluoresce when excited by UV light. Pollen grains fluorescing in different colors on the bodies of pollinators or on the stigmas of flowers can then be observed with a custom-made viewing box (see Minnaar and Anderson, 2019 for details). Quantum dots (2–10 nm) are several orders of magnitude smaller than pollen grains (10–100 µm) and are not known to affect the transport or adherence of the grains (Minnaar and Anderson, 2019). Unlike fluorescent powders that act as pollen analogues and can only be used to assess the presence or absence of transfer, quantum dots can be used to mark and track individual pollen grains, allowing us to quantify proportional differences in the numbers of labeled and unlabeled pollen grains (Minnaar and Anderson, 2019; Anderson and Minnaar, 2020).

We labeled all three newly dehisced anthers per flower with the same color of q-dot solution. Between 100 and 200 µL was required to visibly saturate each anther. Two colors of q-dot solution (green: 550 nm, orange: 620 nm) were used to label an equal number of flowers. Green- and orange-labeled flowers were placed closely together in pairs to increase the likelihood of pollinators moving from one to the other. Each pair was situated among wild *M. lurida* flowers, which attracted foraging flies to the general vicinity. Twelve to 15 flower pairs were set out for each day of observations with a total of 84 labeled flowers used in the experiments. Observers were able to see all pairs simultaneously so that visits could be monitored. As pollinators visited the labeled flowers, we recorded the sequence (and q-dot color) in which the flowers were visited. Pollinators were enclosed in a potassium cyanide-loaded killing jar as they left the second labeled flower or if they appeared to be leaving a labeled flower on the periphery of the study area such that they were not likely to visit a second labeled flower. Many of the flies had pre-existing pollen on their thoraxes from prior visits to unlabeled flowers. Pollinators were killed quickly with little disturbance to pollen loads. As soon as pollinators died, we pinned them, being careful to avoid the top of the thorax where pollen loads were situated. *Moraea lurida* flowers are visited by many fly species from several fly families; however, their effectiveness as pollinators varies greatly with body size because they need to be large enough to come into contact with the anthers and stigma while walking on the lower tepal (Moir et al., 2021). Our experiments only included flowers that were visited by flies large enough to make contact with the anthers and stigmas of *M. lurida* (as outlined by Moir et al., 2021), and these have been identified to family level in this manuscript. A full list of visiting species, identified to genus and species level where possible, was reported by Moir et al. (2021). Once a labeled flower had been visited, the flower was removed and replaced with a new virgin, labeled flower. A total of 26 flies were captured after visiting 38 labeled flowers.

Pollen preclusion

We used a UV q-dot excitation box and a Leica M125 dissecting microscope to view the labeled *M. lurida* pollen grains (Minnaar and Anderson, 2019) on the fly pollinators (Figure 1B). A Leica MC190 HD camera was used to photograph the undisturbed pollen landscapes on the focal area (head and thorax bearing pollen grains) of pollinators at varying depths of field. These photographs were stacked into a single composite image with Helicon Focus Pro software (www.heliconsoft.com). We then counted the number of pre-existing and q-dot labeled pollen grains on each pollinator from the stacked images. Labeled pollen could be distinguished from unlabeled pollen as labeled pollen fluoresces under UV light, whereas unlabeled pollen does not.

Pollen layering

In search of evidence for pollen layering on pollinators (Figure 1B), we selected 14 of the 26 captured flies with large pollen loads that had visited labeled flowers as their last visit. We selected these specimens because they were the most likely candidates for carrying layered pollen loads that would be detectable with the methods used here. Eleven of the 14 selected flies had visited a single labeled flower, while the remaining three had visited two labeled flowers.

Pollen from the pollen loads of these flies was removed layer by layer, using the following methods: For each fly, four 3 × 3 mm squares of double-sided tape were attached to a glass microscope slide (one slide per fly, with four labeled tape squares on each slide). Each of the tape squares on the slide was then successively pressed (lightly and evenly) onto the fly thorax at the same location on the fly, allowing us to retrieve four pollen layers from the same area. For all flies, this resulted in all pollen being removed from the target area. All samples were collected by one investigator (M.M) to ensure consistency in the technique and pressure applied. To determine whether the proportion of labeled pollen decreases with each subsequent sample (from the top to the bottom of the pollen load), we viewed the successive tape samples on microscope slides (with pollen loads facing up) within a UV q-dot excitation under a Leica M125 dissecting microscope at a magnification of 60×10 (See Minnaar and Anderson, 2019). We then counted the number of unlabeled (pre-existing) and labeled pollen grains on each tape square/sample. The proportion of labeled grains in each sample were calculated as labeled pollen in each sample/total number of labeled grains counted in all four samples, thus keeping the denominator constant.

