

Novel insights into pollen movement and floral evolution revealed by quantum dots

Corneile Minnaar

Supervisor: **Prof. Bruce Anderson**, Department of Botany and Zoology, Faculty of Science, Stellenbosch University



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DECLARATION

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This dissertation includes one peer-reviewed paper (Chapter 5) published in the peer-reviewed journal, *Annals of Botany* (doi: 10.1093/aob/mcy167). The development and writing of the paper were the principal responsibility of myself, and the following co-authors contributed: Marinus L. de Jager, Jeffrey D. Karron and Bruce Anderson.

This dissertation also includes one paper posted on a preprint server (Chapter 2), *BioRxiv* (doi: 10.1101/286047). The development and writing of the paper were the principal responsibility of myself, with contributions from Bruce Anderson.

ABSTRACT

To understand the evolution of flowers and mating systems in animal-pollinated plants, we have to directly address the primary function for which flowers evolved—the movement of pollen from anthers to stigmas. Yet, despite a long and distinguished history of making significant advances in understanding of natural selection and evolution, the field of pollination biology has largely studied pollen movement indirectly (e.g., pollen analogues or paternity assignment to seeds) due to a lack of suitable pollen tracking methods. Consequently, understanding of pollen export mechanisms and male reproductive strategies has been limited. In Chapter 2, I describe and test a novel technique to label and track the movement of pollen grains using quantum dots. I show that quantum dots can be attached to pollen grains of several different species and that their attachment to pollen appears not to affect pollen dispersal. In Chapter 3 I employ quantum dot pollen-labelling to test the placement and transfer of pollen in a unique population of *Lapeirousia anceps* (Iridaceae) with a bimodal distribution in floral tube length. I find that floral-tube length acts as a strong reproductive isolation barrier between plants with short-tubed flowers and long-tubed flowers. In Chapter 4 I use quantum dots to explore the function of floral handedness in *Wachendorfia paniculata*. Based on pollen transfer experiments, pollen moves predominantly between left- and right-handed flowers, rather than between flowers of the same type. These experiments allowed the creation of the first map of anther-level pollen grain placement on the bodies of bees. Pollen placement maps revealed pollen quality heterogeneity across pollinator bodies, and that stigmas of *W. paniculata* aligned with areas on bee bodies where the capture of outcrossed pollen is most likely. This led to greater than expected outcross pollen movement. These findings underline the importance of studying micro-scale pollen landscape composition on pollinator bodies and how stigmas interact with them. The thesis concludes with a review which assesses the history of studying male function in plants and identifies critical gaps in our understanding of the ecology and evolution of pollen transport. I explore male reproductive

function along the male fitness pathway, from pollen production to ovule fertilization. At each step of the pathway to paternity, I discuss evolutionary options to overcome barriers to siring success. In particular, I highlight a newly emerging idea that bodies of pollinators function as a dynamic arena facilitating intense male–male competition, where pollen of rival males is constantly covered or displaced by competitors. This perspective extends the pollen-competitive arena beyond the confines of the stigma and style, and highlights the opportunity for important new breakthroughs in the study of male reproductive strategies and floral evolution.

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CH. 1. INTRODUCTION

The transition from abiotic to biotic gamete vectors has led to the adornment of plants across earth with spectacular and diverse displays of flowers. For centuries philosophers, monks, and biologists, beguiled by their beauty and complexity, studied flowers and their interactions with animals with various hypotheses put forward to explain the function of flowers. These hypotheses developed over time from the patently absurd [e.g., insects do nothing but steal the sweet seed-nourishing juice (nectar) from flowers or, flowers are beautiful because they are the sacred sites of marriage between male and female parts of the flower (see historical references in Lorch 1978; Baker 1979; Taiz and Taiz 2017 and references therein)], towards a more reasonable biological understanding of floral function [flowers attract insects and exploit them as pollen vectors (Sprengel, 1793)]. However, it was only after Darwin's body of work (Darwin, 1862, 1876, 1877) shed light on the evolutionary context of flowers that their diverse forms began to make sense—floral traits evolve to recruit and manipulate pollinators to transport pollen between flowers, thereby increasing outcrossing and reducing the negative consequences of self-pollen transfer.

The next century of work on floral evolution focused on floral traits as vector-mediated outcrossing mechanisms until the unapologetic and compelling arguments presented by Janzen (1977) and Willson (1979) forced biologists to consider separate avenues of reproductive fitness through male and female sexual functions of hermaphroditic flowers. In particular, biologists were compelled to address the long-neglected role of male fitness in floral evolution. As with any new investigation into evolutionary processes, researchers aimed to establish functional links between traits displayed by individuals and components of their fitness. On average, less than 2% of pollen grains produced by flowers successfully reach conspecific stigmas (Harder and Thomson, 1989; Gong and Huang, 2014). The remaining 98% are lost to multiple potential fates (Inouye *et al.*, 1994), with each avenue of pollen loss representing an opportunity for selection

to act on male reproductive traits that reduce the amount of wasted reproductive potential (Harder and Thomson, 1989; Harder and Barrett, 1996). The transport of pollen from anthers to stigmas is clearly an important phase for selection on male reproductive traits. However, the functional bridge between observed traits and realised male fitness—pollen movement—has proved exceedingly difficult to study because of a lack of techniques to track the movement of pollen.

The lack of suitable techniques to track pollen has also limited our understanding of important aspects of pollination that go beyond the male fitness consequences of pollen export. The massive diversity of angiosperms is often attributed to the ease with which pollen movement, and therefore gene flow, can be altered by relatively small changes in floral traits. For example, minor mutations in a single allele can lead to a novel flower-colour phenotype which, in turn, attracts novel flower visitors (Bradshaw and Schemske, 2003). If the ancestral and novel colour morphs attract different sets of pollinators, reproductive isolation, and eventually speciation may ensue. Divergence in various other floral traits may similarly result in reproductive isolation (Grant, 1949). For example, divergence in floral-tube length may cause mechanical isolation between short-tubed and long-tubed plants, since pollen placement, and therefore transfer, may occur on different parts of pollinators (Kay, 2006). Floral trait divergence and subsequent pollen movement isolation may represent one of the most important diversification mechanisms on earth. Unfortunately, studies of these mechanisms have largely relied on indirect, and confounding, proxies of pollen movement isolation (e.g., pollen analogues or post-fertilisation measures mating patterns). The ability to track pollen grain movement among divergent floral phenotypes will finally allow us to fully explore the importance of pollen movement isolation in contributing to angiosperm diversity.

More than two centuries after Sprengel first recognised the function of flowers in promoting pollen movement by insects (animals), we still know very little about the dynamic process of animal-mediated pollen transport. While increasingly affordable and accurate

paternity analyses allow us to identify which plants sired which seeds (Jones *et al.*, 2010), this information confounds mechanisms that determine the outcomes pollen export with post-pollination effects of fertilisation and seed-development processes. A complete understanding of floral function and evolution therefore requires the ability to track all pollen grains, not just those that successfully fertilise ovules.

Pollen tracking methods to date

While generally applicable pollen tracking methods remain limited, successful pollen tracking has been achieved for a small proportion of flowering plant species. The best success in tracking pollen comes from a single plant family, the Orchidaceae. Orchid pollen grains are contained in large pollen packets called pollinaria, which can be stained using dyes (Peakall, 1989) or labelled using uniquely coded microfilm tags (Nilsson *et al.*, 1992). The dyed massulae (subunits of a pollinarium) can then be recovered and counted from other flowers once transferred (Peakall, 1989; Johnson and Harder, 2018). However, most angiosperms produce granular pollen, and attempts at staining entire anthers containing granular pollen grains with dye have largely failed, as pollen grains do not absorb dyes well in situ. Consequently, this method of pollen staining has only been used only three times to our knowledge (Huang and Guo, 1999; Huang and Shi, 2013; Armbruster *et al.*, 2014). Pollen grains have also been labelled with radioactive elements (Colwell, 1951), neutron-activated elements (Gaudreau and Hardin, 1974; Handel, 1976), and ¹⁴C labels (Reinke and Bloom, 1979; Pleasants *et al.*, 1990). However, concerns about environmental exposure to radioactive labels, the complicated and time-consuming process of detection of neutron-activated and ¹⁴C labels (up to 14 weeks), and the limited number of unique labels (usually just one), ultimately rendered these methods ineffective.

Instead of attempting to directly label pollen grains, some researchers have used fluorescent dye particles as a pollen proxy (Stockhouse II, 1976; Price and Waser, 1982; Waser

and Price, 1982). In some cases, fluorescent dye particle deposition on stigmas correlates relatively well with pollen grains deposited per visit (Waser and Price, 1982; Fenster *et al.*, 1996; Van Rossum *et al.*, 2011). However, pollen grains are often found on stigmas when dye is not, and vice versa (Waser and Price, 1982). In other studies, dye particles significantly over- or underestimated pollen transfer (Thomson *et al.*, 1986; Waser, 1988; Campbell, 1991; Adler and Irwin, 2006). Micronized metal (Zn and Sn) dusts have also been applied to dehisced anthers (Wolfe *et al.* 1991). While some of the metal particles labelled grains directly, their presence on pollen grains was likely superficial. Moreover, to detect metal dust particles, samples have to be gold-plated for subsequent scanning electron microscopy. To our knowledge, this method has never been applied outside of the original study which reported it.

In rare cases, intraspecific colour variations of pollen grains have been used to track pollen (e.g., Thomson and Plowright, 1980; Holsinger and Thomson, 1994). Similarly, intraspecific variation in pollen size associated with different anther levels in heterostylous morphs have been exploited to quantify pollen movement (Nichols, 1985; Harder and Barrett, 1995; Stone, 1995). Unfortunately, these methods are limited in their applicability, as most other systems do not have pollen-colour or -size polymorphisms which can be exploited. Recently, researchers have been able to genotype individual pollen grains using microsatellite markers (Matsuki *et al.*, 2007) allowing researchers to identify the individual plant origin of pollen grains found on floral visitors (Matsuki *et al.*, 2008; Hasegawa *et al.*, 2009, 2015) and stigmas (Hasegawa *et al.*, 2009). However, this technique is labour intensive and expensive, requiring careful pollen isolation, DNA extraction, and sequencing of individual pollen grains—a simpler, less expensive method is needed to make the study of pollen movement accessible to more pollination biologists.