Statistical analyses

Pollen preclusion

To test for a difference in the pollen counts of labeled pollen deposited on flies that had visited two labeled flowers, of which there were 12 samples, we performed a paired *t*-test on the log-transformed counts (first labeled versus second labeled pollen counts) using R version 4.2.0 (R Core Team, 2020).

We also tested for the preclusion of newly added pollen grains by previously deposited grains using a generalized linear mixed model explaining variation in the log-transformed counts of the most recently deposited pollen grains (q-dot-labeled grains) with the glmmTMB function in the R package glmmTMB (Brooks et al., 2022). Fixed predictor variables included in the model were the log number of the sum of previously deposited pollen count, fly family, and whether the observation was the first or second labeled flower visited (coded as a categorical variable). Fly individual was included as a random factor nested within fly family. Flies that had visited both one and two labeled flowers were included in the data set; for flies that had visited one labeled flower, the count of pre-existing pollen was considered as the previously deposited pollen and labeled pollen as the most recently deposited. For the second observation for flies that had visited two labeled flowers, the count of labeled pollen from the first labeled flower was summed with pre-existing pollen to quantify the previously deposited pollen count, and the labeled grains from the second labeled flower visited was considered as the newly deposited pollen. The r.squaredGLMM function in the MuMIn package v1.47.1 (Barton, 2022) was used to calculate a marginal and conditional R^2 estimate. The marginal R^2 represents the variance explained by the fixed effects in the model and the conditional R^2 is interpreted as a variance explained by the entire model, including both fixed and random effects. Additionally, we generated a conditional density plot with the cdplot function in base R 4.2.0. Conditional density plots describe how the conditional distribution of a categorical variable (*y*-axis) changes over a numerical variable (*x*-axis). In this study, we used it to visualize the relationship between floral visit sequence (as the categorical variable) and pollen count (numerical *x*-axis) for *M. lurida*.

All statistical analyses were performed with RStudio version 2022.07.1 (RStudio Team, 2022), and plotting was done with ggplot2 version 3.3.6 (Wickham, 2016).

Pollen layering

We determined whether the proportion of labeled pollen from the last-visited flower decreases successively with each sticky tape sample, as expected if pollen is layered. Here, the slopes between successive pollen samples (labeled pollen proportion from sample 1 to sample 2, sample 2 to sample 3, and sample 3 to sample 4) on each fly were calculated, then the three slopes were averaged for each of the 14 flies, yielding a total of 14 slopes. A one-sample Wilcoxon test (rstatix R package version 0.70; Kassambara, 2021) was used to determine whether a null slope of zero differed from the population of 14 fly slopes.

We also tested for evidence of pollen layering using a generalized linear mixed model and the glmmTMB function in the R package glmmTMB version 1.1.4 (Brooks et al., 2022) and a beta-binomial distribution and logit link function to describe the proportion of labeled pollen in each sample. In this model, we tested for the effects of the following predictor variables: the number of pre-existing pollen grains per sample, the total number of labeled pollen grains for the fly individual, sample sequence and fly identity as a random effect in the model. Since the data set comprises sequentially repeated sampling of each fly's pollen load, the samples from individual flies cannot be considered as statistically independent samples (Fitzmaurice et al., 2011), but rather they represent identical correlations between pairs of samples. We account for this autocorrelation in the model with the ar1 function of sample and fly individual. The data set employed for this analysis included flies that visited both one and two quantum-dot-labeled flowers. As such, the number of visited labeled flowers was included as a categorical independent variable in the model. The model equation is summarized as Proportion of labeled pollen grains per sample ~ Number of pre-existing grains in corresponding sample × Sample + Total count of labeled pollen + Number of labeled flowers visited (one or two) + ar1(Sample + 0 | Fly).

RESULTS

We captured 25 flies from five families, and a single honey bee (Apidae), from *M. lurida* flowers, of which 14 had visited one labeled flower and 12 had visited two labeled flowers (of differently colored q-dots). Pollen counts from the honey bee were excluded from all analyses because their grooming behaviors differ markedly from flies and they are not regular visitors in this system (Moir et al., 2021).