The primary aim of this thesis was to begin to bridge the pollen movement gap in pollination biology. To do this, I first developed and validated an inexpensive method to label pollen grains using fluorescent nanocrystals (quantum dots) which would allow researchers to

track the movement of individual pollen grains in most angiosperms (Chapter 2). I then used this method to explore pollen movement in a population with a bimodal distribution in tube length to determine the importance of mechanical isolation as a gene-flow barrier and potential speciation mechanism (Chapter 3). My final data chapter (Chapter 4) investigates the function of handed flowers in promoting outcrossing, by generating detailed, individual pollen-grain-placement maps on the bodies of pollinators combined with population-level pollen movement between left- and right-handed flower morphs. I conclude my thesis with a perspective review (Chapter 5) stimulated by the findings in my data chapters. In particular, the finding that pollen grains from different donor plants combined on pollinator bodies to form heterogenous pollen landscapes led me to explore the practically ignored concept of pollen grain competition for space on vector bodies. In this final, and admittedly speculative, chapter, I explore how different pollen placement strategies may lead to the formation of various pollen landscapes on pollinator bodies, and hypothesise various mechanisms through which the male function in plants could alter these pollen landscapes to increase reproductive success.

CH. 2. A NOVEL METHOD TO TRACK THE FATE OF INDIVIDUAL POLLEN GRAINS USING QUANTUM DOTS AS POLLEN LABELS

INTRODUCTION: QUANTUM DOTS AS POTENTIAL POLLEN LABELS

Quantum dots are extremely small nanoparticles of semiconductor metals. They range in diameter from 2 to 10 nm (10 to 50 atoms across) (Neeleshwar *et al.*, 2005) causing atom-like confinement of electrons and electron-holes in bound discrete states (Gammon, 2000). When relatively large semiconductor metal objects are excited (e.g., when an electrical charge is applied) electrons bound to atoms (in the valence band), become free (jump to the conduction band) and can move within the crystal lattice of a large semiconductor object (i.e., electrical conduction) (Cho, 1979; Dean and Herbert, 1979). Electrons behave in this way as long as the semiconductor object is large relative to the wavelength of the electrons (Brus, 1984). In contrast, when the semiconductor object is reduced to the nanoscale (quantum dots), the valence and conduction energy bands that the electrons can occupy become discrete (Yoffe, 2001). In a spherical quantum dot, this can be theoretically visualised as a ball with discrete layers: the outer layer is the conduction band, and the inner layer is the valence band. When electrons become excited (usually through UV radiation), they jump from the valence band to the conduction band (Ekimov, 1991), leaving behind an electron-hole (a quasiparticle with a positive charge relative to electrons) (Yoffe, 2001). Normally, in a large semiconductor object, electrons can move freely, independent of the electron-hole. But, because of the tight confinement of electrons, the close proximity of the electron and the electron-hole form an exciton which jumps to the conduction band (Cho, 1979; Dean and Herbert, 1979; Kusrayev, 2008). When the exciton returns to the ground state (valence band), it emits light energy, causing the quantum dot to fluoresce (Ekimov, 1991). Therefore, quantum dots can emit bright light in the visible spectrum when excited with UV radiation (Ekimov and Onushchenko, 1981, 1982; Brus, 1984). The size of the quantum dot determines the radius of the two energy bands, and therefore the exciton's light emission wavelength (Yoffe, 2001). The emission colour of quantum dots can therefore be tuned precisely by altering the size of the quantum dot.

Quantum dots were first employed as bio-labels two decades ago (Bruchez Jr., 1998; Chan, 1998) and offer several advantages over traditional bio-labels (Jaiswal and Simon, 2004): (1) Their emission colour can easily be manipulated to specification by controlling the size of quantum dots produced. (2) They have much greater photostability than traditional fluorescent markers which lose their fluorescence comparatively quickly under excitation. (3) They have very large Stokes-shifts (difference between excitation and emission wavelengths), and therefore multiple different coloured quantum dots can be excited by a single light source and detected simultaneously. (4) The ease with which bio-functional groups can be attached to quantum dots allows them to be used as bio-labels for virtually any biomolecule, potentially including pollen grains.

Initially, quantum dots were made from toxic heavy-metal semiconductor cores (primarily Cadmium), which precluded their use in natural environments (Hardman, 2006). However, several commercially available, non-toxic alternatives have recently been developed (Xu *et al.*, 2016). Most commercially available quantum dots carry oleic-acid ligands which allow them to be dissolved in non-polar solvents. Since oleic acid is lipophilic, it may bind to lipid-rich pollenkitt (Pacini and Hesse, 2005) surrounding pollen grains, allowing attachment of quantum dots to pollen grains. I evaluated the potential of quantum dots as pollen labels using three important functional criteria: (1) quantum dots must attach directly to pollen grains; (2) the application of quantum dots to an anther should result in most pollen grains being labelled; (3) quantum-dot labels should not affect pollen grain transport.

MATERIALS AND METHODS

Proposed Method

Quantum dots

For all experiments, I used heavy-metal-free CuInSe_xS_{2-x}/ZnS (core/shell) quantum dots (UbiQD, Los Alamos, USA) with zinc oleate ligands (zinc complex with oleic acid). These quantum dots are commercially available in four colours in the visible range with peak fluorescence (± 10 nm) at 550 nm (green), 590 nm (yellow), 620 nm (orange), and 650 nm (red). I dissolved quantum dots in hexane to make a dispensable quantum-dot solution. The concentration and volume of quantum-dot solutions applied to anthers were tailored to suit pollen and anthers of each plant species tested (see below). Quantum-dot solutions were stored in complete darkness below 30°C inside small 2 ml clear-glass vials (9 mm thread; 12 x 32 mm, product number—29371-U; Supelco, Bellefonte, PA, USA) closed with plastic caps containing PTFE/silicone septa (9 mm polypropylene cap; PTFE/silicone septum; product number—29319-U; Supelco, Bellefonte, PA, USA). The vial septum composition is necessary for safe long-term storage of quantum-dot solutions as non-PTFE/silicone septa and plastic caps are eroded by hexane fumes.

Quantum dot application to pollen

I applied quantum dots directly to individual dehisced anthers using a micropipette (0.1–2.0 μ l; product code—p3942-2; Biopette, Labnet International, Edison, NJ, USA) and extra-long 10 μ l pipette tips (SuperSlik™ 10 μ l Extra Long pipette tips; product code—1165-800; Labcon, Petaluma, CA, USA). The very narrow inner diameter of these pipette tips prevents volatile hexane from flowing out of the tip before the quantum-dot solution can be applied to pollen. When applying quantum-dot solution to anthers, I was careful to avoid direct contact between pollen and the pipette tip. I held the pipette tip as close as possible to the upper edge of an anther and ejected the quantum-dot solution slowly onto the anther, allowing it to gently flow across the anther and cover all pollen grains. Hexane is highly volatile (boiling point: 68°C), and

therefore evaporates seconds after application, leaving behind quantum dots which putatively bind to the pollenkit of pollen grains through lipophilic ligands.

To visualise or "read" potential quantum-dot pollen-labels, I designed and built an inexpensive quantum-dot excitation box which can be placed under any standard dissection microscope allowing visualisation of quantum dots on stigmas as well as insects (Fig. 2.1) (see Supplementary information for 3D-printable design files).

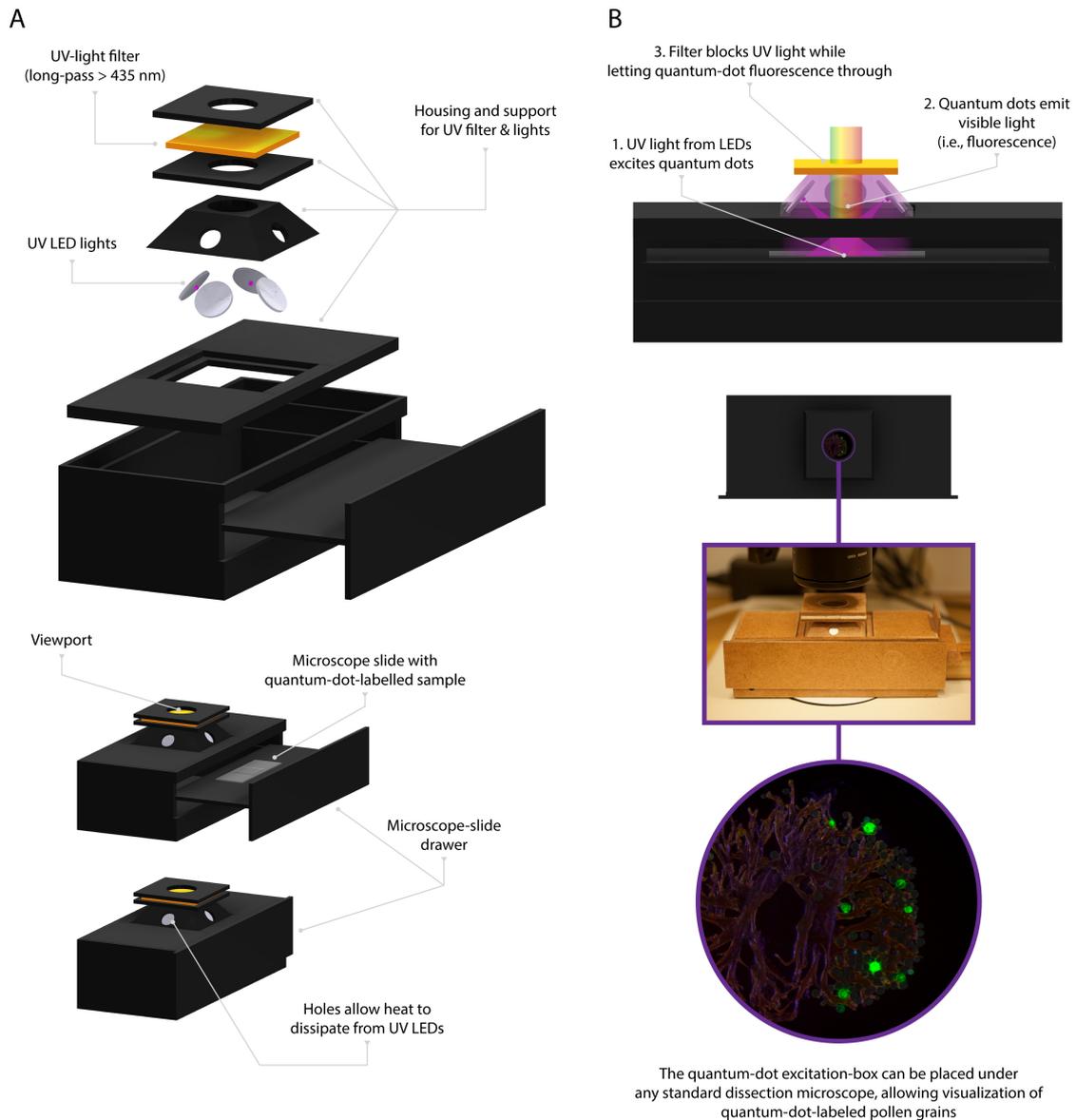


Figure 2.1. Diagram showing the components (A) of the quantum-dot excitation box and how it functions (B). The box contains four LED lights (Intelligent LED Solutions, C3535 1 Powerstar Series UV LED, 390 nm, 400 mW, 125° light angle, 4-Pin) as the UV excitation source. Quantum dots are viewed through a viewport containing a UV blocking, long-pass filter (blocking wavelengths < 435 nm; Schott GG435, 50 x 50 mm) which is aligned underneath the microscope objective. The box can hold microscope slides or insects on a drawer which slides in and out of the box.