Pollen preclusion

The mean pollen counts from the second labeled flower (i.e., the last flower the fly visited) was consistently lower than the means from first labeled flower visits (Figure 2A). The log-transformed pollen counts of the second labeled flowers visited were significantly smaller than the first labeled flowers visited by the flies ($t = 3.084$, $df = 10$, $P = 0.012$) with a mean difference of 0.632 (95% confidence interval: 0.175–1.089). The conditional density plot (Figure 2B) suggests that the probability of receiving labelled pollen may be negatively influenced by the amount of pre-existing

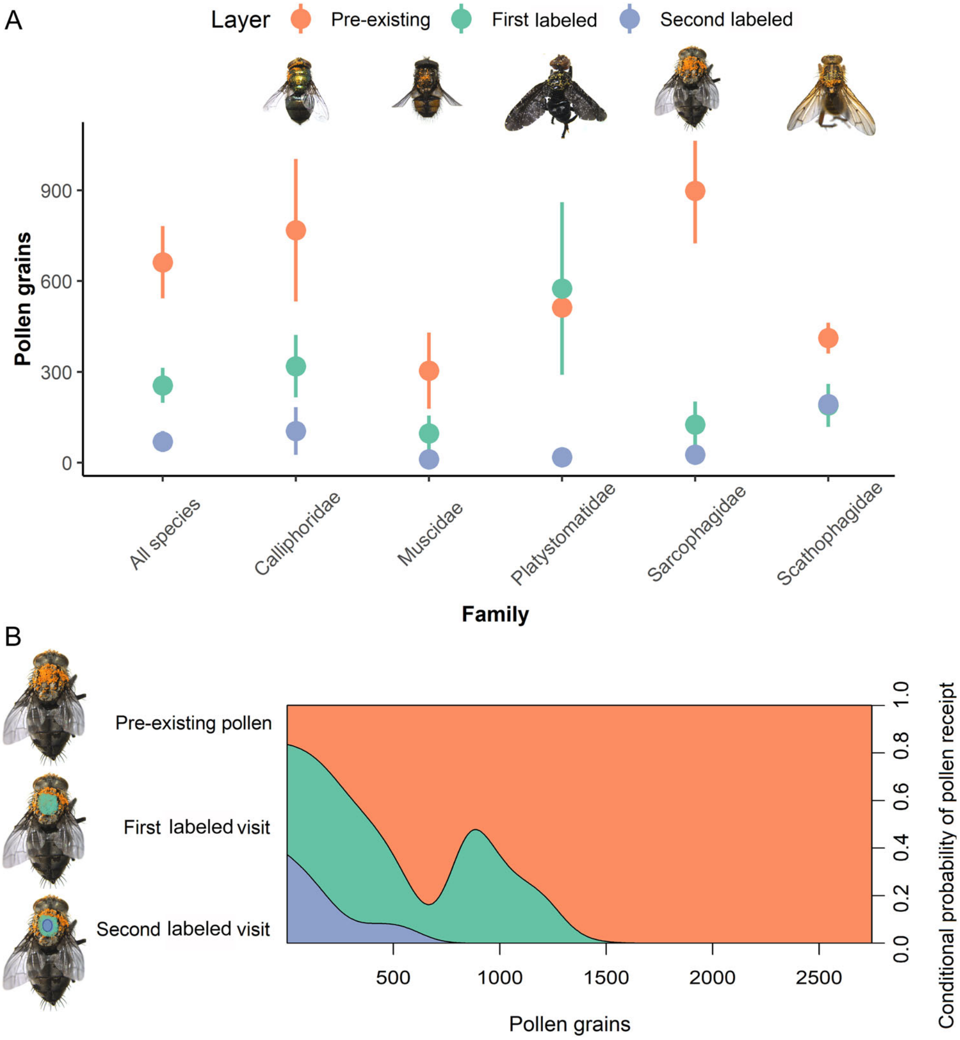

FIGURE 2 (A) Mean number of *Moraea lurida* pollen grains (with SE bars) on pollinators in sequence of floral visits from pre-existing pollen (orange), to first (green) and then second visits (blue) to labeled flowers. The pollen counts are displayed cumulatively for all insect pollinators and per family group. (B) Conditional density plot of *M. lurida* pollen grain counts with floral visit sequence (for all sampled Diptera pollinators). Conditional probabilities are displayed on the right-hand *y*-axis; flower visit sequence (pre-existing: orange, first labeled: green, second labeled: blue) is depicted on the left *y*-axis. This plot shows how large pre-existing pollen loads reduce the probability of pollen load deposition on pollinators from subsequently visited flowers for the first labeled flower visit and even more so for the second labeled flower visit.

TABLE 1 Generalized linear mixed model results, df(Residual) = 11, of the log number of newly deposited pollen grains with (A) the random effect of fly identity nested within fly family predictors and (B) fixed effect predictor results (marginal $R^2 = 0.284$, conditional $R^2 = 0.616$).