Method Validation

I evaluated criteria 1 (quantum dots must attach directly to pollen grains) and 2 (quantum dots should label most pollen grains in the anther) using four different plant species from four different families: *Wachendorfia paniculata* Burm. (Haemodoraceae), *Sparaxis villosa* (Burm. f.) (Iridaceae), *Arctotheca calendula* (L.) Levyns (Asteraceae), *Oxalis purpurea* (L.) (Oxalidaceae). Flowers were collected from urban parks and gardens in Stellenbosch, Western Cape, South Africa. These four species were selected as representative of typical plants to which the quantum-dot labelling technique may be applied. For each species, I determined the appropriate volume for quantum dot application: I started by applying a 0.1 μl dose of pure hexane to an individual anther and checked whether the volume was sufficient to cover the entire anther. If the volume was too small, I increased the dosage incrementally by 0.05 μl until a single dose was sufficient to cover the entire anther, but not flow beyond anther tissue. Suitable dosage volumes for the four different species were as follows: 0.30 μl per anther for *W. paniculata*; to 0.50 μl per anther for *S. villosa*; 0.35 μl per anther for *A. calendula*; and 0.15 μl per anther for *O. purpurea*. This roughly equated to the anther volume (measured as product of the length, height, and width of the anther to the closest 0.5 mm) divided by five. Volumes may be increased in hot weather as heat increases evaporation rates of quantum dot solutions from pipette tips as well as anthers. The effects of heat can also be ameliorated by keeping quantum dot solutions cold using ice packs in the field.

Next, I determined the appropriate quantum-dot concentration for quantum-dot application to pollen. I initially applied a 2 mg/ml (quantum-dot/hexane) solution at the ideal volume determined for each species. I then placed labelled pollen next to unlabelled pollen on microscope slides using two separate, sterile pipette tips. I examined the grains using the quantum-dot excitation-box to check whether labelled grains were easy to distinguish from unlabelled grains. Although labelled grains were distinguishable from unlabelled grains at 2 mg/ml (quantum-dot/hexane) solution, I increased the concentration of the quantum-dot

solution to 5 mg/ml for all species to ensure that labelled grains were clearly distinct from unlabelled grains.

Do quantum dots attach to pollen grains (criterion 1)?

To test whether quantum dots physically attach to pollen grains, I applied quantum-dot solutions to five different anthers for each of the four species. I then removed the anthers and placed each inside a small centrifuge tube (0.3 ml) containing 100 μ l of 70% ethanol–distilled water solution. To dislodge pollen from anthers into the ethanol solution, I vortexed each tube for two minutes. The 70% ethanol solution acts as a polar solvent and will therefore not remove pollenkit, which is primarily hydrophobic (Pacini and Hesse, 2005), from pollen grains. If dots are physically attached to pollen grains through lipophilic interactions, they should remain on pollen grains when agitated in ethanol. However, if quantum dots are not physically attached to pollen grains, they would likely be removed from pollen grains during agitation in ethanol. After vortexing, I waited two minutes to allow unattached quantum dots to separate from pollen grains suspended within the solution. I then took five 10 μ l subsamples of the pollen-ethanol suspension using a micropipette and expelled each subsample onto a separate microscope slide to see if quantum dots remained attached to pollen grains using the quantum-dot excitation box.

What proportion of grains in an anther are labelled (criterion 2)?

In addition to confirming quantum dot attachment in the experiment above, I also determined the proportion of labelled to unlabelled grains by counting every labelled and unlabelled pollen grain in each 10 μ l subsample. While not all pollen grains present within anthers were counted, I assumed the random sampling of pollen grains after vortexing was representative of all pollen grains within an anther.

Do quantum dots influence pollen transport (criterion 3)?

If quantum dots successfully attach to pollen grains, the presence of quantum dots may still influence how grains get transported, limiting their utility for biologically realistic estimates of pollen movement. To test whether quantum-dots influence pollen transport, I conducted multiple pollen-transfer trails with labelled and unlabelled *S. villosa* pollen using honey bees *Apis mellifera capensis* Eschscholtz, 1822 as pollen vectors.

Comparing pollen transfer dynamics of labelled and unlabelled pollen under natural conditions is impossible because pollen transferred from target donors cannot be distinguished from pollen already on the vector from previous donors. Therefore, comparisons of labelled and unlabelled pollen transfer require captive vectors which are clean of any pollen.

Honey bee maintenance and training

I obtained ca. 400 newly-emerged adult honey bees from brood frames placed inside an incubator at 36°C for 48 hours. All adult honey bees were removed from brood frames before incubation to ensure that honey bees taken from frames after incubation were newly-emerged. I placed the honey bees inside a polystyrene mini-nucleus hive (Apidea: Bruck-enstrasse 6 CH-3005, Bern, Switzerland) containing preformed wax comb with bee bread and a constant supply of 50% sugar solution. The mini-nucleus hive was kept inside a flight cage (70 x 70 x 140 cm) with a central partition dividing the cage into two equal halves. One half of the flight cage housed the bees for training and maintenance, while the other half was reserved for pollen transfer experiments (see below). The flight cage was kept indoors at 25-30°C and a 12:12 hour, light:dark cycle.

Once bees were actively flying within the flight cage (one week), I trained them to collect nectar from *S. villosa* flowers. To train bees, I placed six emasculated *S. villosa* flowers inside the flight cage for at least four hours per day. Each flower was securely attached to the top of 30 cm long bamboo skewers secured to the cage floor. Flower stems were held inside

small centrifuge tubes (0.3 ml) containing water. I supplemented nectar (20% w/w sucrose/tap water added to flowers in 5 μ l doses) in flowers when empty to ensure that flowers remained rewarding irrespective of honey bee foraging rate. Honey bees foraged consistently from flowers after three days of training, at which point I commenced pollen transfer experiments. Training continued throughout experiments.

Pollen transfer experiments

At the start of each experimental day, I picked unopened *S. villosa* flowers in the morning and randomly split them into two groups, donors and recipients, in a 1:10 ratio. Recipients were emasculated in the morning prior to anther dehiscence and used the following day once stigmas were mature and receptive. In addition, I checked the stigmas of all recipients for any pollen grains under a dissection microscope. All flowers with stigmatic pollen were discarded. I removed the stigmas of donor flowers in the morning prior to anther dehiscence and assigned them randomly to one of two treatments: labelled or unlabelled pollen. Once anthers were fully dehisced, I either left the flowers as they were (unlabelled pollen) or applied quantum-dot solution to the anthers as described before (labelled pollen).

For each pollen transfer trial, I placed 11 *S. villosa* flowers in a line perpendicular to the cage partition (spaced 5 cm apart) on the experimental side of the flight cage. The first flower in the line acted as the pollen donor, while the next 10 flowers acted as pollen recipients. The donor flower was placed 2 cm away from a small door (5 x 10 cm) in the cage partition. This door could be opened and closed from the outside of the cage, to allow or prevent bees passing from one part of the cage to the other. Flowers were attached to bamboo skewers as before, but I allowed part of the bamboo skewer to extend above flowers which enabled me to cover individual flowers with a small plastic cup to prevent bees from visiting any flower more than once.

To start a pollen transfer trial, I opened the door in front of the donor flower. At the same time, I held a piece of cardboard behind the donor flower so that honey bees could not see the recipient flowers. I waited for a honey bee to fly through the door and visit the donor flower, after which I closed the door and removed the cardboard blocking the recipient flowers. Once the bee finished visiting the donor flower, I covered it with a plastic cup to prevent repeat visitation. Thereafter, I ensured that all flowers received only one visit by covering flowers with a plastic cup (numbered to indicate visit sequence) immediately after being visited. Once a bee finished visiting all of the flowers, I captured and killed it to ensure that pollen was not transported back to the training area. I completed 15 pollen-transfer trials for each treatment. Only three trials out of 30 resulted in visits to 9 recipients instead of all 10 (unlabelled: 2; labelled: 1).

After each trial, I recorded the position in the transfer sequence for each flower and the treatment applied to the donor flower. To account for potential effects of flower morphology on the likelihood of stigma and anther contact with bees, I measured the closest distance between each recipient stigma and the lower lip of the flower (stigma height), and the closest distance between the donor's anthers and the lower lip of the flower (anther height). I harvested the stigmas of each recipient and placed them on individual microscope slides. To prepare stigma slides, I squashed unlabelled stigmas under a cover slip with melted Fuschin gel, while labelled stigmas were squashed under a cover slip without a mounting medium and the edges of the cover slip secured and sealed using transparent sticky tape. Stigma slides were stored at -20°C until pollen could be counted. I counted pollen using a standard dissection microscope with labelled pollen visualised inside the quantum-dot excitation box.

I modelled pollen transfer using a non-linear, mixed-effects regression of the number of pollen grains deposited on stigmas (pollen count) as an exponential decay function of visit-sequence number (*visit seq.*), stigma height (*stigma h.*), and anther height (*anther h.*):

$$\text{pollen count} = [a + b(\text{anther h.}) + c(\text{stigma h.})] \cdot e^{-d(\text{visit seq.})}$$

The expression within the square brackets determines the number of pollen grains at the first visit in the sequence, while the expression to the right of the square brackets determines the rate of decay (pollen depletion) as a function of visit sequence number. Therefore a , represents an independent intercept, and b and c are expected to be negative because increased stigma and anther heights may result in decreased transfer of pollen due to poor contact with honey bees. The parameter d is expected to be positive and controls the magnitude of pollen-transfer decay with increasing visit sequence number. I accounted for potential effects of individual bees on pollen transfer by allowing a and d to vary for each individual bee (i.e., individual bee ID was included as a random effect on a and d).

To determine if treatment had an effect on pollen carryover, parameter estimates for a and d for each treatment were computed separately and compared using Wald-type t-tests (Pinheiro and Bates, 2000). Models were computed in R (R Core Team, 2017) using the "nlme" function (package nlme: Pinheiro *et al.* (2017)).

RESULTS

Do quantum dots attach to pollen grains (criterion 1), and what proportion of grains in an anther are labelled (criterion 2)?

Quantum dots remained attached to pollen grains even after agitation in 70% ethanol, in all subsamples, for all four species (25 subsamples per species). Moreover, the majority of pollen grains in each subsample were labelled by quantum dots. No unlabelled grains were found in any of the subsamples for *A. calendula*, while nearly all grains were labelled for *W. paniculata* (mean proportion \pm SE: 0.97 \pm 0.01) and *S. villosa* (0.92 \pm 0.01) (Fig. 2.2). The proportion of grains labelled for *O. purpurea* was comparatively low, but most grains were still labelled (0.74 \pm 0.02).

Do quantum dots influence pollen transport (criterion 3)?

Parameter estimates for a and d for unlabelled (a : mean \pm SE=876.70 \pm 71.30; d : mean \pm SE=0.28 \pm 0.03) and labelled (a : mean \pm SE=886.44 \pm 70.24; d : mean \pm SE=0.25 \pm 0.02) treatments did not differ significantly (a : $t_{(262)}=-0.60$; $p=0.55$; d : $t_{(262)}=0.36$; $p=0.72$). Stigma height (c) had a significant effect on pollen transfer (mean \pm SE=-90.01 \pm 8.48, $t_{(262)}=-10.61$, $p<0.0001$), while anther height (b) had a marginal effect (mean \pm SE=-13.40 \pm 6.82, $t_{(262)}=-1.97$, $p=0.05$). These results indicate that quantum dot application to anthers of *S. villosa* did not affect pollen carryover (Fig. 2.3).

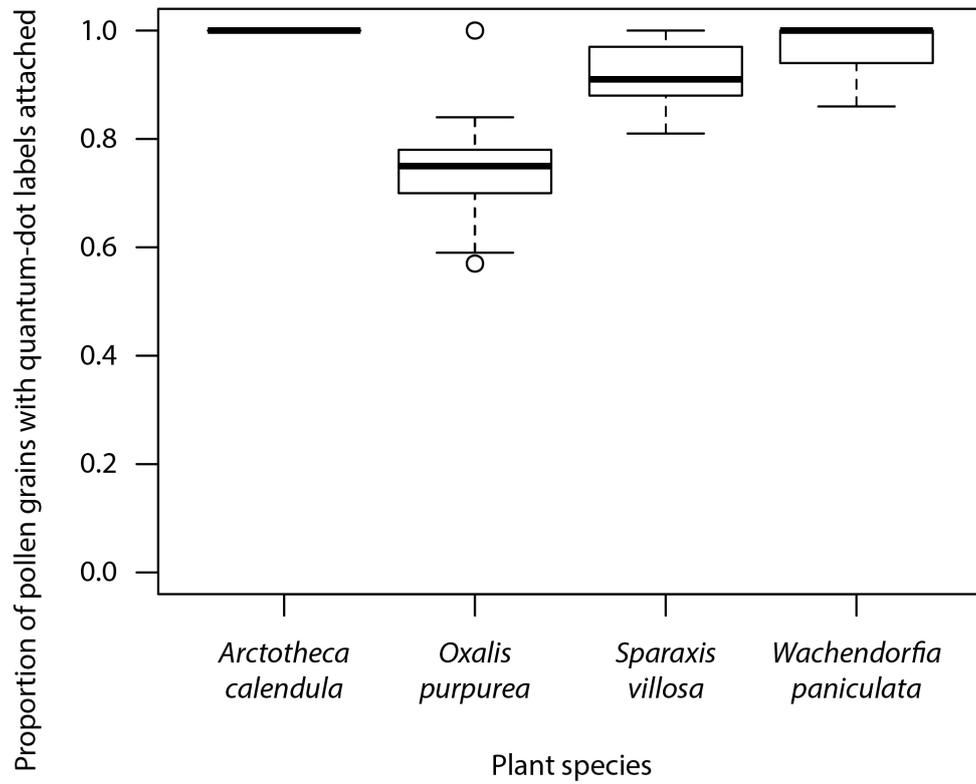


Figure 2.2. Boxplots of the proportion of pollen grains with quantum dots attached after application of quantum-dot solution to anthers of four species and agitation of pollen and anthers in 70% ethanol.

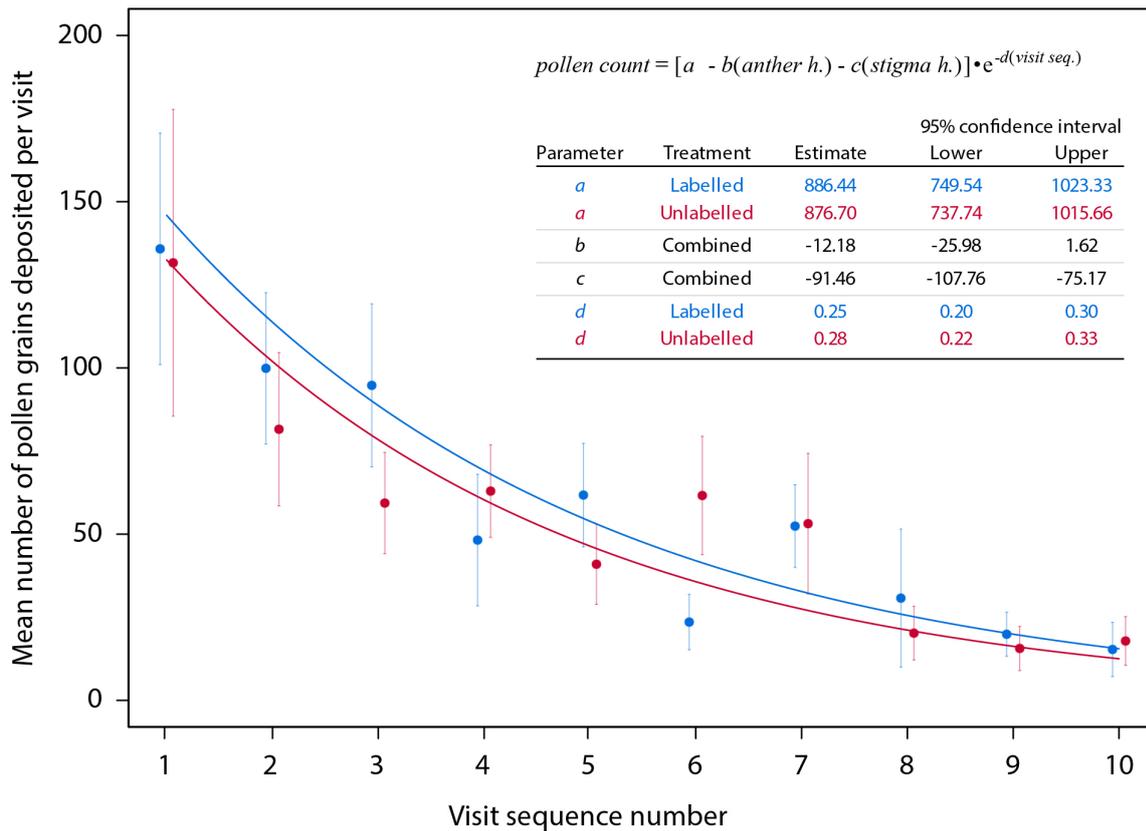


Figure 2.3. The mean \pm SE number of unlabelled (blue) and labelled (red) donor pollen grains deposited per visit as a function of visit sequence number during pollen transfer trials. The curves indicate the predicted mean pollen deposition from a non-linear mixed-effect regression model (equation 1, shown above). The embedded table shows treatment-specific parameter estimates from this model with 95% confidence intervals.

DISCUSSION

Do quantum dots attach to pollen grains (criterion 1), and what proportion of grains in an anther are labelled (criterion 2)?

The finding that quantum dots remain on pollen grains after agitation in 70% ethanol suggests that quantum dots attached to pollen grains, potentially through a lipophilic interaction between their oleic-acid ligands and the lipid-rich pollenkitt surrounding pollen grains. Nearly all animal-pollinated angiosperms have pollen grains surrounded by pollenkitt (Pacini and Hesse, 2005). Although pollenkitt composition varies, lipids are the primary constituent. Therefore, quantum dots could potentially bind to pollen grains of most animal-pollinated species. A possible exception may be members of Brassicaceae which produce tryphine to coat pollen grains instead of pollenkitt (Pacini and Hesse, 2005). While tryphine is functionally similar to pollenkitt, it is composed of both lipophilic and hydrophilic substances (Dickinson and Lewis, 1973), which may reduce the efficiency of quantum-dot attachment. The exact mechanism of quantum-dot attachment to pollen grains requires further exploration.

Most pollen grains present in anthers were labelled after application of quantum dot solution for the species tested in this study. However, a lower proportion pollen grains were labelled in anthers of *O. purpurea*. Anthers of *O. purpurea* release pollen through very narrow apertures and do not fully open upon dehiscence. This semi-closed anther structure may explain why a smaller proportion of *O. purpurea* grains were labelled. It may therefore be difficult to label all pollen grains in species with closed anther structures (e.g., poricidal anthers). However, for most applications, labelling all pollen grains in an anther may be unnecessary, since comparisons of pollen transfer will likely be relative. For studies comparing pollen transfer among species, I recommend quantifying the proportion of pollen grains labelled in anthers following methods in this paper and applying these proportions as species-specific correction factors for quantitative assessments of pollen movement.

Do quantum dots influence pollen transport (criterion 3)?

Pollen transport experiments revealed no effect of quantum dot labels on pollen transport for *S. villosa*. This may be a consequence of the small amount of quantum dots required to label almost all pollen grains in anthers. For *S. villosa*, only 2.5 µg of quantum dots were applied to an entire anther. The mass attached to pollen grains is likely even less than that (some quantum dots remain on anthers and in the pipette tip).