(A) Group	Family	Variance	SD
Fly identity	(Intercept)	0.318	0.564
	Muscidae	0.318	0.564
	Platystomatidae	0.318	0.564
	Sarcophagidae	0.489	0.699
	Scathophagidae	0.318	0.564
Residual		0.167	0.408

(B)	Estimate	SE	*z*	*P*
(Intercept)	2.663	0.949	2.805	**0.005**
Previously deposited grains	−0.185	0.346	−0.535	0.593
Muscidae	−0.375	0.314	−1.195	0.232
Platystomatidae	0.144	0.287	0.502	0.616
Sarcophagidae	−0.226	0.269	−0.837	0.402
Scathophagidae	0.129	0.271	0.475	0.634
Labeled flowers (two)	−0.653	0.172	−3.798	**<0.001**

pollen already present on the fly. For example, if a pollinator is carrying more than 1500 pollen grains, there is a highly reduced probability that additional labeled grains will be deposited on the pollinator. The probability of pollen receipt appears to increase when pollen load size decreases below 1500 grains so that when flies carry no pre-existing pollen, the probability of labeled pollen receipt is large (0.8). The probability of pollen receipt by a second labeled flower is always lower than the probability of pollen receipt by the first labeled flower (Figure 2B).

An influence of pre-existing pollen on pollen receipt was not supported by the generalized linear mixed model, which was suggestive of a nonsignificant ($z = 0.535$, $P = 0.593$), weakly negative (−0.185) relation between pre-existing pollen and pollen receipt (Table 1). Fly family and individual fly identity also had weak, nonsignificant effects on newly deposited grains counts. These weak relationships are displayed in Appendix S2 with a gradual downward trend in the model trendline. The only statistically significant model predictor was whether the newly deposited grains emanated from the first or the second visited labeled flower ($P > 0.001$), supporting earlier results (Figure 2A) that pollen loads of from the first labeled flower were higher than pollen loads from the second labeled flower ($z = -3.798$, $P < 0.001$).

Pollen layering

We counted pollen from 14 individual flies with four samples per fly ($N = 56$ samples). Eleven flies had visited a single labeled flower, while three flies had visited two labeled flowers, providing pollen counts for a total of 17 observations. All 14 flies had negative slopes suggestive of decreasing proportions of labeled pollen with each successive sample (Figure 3), and taken together, these differed significantly from a null slope of zero ($z = -3.3$, $N = 14$, $P < 0.001$) with a relatively large effect size ($r = 0.881$). See Appendix S3 for the pollen count data for labeled and pre-existing pollen types across all flies.

Model results showed that the number of pre-existing pollen grains per sample had a very small (statistically nonsignificant) negative effect on the proportion of labeled grains per sample and on the interaction terms with sample as an ordinal variable (Table 2). Additionally, the total count of labeled pollen per fly and number of labeled flowers visited (one or two) were not significant predictors

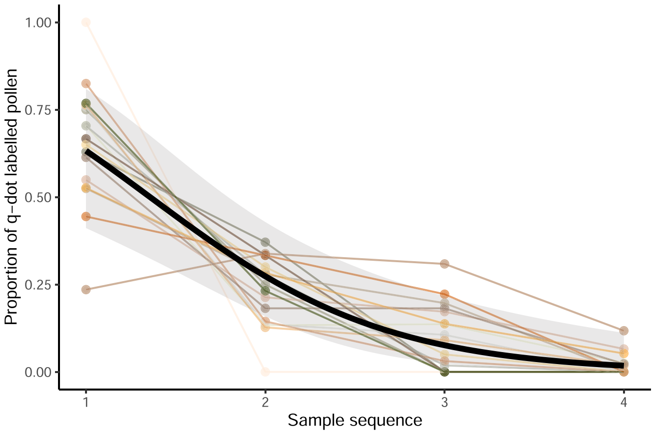

FIGURE 3 The proportions of quantum-dot-labeled *Moraea lurida* pollen within four sequential samples, from the top of the pollen load (sample 1) to the bottom of the pollen load (sample 4). Colored lines represent the proportions of pollen per sample for each pollen count observation; the black line represents a generalized linear model with grey shading as 95% confidence intervals.

TABLE 2 Generalized linear mixed model results df(Residual) = 43, predicting the proportion of labeled pollen grains on successive sticky tape samples (response variable) removed from flies. Random effects model of fly individual: $\sigma^2 = 1.46 \times 10^{-6}$, SD = 0.001.