Statistically identical intercepts estimated for labelled and unlabelled pollen transfer curves also suggest that the amount of pollen picked up by bees is not affected by quantum dot labelling. Furthermore, the total amount of pollen transferred by individual bees during trials did not differ between treatments [untagged (mean±SE)=542.33±68.31; tagged (mean±SE)=581.33±45.63; $t_{(28)}=-0.47$; $p=0.64$]. I conclude that the ability of pollen grains to attach to bees as well as their ability to attach to stigmas was unaffected by quantum dot labelling.

Limitations, unknowns, and future improvements

Limited colours

Currently, there are only four commercially available, distinguishable quantum dot colours, although there is ongoing research in the development of non-toxic colours of short-wavelength (blue and violet). This may limit simultaneous assessments of pollen movement from more than four individual plants in close proximity.

The effect of quantum-dot labelling on other pollen characteristics

It remains unclear if brief exposure to hexane, or quantum dots themselves, affects pollen viability. If pollen viability is unaffected, it may be possible to identify individual pollen grains on

stigmas as well as their pollen tubes from specific individual plants, and therefore study both pollination and post-pollination components of male reproductive success.

I also did not determine whether pollen grains were structurally, or chemically altered by the quantum-dot labelling process and so it is possible that other aspects of pollination (other than transport) are affected by labelling. For instance, the scent profile of pollen grains may be altered if the hexane application draws volatiles out of pollen grains. To the human eye, quantum-dot labelling may change the colour of pollen grains very slightly for some species under visible light and it is unclear how this may affect foraging behaviour of pollinators.

CONCLUSION

Quantum-dot nanotechnology may allow direct assessments of pollen movement in most angiosperms. I anticipate that this will help to quantify (among other things): the magnitude and frequency of pollen loss during various stages of the pollen export process (Inouye *et al.*, 1994); the importance of vector-mediated pollen-movement isolation as a speciation and diversification mechanism in angiosperms (Armbruster, 2014); the structure and competitive implications of the various pollen landscapes that form on vectors as a result of sequential visits to competing conspecifics and heterospecifics (Minnaar *et al.*, 2018); and the importance of specific pollinators in facilitating reproduction and persistence of plants in vulnerable ecosystems of and economically important agricultural systems (Potts *et al.*, 2016).

SUPPLEMENTARY INFORMATION

3D-printable design files (STL: step files) for mechanical components of the quantum-dot excitation box used in this paper:

Qdot excitation box design file 1_Base_SupplInfo.STL:

https://1drv.ms/u/s!Au0ibLocAa_sirdsyegJliLciNaTUw

Qdot excitation box design file 2_Drawer_SupplInfo.STL:

https://1drv.ms/u/s!Au0ibLocAa_sirdtkP4ex96DpFScQw

Qdot excitation box design file 3_LED and filter housing platform_SupplInfo.STL:

https://1drv.ms/u/s!Au0ibLocAa_sirdruFKB8Rj-OR7UBQ

Qdot excitation box design file 4_LED and filter housing_SupplInfo.STL

https://1drv.ms/u/s!Au0ibLocAa_sirdoBazcEzOnZixFkw

CH. 3. FLORAL-TUBE LENGTH ACTS AS A STRONG, BUT ASYMMETRICAL MAGIC TRAIT

INTRODUCTION

Geographic models of speciation view the speciation process as an "inevitable" (Mayr 1963, p. 581) consequence of neutral divergence through geographic isolation, with or without selection (e.g., Singhal and Moritz 2013). In contrast, ecological speciation (*sensu* Schluter 1996; Funk 1998) explicitly recognises that divergent selection in different ecological environments can promote the evolution of reproductive isolation and speciation (Nosil, 2012). Ecological speciation is therefore expected to drive speciation at a faster and more consistent pace because divergence is driven by selection and not a random process (Gavrilets 2004; e.g., Feulner *et al.* 2015)¹.

Speciation can be further accelerated if traits under divergent selection also influence mating patterns—i.e., phenotypic divergence leads to reproductive isolation (Gavrilets, 2004; Servedio *et al.*, 2011; Smadja and Butlin, 2011). For example, wing colour-patterns of mimetic *Heliconius* butterflies are under divergent selection to match existing, but geographically variable, mimicry rings (Mallet and Barton, 1989; Merrill *et al.*, 2012). The divergence in wing colour-patterns also results in non-random mating because butterflies prefer to mate with individuals that match their own colour pattern (Jiggins *et al.*, 2001). Consequently, the more wing colour diverges, the more reproductively isolated the phenotypes become. Traits which couple divergence and mating in this way (e.g. *Heliconius* wing patterns) have been termed “magic traits” (Gavrilets, 2004) because their coupling is expected to increase the speed of the speciation process, even in the face of gene flow (Maynard-Smith, 1966; Servedio *et al.*, 2011; Smadja and Butlin, 2011).

The study of magic traits has received considerable, well-deserved attention in the last decade (Servedio *et al.*, 2011; Smadja and Butlin, 2011); however, very few examples of true

¹ However, in small populations, genetic drift, as a result of founder effects, may cause rapid divergence (Lanfear *et al.*, 2014)

magic traits have been demonstrated. Putative evidence for magic traits suggests that they are more common (Servedio *et al.*, 2011; Nosil, 2012) than once thought (Maynard-Smith, 1966; Gavrillets, 2004) and that the paucity of confirmed magic traits may simply be a reflection of the difficulty in determining whether a specific trait is magic or not. To validate a trait as magic, experiments need to test and confirm two hypotheses: (1) the trait itself (not a covariate of it) is under direct divergent selection and (2) trait variation caused by divergent selection generates assortative mating (Servedio *et al.*, 2011; Nosil, 2012). Despite their seeming simplicity, these conditions can be difficult to test experimentally. For example, a recent study on *Heliconius* revealed the importance of scent in male mate-preference (Mérot *et al.*, 2015). Because female scent appears to diverge along with wing colour patterns, it is possible that assortative mating patterns based on wing colour are also driven by scent. Consequently, the classic example of magic-trait speciation in *Heliconius* (Mallet and Barton, 1989; Jiggins *et al.*, 2001; Merrill *et al.*, 2012) may not be as clear as once thought and illustrates the difficulties in isolating the effect of a specific trait on assortative mating. As a consequence of this difficulty, most putative examples of magic traits remain unconfirmed (Servedio *et al.*, 2011).

While most of the theory and research on magic traits involves divergent selection on animal mating cues or mating preferences, only a few examples of putative magic traits have been considered in plants (Servedio *et al.*, 2011). This is surprising since the link between trait divergence and reproductive isolation has long been considered an important aspect of pollinator-driven speciation in plants (Grant, 1949; Grant and Grant, 1965; Stebbins, 1970). That is, geographic variation in pollinator fauna drives floral divergence and this floral divergence potentially affects reproductive isolation upon secondary contact.

Many floral traits appear to hold the promise of magic because any evolutionary change in floral traits could potentially alter pollen movement by attracting different sets of pollinators or by placing pollen in discrete locations on pollinators' bodies (Grant, 1994; Armbruster, 2014). For example, floral colour variation in *Mimulus* affects which species visit flowers and

divergence in colour could therefore result in reproductive isolation (Bradshaw and Schemske, 2003). By substituting alleles at a single locus in sister species of *Mimulus*, Bradshaw and Schemske (2003) demonstrated that a mutation affecting flower colour could cause strong reproductive isolation because different floral colours attracted different pollinator communities. Similarly, divergence in flowering phenology could lead to temporal reproductive isolation (Savolainen *et al.*, 2006). Floral traits such as these have been termed "automatic" magic traits because divergent selection acts directly on a trait that only functions in mating, thereby leading to reproductive isolation as an "automatic by-product" (Servedio and Kopp, 2012). Conceivably, almost any floral trait (e.g., floral scent, size, and morphology) under divergent selection could be considered a putative magic trait because divergence in floral traits could affect pollinator interactions with flowers and result in non-random movement of pollen. While floral traits potentially represent an endless pool of magic traits that activate as soon as divergent-forms establish contact in sympatry, studies of pollinator-mediated speciation seem oddly divorced from magic trait theory: reviews on pollinator-mediated speciation rarely use the term "magic trait" (e.g., Kay & Sargent 2009; Ellis & Anderson 2011; Armbruster 2014; Van Der Niet, Peakall & Johnson 2014). To my knowledge, flower colour in *Mimulus* remains the only floral trait that meets both criteria for a magic trait.

Evidence for divergent selection on floral traits is common, but varies in quality (reviewed in Kay and Sargent 2009; Armbruster 2014). In the last two decades, convincing evidence of pollinator-mediated divergence in floral traits has emerged from studies exploring associations between local pollinator proboscis length and flower morphology (e.g., Anderson and Johnson 2008, 2009; Pauw *et al.* 2009; Anderson *et al.* 2014; Boberg *et al.* 2014; Newman *et al.* 2014, 2015). For example, *Lapeirousia anceps* (L.f.) Ker Gawl (Iridaceae) occurs across a soil and altitude mosaic associated with different species of long-proboscid flies that vary in proboscis length (Goldblatt *et al.*, 1995; Manning and Goldblatt, 1997; Pauw *et al.*, 2009). High altitude populations are associated with horse flies which have relatively short proboscides (20–

33mm) whereas low-lying populations are visited only by tangle-wing flies which can have very long proboscides (up to ca. 95mm) (Goldblatt *et al.*, 1995; Pauw *et al.*, 2009). Like many other long-tubed plants (predicted by: Darwin 1859, 1862; experimentally shown by: Nilsson 1988; Alexandersson and Johnson 2002; Anderson and Johnson 2008; Muchhala and Thomson 2009; Sletvold *et al.* 2012), the match between the floral-tube length and pollinator proboscis length has dramatic fitness consequences for *L. anceps* (Pauw *et al.*, 2009). Consequently, shifts between different pollinator species have caused geographically divergent selection on *L. anceps* tube length. This has resulted in local plant ecotypes with tube lengths that match the proboscis lengths of their local pollinator species. In addition, *L. anceps* appears to be locked into a coevolutionary race of tube length and proboscis length matching with at least one of its pollinators, the tangle-wing fly (*Moegistorhynchus longirostris* Wiedemann, 1819). Pauw *et al.* (2009) have demonstrated that pollinator proboscis and plant tube length select reciprocally on one another. These races occur at a local scale and vary geographically in intensity, so that populations differ in magnitude of elongation (Anderson *et al.*, 2010). The result is a geographic mosaic of local pollinator-driven tube-length variation ranging from 40-100 mm (Pauw *et al.*, 2009; Zhang *et al.*, 2013; Anderson *et al.*, 2016). This system provides strong evidence that pollinators drive floral trait divergence (either through pollinator shifts or coevolution), thus clearly satisfying the first criterion for magic traits—divergent selection on the trait itself (Servedio *et al.*, 2011). However, floral-trait divergence in allopatry, even if driven by pollinator-mediated selection, does not necessarily equate to ecological speciation driven by pollinators as, in most cases, gene flow is still ultimately limited by geographic barriers and not differences in the trait itself. Unless divergent populations make secondary contact or are close enough in proximity for potential gene flow (Nosil, 2012), we can only consider cases of geographic variation as potential, or “sleeping”, magic traits. For example, if secondary contact were to occur between variable *L. anceps* populations, tube length may act as a gene-flow barrier if

short- and long-tubed flowers place and receive pollen on discreet locations along the pollinators' proboscis and body (Stiles, 1975; Grant, 1994).

Evidence for the second magic-trait criterion (divergent traits generate reproductive isolation) is, in contrast, very uncommon (Armbruster, 2014) and is perhaps the reason why most divergent traits have not yet been confirmed as being magic. In a comprehensive study of reproductive isolation barriers in closely-related *Costus* species, Kay (2006) found strong mechanical isolation between short-tubed *C. scaber* and long-tubed *C. pulverulentus* by measuring dye transfer by hummingbirds. While this study was not conducted on incipient species, it demonstrates the potential for tube-length to influence mating patterns through mechanical isolation. Our lack of progress in demonstrating magic traits in plants has been hampered, in part, by a lack of opportunities to study early stages of speciation in extant populations because divergent tiles in floral-trait mosaics are often widely separated in space (Newman *et al.*, 2015). However, highly contrasting tiles in geographic mosaics occasionally overlap: in October 2003, Anderson *et al.* (2016) discovered a unique population of *L. anceps* (Iridaceae) displaying a bimodal distribution in tube length (short-tubed plants: ca. 28 mm; long-tubed plants: ca. 54 mm). Initial work on this population suggests that gene flow between short- and long-tubed flowers is restricted, but many factors could contribute to this (Anderson *et al.*, 2016). To directly demonstrate that physical differences in tube length result in assortative mating, we need to quantify pollen movement directly. However, until recently, no reliable method existed to track the movement of individual pollen grains making it impossible to confirm how, or if, divergent floral traits promote assortative pollen movement.

In this chapter, I aim to test the function of floral-tube length as a magic trait in this unique population in the first field application of the newly developed quantum-dot pollen-labelling technique. I specifically consider both pollen placement on long-proboscid flies and subsequent pollen transfer among short- and long-tubed plants. I hypothesise that tube-length

will contribute significantly to reproductive isolation through the spatially separated placement and transfer of pollen between short- and long-tubed plants.

METHODS

Study site and population trait distributions

The bimodal *L. anceps* population is located near Mamre, Western Cape, South Africa (33°31'S, 18°28'E) within a relatively small patch of sand-plain fynbos (200 x 300 m) (Anderson *et al.*, 2016). This population is primarily composed of two tube-length phenotypes: short-tubed flowers with tube lengths < 32 mm; long-tubed flowers with tube lengths >42 mm (Anderson *et al.*, 2016). I conducted experiments during November 2015 and 2016, during which time I randomly measured single flowers from 90 individual plants in the population to characterise a current distribution of floral tube length. I also characterized fly proboscis-length distribution from 19 flies caught during experiments.

Pollen transfer experiments

To capture ecologically realistic pollen-transfer sequences among flowers of varying tube lengths, I randomly selected approximately 30 virgin flowers from the population in the morning before flies were active. Flowers were placed inside a mesh cage to prevent pollinator visits. I then took eight flowers from the pollinator-exclusion cage, secured them on thin bamboo sticks attached to plastic centrifuge tubes for water, and arranged them at 10 cm intervals along the end of a 1.5 m wooden dowel. I then uniquely labelled pollen of the first, fourth, and seventh flower (i.e., every third flower) in the sequence by applying either green, yellow or red quantum dots (colours chosen at random). Quantum dots were dissolved in hexane (10 mg quantum dots per ml hexane) and applied to anthers in 0.35 µl doses per anther following methods presented in Chapter 2. Flowers with labelled pollen acted as potential pollen donors among the eight flowers. All eight flowers could act as recipients. The flowers were then presented to flies within the population and the sequence of visits to experimental flowers recorded. After a single visit sequence, I recorded the tube length of each visited flower (pollen donors and receivers) and mounted the stigmas of each visited flower on a separate microscope slide without a mounting

medium (see Chapter 2). Completed microscope slides were stored at -20°C . Visited flowers were replaced with virgin flowers from the pollinator-exclusion cage, and quantum dots were applied to the replacement flowers if the previous flowers were labelled pollen donors.

Pollen placement

When possible, I caught flies after they visited experimental flowers to determine the relationship between flower tube length and pollen placement on flies. To catch and kill flies, I used a modified butterfly net as follows: I modified a standard butterfly net by cutting a hole at the tip of the net and placing an open jar inside the hole, securing it to the net with rubber bands. To capture a fly, I placed the modified capture net over a fly with the loop of the net (i.e., the net opening) securely in contact with the ground and the jar held as high as possible. The result of this procedure is that the fly finds itself trapped in a teepee-like structure and invariably tries to escape by flying upwards and into the jar. Immediately after capture, a killing jar containing potassium cyanide (KCN) was attached to the jar containing the fly. The attachment of the two jars was facilitated by a male-to-male jar connector consisting of two jar lids glued together (top-to-top) with a hole cut into the middle to allow the KCN gas to pass through. Flies were pacified within seconds after capture. This modified net method allowed me to capture flies while avoiding excessive contact between the fly and the net (which may result in pollen being dislodged from the fly). After capture, I secured flies to a foam board with an insect pin inserted through the lower part of the thorax. I carefully attached the tip of the fly proboscis to the head of an insect pin using a small drop of cyanoacrylate. This allowed me to extend and straighten the fly proboscis parallel to the fly body and secure it above the foam board free from contact with anything that might displace pollen. Flies were then fixed in this position by drying them in a warm, dark cupboard for two days after which they were stored at -20°C .

To determine placement of pollen from donor flowers, I measured the position of quantum-dot labelled grains along the length of the fly's body as follows: First, I pierced an insect pin into the back of the fly's abdomen. I then attached the insect pin to a long rod extending through the side of the quantum dot excitation box which allowed me to move the fly along the length of the box. The fly was positioned so that its body and proboscis were aligned parallel to the length of the box. I then placed the box under a standard dissection microscope and moved the fly until the tip of the fly's proboscis was aligned with the midline of the microscope's eyepiece grid. I then recorded the position of the end of the rod outside of the box as a reference point for the proboscis tip. I then slowly moved the rod so that the fly's proboscis and body passed through the midline of the eyepiece grid, stopping whenever a quantum-dot labelled pollen grain was detected, and recorded the colour and distance of each quantum-dot-labelled pollen grain from the proboscis tip (to the nearest millimetre) so that pollen grains and their positions could be assigned to their respective donors.

Data analyses

Pollen transfer

Most visits resulted in zero pollen transferred (79.7%; n=222). I therefore used hurdle-regression models (Cragg, 1971) to analyse pollen transfer. Hurdle models allow the probability (on the logit scale) of a zero response (i.e., no pollen transferred) to be modelled separately from the magnitude/count of the response (i.e., amount of pollen transferred) (Hadfield, 2010). I fit generalised linear mixed-effects hurdle models (GLMM-hurdle) in R (R Core Team, 2017) using the Markov-chain Monte Carlo technique in the package MCMCglmm (Hadfield, 2010). The Bayesian approach implemented in MCMCglmm allows for highly flexible model specification. For hurdle models, coefficients for fixed and random effects can be estimated for both the zero-response probability and the count portion of the model separately.

My primary hypothesis was that large differences in tube length between donor and recipient flowers would result in poor pollen transfer and, therefore, reproductive isolation between short- and long-tubed plants. Therefore, the absolute difference in tube length between donor and recipient flowers for a given visit (hereafter: tube-length difference), should influence both the zero-transfer probability and the quantity of pollen transferred. However, I measured pollen transfer beyond the first visit after the donor and therefore the position of a particular recipient in a visit sequence (hereafter: visit sequence number) should be taken into account. The amount of a donor's pollen transferred is likely to decrease with each successive visit as a result of pollen displacement or loss in flight (Thomson *et al.*, 1986). I therefore included visit sequence number as a separate term in the model as well as the interaction between tube-length difference and visit-sequence number, with coefficients for each term in the model estimated for both the zero-response probability and the count portion of the model. Since donors transferred pollen to several recipients, donor identity was added to the model as a random effect of the zero-inflation process.

Pollen placement quantity

I calculated total pollen placement from a specific donor onto a fly as the sum of the total amount pollen found on the fly's body and the total amount of pollen transferred to stigmas of flowers before capture. A substantial proportion of flowers visited by flies did not place any pollen on fly bodies (37.1%; n=35), while the 22 flowers that did place pollen on flies, placed a total of 330 pollen grains. Therefore, as with pollen transfer, these zero-inflated data are most appropriately modelled by accounting for the probability of zero-pollen placement and the total amount of pollen placed when not zero as separate processes using a GLMM-hurdle model (Hadfield, 2010).

I modelled the quantity of pollen placed (count component of the response) and the likelihood of pollen placement on flies (the likelihood of a zero response) as a function of floral-

tube length. Since several flies visited multiple donors, fly identity was added to the model as a random effect of the zero-inflation process.

Pollen placement position

Variation in placement position among individual pollen grains from a single donor flower was minimal (mean range \pm SE= 0.64 \pm 0.29 mm; maximum range= 3 mm). I therefore used the mean position of pollen grains placed by a donor flower to represent that flower's placement position. I modelled placement position as a linear function of donor tube-length using least-squares regression in R (R Core Team, 2017).