	Estimate	SE	*z*	*P*
(Intercept)	1.078	1.398	0.771	0.441
Pre-existing pollen per sample	−0.001	0.001	−0.431	0.667
Sample 2	−2.256	1.386	−1.628	0.104
Sample 3	−2.996	1.504	−1.992	**0.046**
Sample 4	−4.665	2.284	−2.043	**0.041**
Total count of labeled pollen on fly	0.000	0.004	−0.026	0.979
Number of labeled flowers visited	−0.148	0.830	−0.178	0.858
Pre-existing pollen: Sample 2	0.001	0.002	0.366	0.714
Pre-existing pollen: Sample 3	0.000	0.003	0.100	0.920
Pre-existing pollen: Sample 4	0.000	0.007	−0.066	0.948

of the proportion of labeled pollen per sample. However, the proportion of labeled pollen found on a sticky tape was dependent on what sample it came from. Here, samples three and four had significantly lower proportions of labeled pollen than sample 1, suggestive of a layering effect.

DISCUSSION

This is the first study to present clear empirical and visual evidence that pollen is layered on the bodies of pollinators after they visit successive flowers with granular pollen. Flies that were captured just after visiting labeled *M. lurida* flowers clearly had quantum-dot-labeled pollen on top of the unlabeled pollen grains from previously visited flowers. Furthermore, we demonstrated that the proportion of labeled pollen grains declines with each successive pollen sample peeled from the pollinator's body with sticky tape. While the application of quantum dots may potentially reduce adherence of labeled pollen grains to pollinators and thus could change the absolute quantities of pollen transferred between insects and flowers, it is not expected to generate the layered pattern discussed above (reduced proportions of labelled pollen as one gets closer to the pollinator's body). Minnaar and Anderson (2019) also found no evidence to suggest that quantum-dot labelling influenced pollen transport efficiency in another flower from the Iridaceae family.

One consequence of structured pollen landscapes is that they may facilitate male–male competition by granular pollen grains, especially if pollen from earlier-visited flowers are overlain by pollen from subsequently visited flowers (Minnaar et al., 2019a). We found some evidence for such competition when pollen loads from the first labeled flower were larger than pollen loads from the second labeled flower, suggestive of interference through pollen preclusion. Nevertheless, the total pre-existing pollen load was a poor predictor of labeled pollen receipt. It is not clear why these results are apparently contradictory, although it may be possible that total pre-existing pollen loads include pollen grains on parts of the fly that have been displaced and are not interacting directly with newly deposited grains. Clearly, more research is needed on the ability of pollen layering to facilitate pollen preclusion in plants with granular pollen.

Higher pollen loads of earlier-visited than later-visited flowers may have occurred if it is harder for pollen grains to adhere to pre-existing pollen surfaces than directly to the hairy body of a pollinator. Hairs and bristles on the bodies of pollinators are likely to provide stability to pollen loads, and if the hairs do not extend all the way past the surface of the pollen layer, then it may be difficult for new pollen to adhere. Protruding hairs on pollinator bodies may also provide a mechanism to scrape pollen from anthers; so when hairs do not protrude from pollen loads, fewer pollen grains may be scraped from anthers during pollinator visits. Consequently, when suitable substrates for pollen deposition become saturated, fewer grains may be deposited per visit, and pollen may be more easily lost from the pollinator after it leaves the flower.

Other kinds of pollen competition

While we looked for evidence of the negative effects of pollen preclusion in this study, pollen layering may also give rise to male–male competition through smothering. Smothering could occur if flowers cover pre-existing pollen from rival males with their own pollen so that their pollen is preferentially transferred to the stigmas of subsequently visited flowers (Minnaar et al., 2019a). Cocucci et al. (2014) demonstrated pollen smothering in some milkweed species; new pollinaria attached themselves to pollinaria already present on pollinators and reduced the success of those pre-existing pollinaria. Pollen smothering and pollen preclusion are expected to have opposite effects on the association between visit sequence and pollen deposition: Smothering should favor the siring ability of the last male flower visited, whereas pollen preclusion should limit the effectiveness of smothering and favor the first male flowers visited. The size of pollen packages has been hypothesized as being constrained by pollinators that groom pollen because they are thought to groom more after receiving large pollen loads (Thomson, 1986; Harder and Thomson, 1989; Harder, 1990). However, smothering is expected to be more effective when pollen is deposited in large loads (Minnaar et al., 2019a) and may thus balance selection for small pollen loads. Consequently, smothering may be especially important if pollinators do not groom or cannot groom the areas of deposition. Another factor that may select for larger pollen loads is that pollen-consuming pollinators may prefer plants or flowers with larger pollen loads (Heiling et al., 2023 [in this issue]). Importantly, our demonstration of pollen preclusion does not mean that smothering is not occurring simultaneously and having opposite effects on plant fitness. Consequently, to quantify whether pollen preclusion or pollen smothering is more important, it is necessary that future studies also investigate the effects of deposition sequence on pollen export (i.e., is more pollen exported by the first or the last males visited).