Modelling reproductive isolation

The numbers of transfer events recorded during experiments were not equal across transfer categories or visit sequence number. I therefore designed a custom bootstrap model in R to estimate the effect of floral tube-length on reproductive isolation (RI). The aim of the model was to estimate the cumulative transfer of pollen among short- and long-tubed plants during a reproductive season using pollen transfer data collected during experiments. To parameterise the model, I assumed that each individual flower in the population received four visits (rounded up from 3.66 visits estimated from unpublished visitation rate data collected by B. Anderson), and that each plant produced 20 flowers in a season. Because sample sizes for visit sequences longer than four visits were low, I estimated pollen receipt from a visit to an individual flower from visits to four preceding donors. I assumed that long- and short-tubed plants were equally abundant in the population to focus on the effect of floral-tube length—running the model based on representative abundances in the population confounds the effect of tube-length on RI. If each flower in the population receives four visits that can deliver pollen from four preceding donors, a total of 16 donor flowers (8 long and 8 short) may contribute to a single flower's pollen receipt. The model estimated pollen transfer to individual short- and long-tubed flowers through structured random sampling (with replacement) of actual transfer events

recorded during experiments. Estimated cumulative pollen receipt per flower was then summed for the entire plant with 20 flowers in total. This procedure was repeated to obtain RI estimates for 1,000 short- and long-tubed plants. I then used the randomly sampled estimates of pollen transfer to calculate RI for individual short- and long-tubed plants using the following equation [based on equation 4A from Sobel and Chen (2014)]:

$$RI_{Si} = 1 - 2 \times \left(\frac{\sum L \text{ pollen}_{Si}}{\sum L \text{ pollen}_{Si} + \sum S \text{ pollen}_{Si}} \right) \quad (3A)$$

where reproductive isolation for short-tubed plant i (RI_{Si}) is calculated from the cumulative sum of long-tubed pollen transferred to short-tubed plant i ($\sum L \text{ pollen}_{Si}$) as a proportion of the cumulative sum of both long and short pollen transferred to short-tubed plant i ($\sum L \text{ pollen}_{Si} + \sum S \text{ pollen}_{Si}$). RI for long-tubed plants was calculated in the same manner. The overall reproductive isolation between short- and long-tubed plants was calculated similarly, with total pollen transfer between long- and short-tubed plants taken as a proportion of all pollen transfer (between and within short- and long-tubed plants). Equation 3A is superficially similar to the equation used in Ramsey *et al.* (2003); however, it ranges from -1 to 1, where -1 is complete disassortative mating, zero is random mating and 1 is complete reproductive isolation (completely assortative). I have opted to use Sobel and Chen's (2014) scaling system because its interpretation is more intuitive than that used by Ramsey *et al.* (2003).

RESULTS AND DISCUSSION

Floral-tube length in the *L. anceps* population is strongly bimodally distributed and has changed little in over a decade, whereas fly proboscis length is still unimodal and matched to the distribution of long-tubed flowers (see Fig. 3.1, *cf* Anderson *et al.* 2016). This suggests that realised mating between long and short-tubed flowers is limited enough that the relative number of intermediate individuals has remained low, at least in the very short term. While other reproductive isolation barriers (e.g., non-random foraging and incompatibilities between long and short-tubed flowers—Anderson *et al.* 2016) have been demonstrated in this population, the direct effect of tube-length variation on pollen movement and reproductive isolation has yet to be explored. Below I discuss the evidence for floral tube-length as a magic trait in this population.

Pollen placement on pollinators

The 19 flies captured after visiting 35 flowers (15 short; 20 long) with quantum-dot-labelled pollen and found that three times more pollen was placed on flies per visit by long-tubed flowers (mean \pm SE: 13.7 \pm 5.4) than by short-tubed flowers (mean \pm SE: 3.8 \pm 2.6). Correspondingly, the GLMM-hurdle model found a significant effect of donor tube-length on the number of pollen grains placed on flies (effective sample size=10529; pMCMC=0.02). While percentage of flowers placing pollen on flies was higher for long-tubed (70%; n=20) than for short-tubed flowers (53%; n=15), the likelihood of pollen placement was not significantly influenced by donor tube length (GLMM-hurdle model: effective sample size=10000; pMCMC=0.34). Therefore, the large difference in total amount of pollen placed on flies by long- and short-tubed flowers cannot be explained simply by rate of contact with anthers alone, but may also reflect differences in available surface area in the predominant placement localities of long and short-tubed flowers: donor tube-length showed a significant, positive, and linear relationship with placement position along the length of flies ($R^2=0.42$, $p<0.01$, $n=14$). Pollen from long-tubed flowers was

predominantly placed on the head, thorax and base of the proboscis, all of which are relatively large surface areas available for pollen placement (Fig. 3.2). In contrast, low pollen placement rates of short-tubed flowers may be related to the small surface area available for pollen placement (the thin mid-proboscis).

The positive relationship between donor tube-length and placement position on flies sets the stage for mechanical isolation through differential pollen placement, and therefore assortative pollen movement. While no long pollen (n=64 grains) was found <40 mm away from fly proboscis tips, some short-tube pollen grains were found on/close to the head of one of the flies captured (Fig. 3.2) where they would likely be available for deposition on stigmas of long-tubed flowers. Based on pollen placement patterns alone, long-tubed flowers are unlikely to transfer pollen to short-tubed flowers; however, whether short-tubed flowers are mechanically isolated from transferring pollen to long-tubed flowers is unclear.

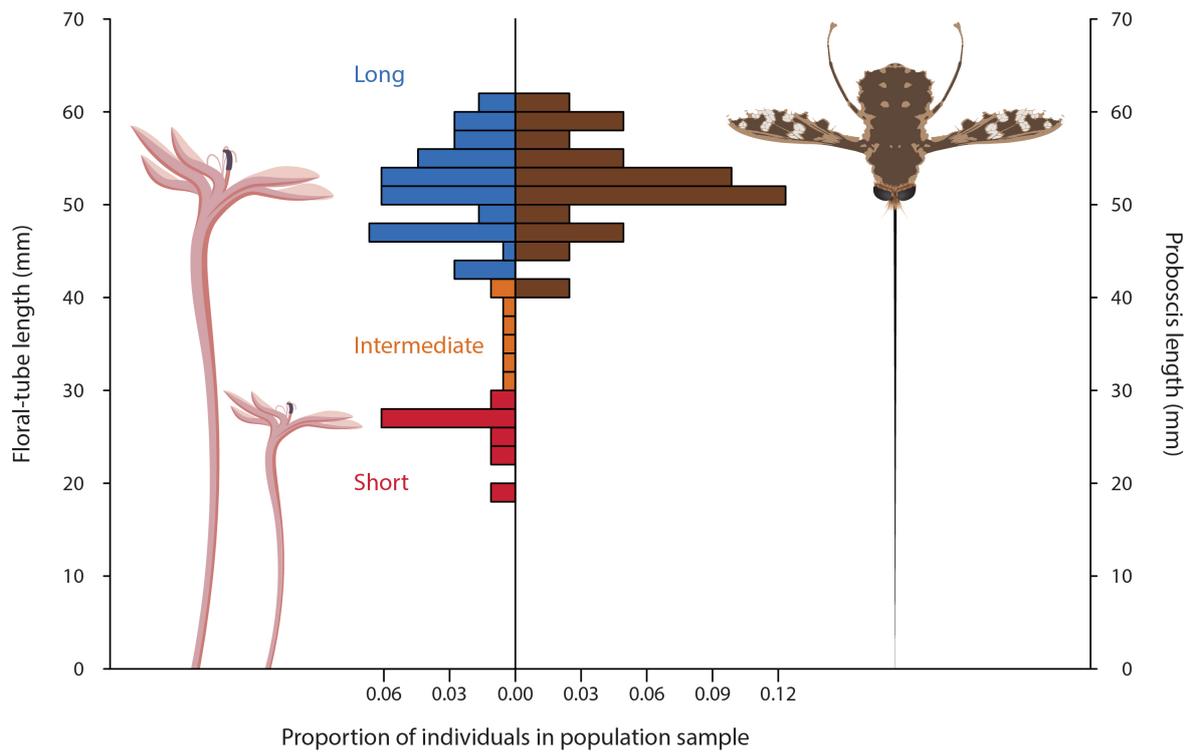


Figure 3.1. Distribution of floral-tube length and fly proboscis length from individuals within the Mamre population. Left: histogram of floral-tube length (n=90) for short (<32mm; red bars), intermediate (>32mm<42mm; orange bars), and long individuals (>42mm; blue bars). Right: histogram of fly proboscis length (n=20).

Pollen transfer amongst long- and short-tubed flowers

Long-tubed flowers transferred most of their pollen to other long-tubed flowers, whereas short-tubed flowers transferred pollen to both long- and short-tubed flowers (Fig. 3.3); however, these patterns do not take visit sequence number into account. GLMM-hurdle models suggest that the amount of pollen transferred to stigmas from a long donor declines significantly with each successive flower visited in the sequence (effective sample size=101; pMCMC=0.01). However, the same reduction trend was not observed for short donors (effective sample size=1709; pMCMC=0.31). For long-tubed flowers there was a significant interaction between tube-length difference and visit-sequence number for the probability of zero pollen transfer (effective sample size=261.69; pMCMC=0.005). This suggests that long-tubed flowers were unlikely to transfer any pollen to short-tubed flowers or to any flowers at the end of the transfer sequence. In contrast, short-tubed flowers were equally likely to transfer pollen to long- and short-tubed flowers and this likelihood did not decrease significantly with visit sequence number (effective sample size=1630; pMCMC=0.35).

The above results support the patterns seen in the pollen-placement and raw transfer data: (1) long-tubed flowers very seldom transfer pollen to short-tubed flowers and are most likely to transfer pollen to other long-tubed flowers; (2) although short-tubed flowers transfer little pollen, they are equally likely to transfer pollen to either long- or short-tubed flowers. Pollen movement from short- to long-tubed flowers may occur when short pollen is unexpectedly placed on the head region of the fly (as observed in a few cases). This could occur if pollen placed on the middle of the proboscis by a short flower is pushed upwards by the rim of the corolla, or other reproductive parts of the flower, after flies visit long-tubed flowers. Pollen movement from short- to long-tubed flowers could also occur if the reproductive parts of long-tubed flowers make contact with the middle of the fly proboscis. However, this appears to be less likely as long to short pollen transfer was rare. A similar mechanism for asymmetrical pollen transfer between long- and short-tubed flowers has been found in two hummingbird-pollinated

Ipomopsis species (Wolf *et al.*, 2001): short-tubed *Ipomopsis arizonica* (*Selaphorus platycercus*) transferred pollen to long-tubed *Ipomopsis aggregata* at a much higher rate than pollen movement in the reverse direction. Although Wolf *et al.* (2001) did not determine pollen placement positions for the two *Ipomopsis* species, they hypothesised that short-tubed flowers place pollen near the tips of bills of hummingbirds which are capable of brushing past stigmas of the longer-tubed species. However, the longer-tubed species places pollen on the heads and faces of hummingbirds which rarely make contact with stigmas of the short-tubed species. Although granular pollen is likely to behave very differently to pollinaria glued to pollinators by orchids, a similar pattern of asymmetrical massulae movement was found in a manipulative experiment on the orchid *Satyrium longicauda*. By artificially shortening nectar spurs, Ellis and Johnson (2010) demonstrated that short- and medium-spurred flowers exported massulae almost as efficiently as flowers with naturally long spurs to other long-spurred flowers in the population. Moreover, they showed that short- and medium-spurred flowers received significantly fewer massulae from other long-spurred flowers in the population. Taken together, these studies, and the pollen transfer findings of this study, suggest that floral-tube length may act as an asymmetrically permeable barrier, allowing short-tubed flower pollen to pass through but blocking pollen from long-tubed flowers. While these studies have all focused on pollen transfer at the flower level, plants have multiple flowers, each of which may receive multiple pollinator visits. Consequently, the strength of tube-length as a barrier to pollen movement should be calculated in the context of cumulative pollen transfer to short- and long-tubed plants.