Possible extent and effects of pollen preclusion

Because grooming is likely to disrupt both the vertical and horizontal structure of pollen landscapes (Minnaar et al., 2019a), we considered pollen layering as a likely outcome when pollinators seldom groom. However, pollen layering may also occur in pollinators that groom if they are unable to reach the areas of pollen deposition (Koch et al., 2017). Pollen grooming may also be linked to pollen edibility, and bumble bees are known to groom pollen from some plant species but not from other species (Hao et al., 2020). Lastly, if grooming pollinators such as bumble bees do not groom after visiting every flower, visiting sequences where pollen is ungroomed may allow layers to build up and affect pollen competition. Consequently, the effects of pollen layering may need to be considered for grooming and non-grooming pollinators.

The importance of pollen preclusion as a consequence of layering may also depend on pollen load size, types of pollinators, and the position and mechanism of pollen placement (See Minnaar et al., 2019a). However, when it does occur, it may be useful to consider its potential ecological and evolutionary effects: Although pollen preclusion is likely to reduce the efficiency of pollen transfer, it is unlikely to have a negative effect on stigmatic pollen deposition, pollen limitation, and female fitness. Pollen preclusion requires pollinators to be carrying large pollen loads, and consequently, it may be most important in systems where there is little or no pollen limitation. While pollen limitation is thought to be an important driver of floral evolution through the female fitness pathway (Knight et al., 2005), pollen competition (i.e., male fitness pathway) may be a more important contributor to floral evolution when pollen is less limited.

Pollen preclusion may also introduce more stochasticity into pollen transfer pathways so that pollinator visitation rates or the mechanical fit between pollinators and flowers is not always the most important aspect governing pollen export. Instead, the size of pre-existing pollen loads on pollinators may have a large impact on whether pollen is deposited on a pollinator or not and many visitations may consequently not result in any pollen deposition onto pollinators. Stochastic pollen transfer could potentially reduce geitonogamy if pollinators visit multiple flowers on a plant but do not transfer pollen with each visit. Furthermore, pollen preclusion may additionally reduce geitonogamy if the pollen transferred is mostly from plants that were visited earlier. Interestingly, male fitness is likely to be negatively affected by pollinators bearing large pollen loads, but female fitness is likely to be positively affected. Several studies have examined pollen deposition onto stigmas, but few of them have examined pollen deposition on pollinators. However, studies of granular pollen transport do suggest that pollen transfer is indeed often very stochastic (Ekimov, 1991).

Many plants appear to control the amount of pollen dispensed (Castellanos et al., 2006), and often, dispensing smaller pollen loads can increase male fitness (Harder and Wilson, 1994), possibly because large pollen loads may be more likely to be groomed or to fall off (Thomson et al., 1986). Pollen preclusion may have similar effects to slow pollen-dispensing strategies because it may slow the rates of pollen dispensing. However, one possible consequence of pollen preclusion is that it may be beneficial to deposit a lot of pollen onto pollinators early in the day (when pollen loads are small), and smaller pollen loads may be more beneficial later in the day when pollinators are more saturated by pollen. Here, one may expect initially rapid anther dehiscence, followed by reductions in anther dehiscence rates. Selection for more showy flowers may also increase the chances of a flower being visited early in the day when pollen loads are still small.

Another strategy that may be used to limit the negative effects of pollen preclusion when pollinators arrive at flowers carrying large pollen loads is to clean pre-existing pollen from pollinators before new pollen is placed (Minnaar et al., 2019a). Minnaar et al. (2019a) envisaged that brush-like structures may have evolved in some flowers such as the lobelias for this purpose. Large stigmas may also fulfill a similar cleaning function, especially if female flowers are typically visited before male flowers. Many flowers develop sequentially, so the top flowers on inflorescences are in the male phase, and the bottom flowers are in the female phase (Harder et al., 2000). Bee pollinators usually visit these flowers from the bottom upward, visiting first the female flowers and then the male flowers. While this sequence is known to reduce geitonogamy (Harder et al., 2000), it may also increase pollen export if the female flowers clean pre-existing pollen from rival pollen donors, allowing the male flowers to place pollen onto relatively pollen-free pollinators.

CONCLUSIONS

Although structured landscapes of granular pollen have been hypothesized to exist on the bodies of pollinators, this study is the first to demonstrate such structuring and one of its consequences: male–male competition. We provide some evidence to suggest that the bodies of pollinators act as arenas, where pollen grains from rival males compete with one another for space via precluding one another from adhering to the surface of the pollinators' body. However, it is not yet clear how the structure of pollen loads is affected by different pollen placement strategies and also how different kinds of male–male competition affect plant fitness and how that varies under different ecological conditions.

AUTHOR CONTRIBUTIONS

M.M. and B.A.: study conception and design; acquisition of data; analysis and interpretation of data; manuscript drafting and revision.

ACKNOWLEDGMENTS

The research was funded by the British Ecological Society (grant no. EA20/1196) and the National Research Foundation (grant no. 137988). We acknowledge CapeNature for permitting the relevant collections (permit numbers CN44-28-15026 and CN35-28-15025). We thank our reviewers and editors who improved this manuscript significantly with their very constructive advice on statistics and contextualizing the study.

DATA AVAILABILITY STATEMENT

All data used in analyses can be found at https://github.com/MonikaMoir/Pollen-layering-and-male-male-competition.

ORCID

Monika Moir http://orcid.org/0000-0003-1095-1910
Bruce Anderson http://orcid.org/0000-0002-7264-4505

REFERENCES

Anderson, B., and C. Minnaar. 2020. Illuminating the incredible journey of pollen. *American Journal of Botany* 107: 1323–1326.

Armbruster, W. S., T. F. Hansen, C. Pélabon, R. Pérez-Barrales, and J. Maad. 2009. The adaptive accuracy of flowers: measurement and microevolutionary patterns. *Annals of Botany* 103: 1529–1545.

Barton, K. 2022. MuMln: Multi-model inference. R package, website: https://cran.r-project.org/web/packages/MuMIn/index.html

Castellanos, M. C., P. Wilson, S. J. Keller, A. D. Wolfe, and J. D. Thomson. 2006. Anther evolution: pollen presentation strategies when pollinators differ. *American Naturalist* 167: 288–296.

Cocucci, A. A., S. Marino, A. P. Wiemer, and S. Alicia. 2014. The buck in the milkweed: evidence of male–male interference among pollinaria on pollinators. *New Phytologist* 203: 280–286.

Duffy, K. J., and S. D. Johnson. 2014. Male interference with pollination efficiency in a hermaphroditic orchid. *Journal of Evolutionary Biology* 27: 1751–1756.

Ekimov, A. I. 1991. Optical properties of semiconductor quantum dots in glass matrix. *Phsica Scripta* 39: 217–222.

Fitzmaurice, G. M., N. M. Laird, and J. H. Ware. 2011. Applied longitudinal analysis, 2nd ed. John Wiley & Sons, NJ, USA.

Goldblatt, P., P. Bernhardt, and J. C. Manning. 2005. Pollination mechanisms in the African genus *Moraea* (Iridaceae, Iridoideae): floral divergence and adaptation for pollinators. *Adansonia* 27: 21–46.

Hao, K., Z. Tian, Z. Wang, and S. Huang. 2020. Pollen grain size associated with pollinator feeding strategy. *Proceedings of the Royal Society, B, Biological Sciences* 287: 20201191.

Harder, L. D., S. C. H. Barrett, and W. W. Cole. 2000. The mating consequences of sexual segregation within inflorescences of flowering plants. *Proceedings of the Royal Society, B, Biological Sciences* 267: 315–320.

Harder, L. D., S. A. Richards, J. Ågren, and S. D. Johnson. 2021. Mechanisms of male–male interference during dispersal of orchid pollen. *American Naturalist* 197: 250–265.

Harder, L. D., and J. D. Thomson. 1989. Evolutionary options for maximizing pollen dispersal of animal-pollinated plants. *American Naturalist* 133: 323–344.

Harder, L. D., and W. G. Wilson. 1994. Floral evolution and male reproductive success: Optimal dispensing schedules for pollen dispersal by animal-pollinated plants. *Evolutionary Ecology* 8: 542–559.

Harder, L. D., and W. G. Wilson. 1998. Theoretical consequences of heterogeneous transport. *Ecology* 79: 2789–2807.

Heiling, J. M., R. E. Irwin, and W. F. Morris. 2023. Conflicting constraints on male mating success shape reward size in pollen-rewarding plants. *American Journal of Botany* 110: e16158.

Kassambara, A. 2021. rstatix: pipe-friendly framework for basic statitical tests. R package version 0.7. Website: https://cran.r-project.org/package=rstatix

Knight, T. M., J. A. Steets, J. C. Vamosi, S. J. Mazer, M. Burd, D. R. Campbell, M. R. Dudash, et al. 2005. Pollen limitation of plant reproduction: pattern and process. *Annual Review of Ecology and Systematics* 36: 476–497.

Koch, L., K. Lunau, and P. Wester. 2017. To be on the safe site – ungroomed spots on the bee's body and their importance for pollination. *PLoS One* 12: 1–16.

Lertzman, K. P., and C. L. Gass. 1983. Alternate models of pollen transfer. *In* C. Jones and R. Little [eds.], Handbook of experimental pollination biology, 474–489. Van Nostrand Reinhold, NY, NY, USA.

Marcelo, V. G., F. M. Darcie Marquitti, M. Vallejo-Marin, and V. L. G. D. Brito. 2022. Pollinator grooming behavior alters pollen landscapes on bees bodies and increases pollen carryover to other flowers. *bioRxiv* 2022–07. https://www.biorxiv.org/content/10.1101/2022.07.14.499901v1. abstract [preprint].

Minnaar, C., and B. Anderson. 2019. Using quantum dots as pollen labels to track the fates of individual pollen grains. *Methods in Ecology and Evolution* 10: 604–614.

Minnaar, C., and B. Anderson. 2021. A combination of pollen mosaics on pollinators and floral handedness facilitates the increase of outcross pollen movement. *Current Biology* 31: 3180–3184.

Minnaar, C., B. Anderson, M. L. De Jager, and J. D. Karron. 2019a. Plant–pollinator interactions along the pathway to paternity. *Annals of Botany* 123: 225–245.

Minnaar, C., M. L. de Jager, and B. Anderson. 2019b. Intraspecific divergence in floral-tube length promotes asymmetric pollen movement and reproductive isolation. *New Phytologist* 224: 1160–1170.

Moir, M., S. D. Johnson, and B. Anderson. 2021. Remarkable floral colour variation in the functionally specialized fly-pollinated iris, *Moraea lurida*. *Biological Journal of the Linnean Society* 200: 218–232.

Morris, W. F., M. Mangel, and F. R. Adler. 1995. Mechanisms of pollen deposition by insect pollinators. *Evolutionary Ecology* 9: 304–317.

Muchhala, N., and J. D. Thomson. 2010. Fur versus feathers: pollen delivery by bats and hummingbirds and consequences for pollen production. *American Naturalist* 175: 717–726.

Muchhala, N., and J. D. Thomson. 2012. Interspecific competition in pollination systems: costs to male fitness via pollen misplacement. *Functional Ecology* 26: 476–482.

R Core Team. 2020. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. Website: http://www.R-project.org/

RStudio Team. 2022. RStudio: integrated development environment for R. RStudio, PBC, Boston, MA, USA. Website: http://rstudio.com

Thomson, J. D., M. V. Price, N. M. Waser, and D. A. Stratton. 1986. Comparative studies of pollen and fluorescent dye transport by bumble bees visiting *Erythronium grandiflorum*. *Oecologia* 69: 561–566.

Wickham, H. 2016. ggplot2: Elegant graphics for data analysis, 2nd ed. Springer International Publishing, Cham, Switzerland.

SUPPORTING INFORMATION

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

APPENDIX S1. Photographs of fly pollinators caught directly after visitation to *Moraea lurida* flowers illuminated in a quantum-dot excitation box. The photographs display evidence of pollen layering in plants; granular and quantum-dot-labeled pollen grains are on top of unlabeled pollen grains from previously visited flowers. Labeled grains fluoresce yellow-green in A–C and orange in D and E; a few grains are indicated with arrows.

APPENDIX S2. Scatterplot displaying the number of newly/recently deposited pollen grains with the number of previously deposited pollen grains per fly family as indicated by color (both counts were log-transformed). Point shapes indicate whether the observation was from the first labeled flower visited by the fly (circle) or the second labeled flower (triangle). The black line represents a generalized linear model with grey shading as 95% confidence intervals.

APPENDIX S3. Boxplots of the log-transformed pollen counts for sample sequence for the two categories of labeled and pre-existing pollen types.

How to cite this article: Moir, M., and B. Anderson. 2023. Pollen layering and male–male competition: Quantum dots demonstrate that pollen grains compete for space on pollinators. *American Journal of Botany* e16184. https://doi.org/10.1002/ajb2.16184