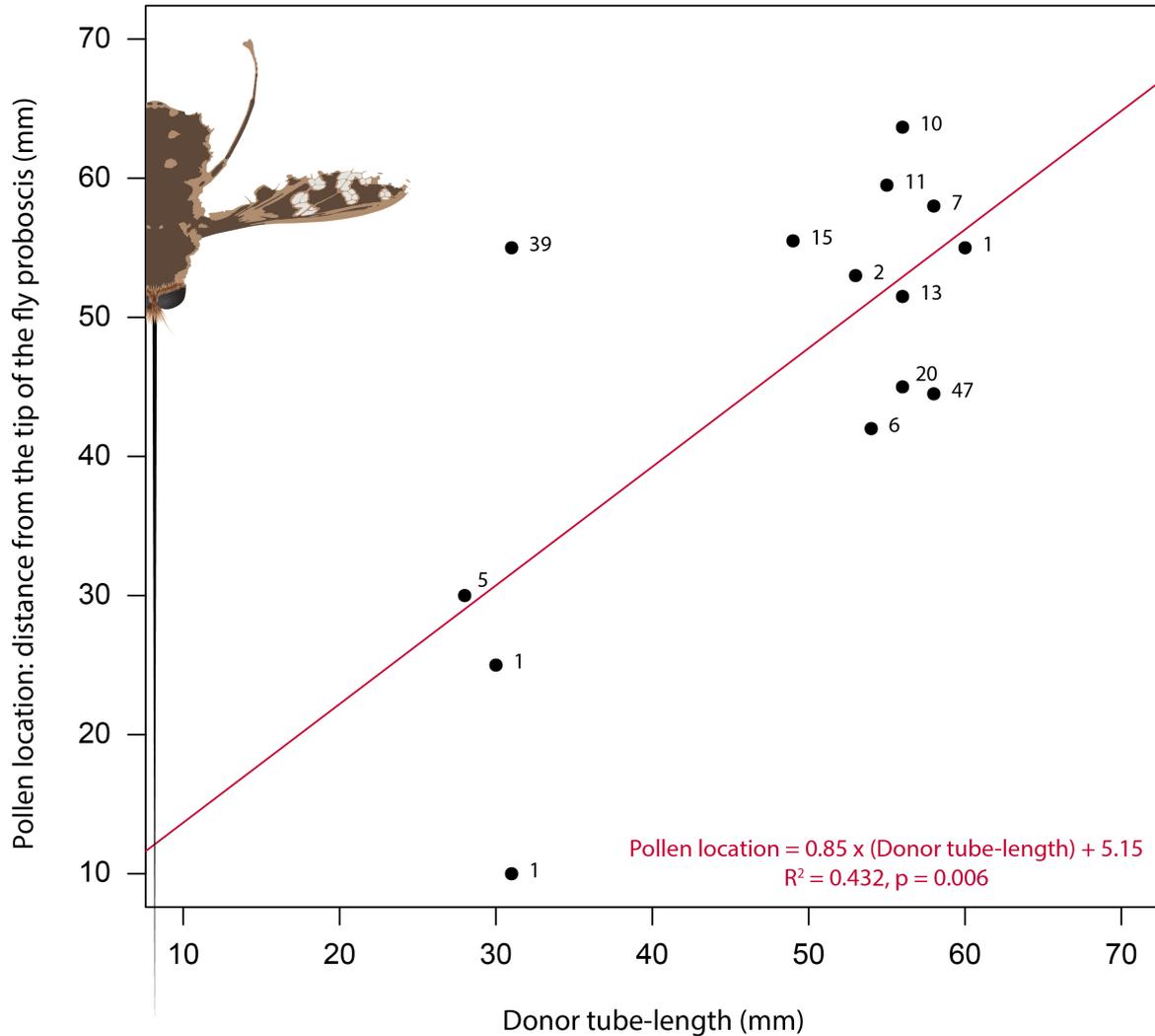


Figure 3.2. Pollen location (distance from the tip of the fly proboscis) as a function of donor tube length. Each black dot represents the mean pollen position and floral tube length of 14 individual flowers. Numbers next to dots indicate the total number of pollen grain placed on flies (pollen found on the body of a fly + any pollen transferred to flowers prior to capture). The red line represents the linear regression model (bottom right) of pollen location as a function of donor tube-length. A typical fly (to scale on the y-axis) is depicted on the left side of the graph to provide a reference for where pollen grains were placed.

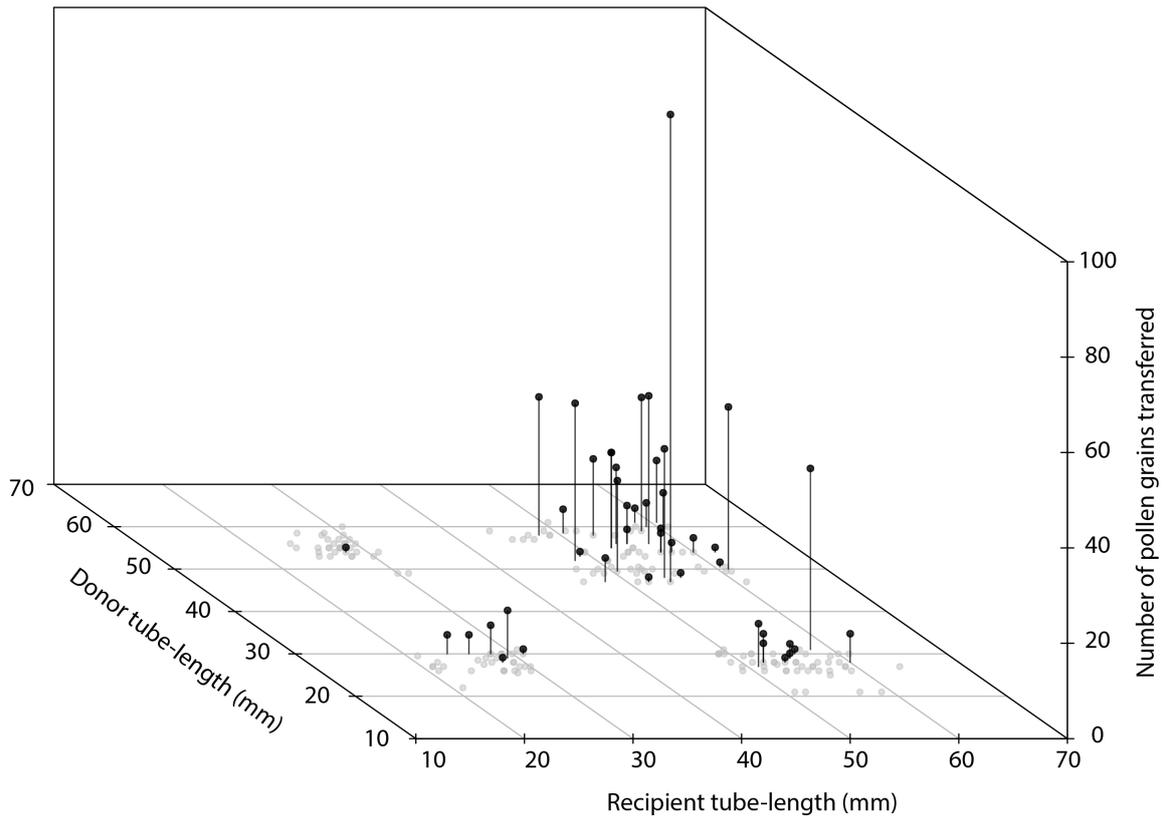


Figure 3.3. Pollen transfer relative to donor and recipient tube length. Each dot represents a transfer event (i.e., a visit to a recipient flower after visitation to a donor) with grey dots indicating zero pollen transferred, and black dots indicating the successful pollen transfer.

Reproductive isolation

Pollen transfer estimates from the bootstrap function show that pollen received by short-tubed plants is almost entirely from short-tubed donors (median | 95% CL: $RI_S = 0.96 | 0.92-0.99$). In other words, the difference in tube-length between long donor flowers and short recipients results in a 96% increase in assortative pollen movement relative to random expectations. In comparison, when short-tubed flowers act as donors, the difference in tube length relative to long recipients results in a 79% increase in assortative pollen movement (median | 95% CL: $RI_L = 0.79 | 0.58-0.93$) (Fig. 3.4). This asymmetry in pollen movement may provide a functional explanation for gene flow patterns previously found in this population: Using allozyme electrophoresis, Anderson *et al.* (2016) found evidence for limited gene flow from short- to long-tubed plants but no evidence for gene flow from long- to short-tubed plants. The genetic fingerprint left on long-tubed plants by short-tubed plants is potentially driven by low, but consistent, pollen transfer from short- to long-tubed flowers. The lack of gene flow from long- to short-tubed plants is also supported by pollen movement patterns found in this study.

The overall effect of tube length in reproductive isolation does not appear to be weakened by this asymmetry—tube length as a trait resulted in an overall increase of 94% in reproductive isolation (median | 95% CL: $RI_{\text{overall}} = 0.94 | 0.02-1.00$). This may seem counterintuitive given the fact that GLMM-hurdle models found that short-tubed flowers were not statistically more likely to transfer pollen to either long or short-tubed flowers (i.e. tube-length presents no barrier to pollen export by short-tubed plants). However, the marked differences in estimates of cumulative pollen received by long- and short-tubed plants from long and short donors ($F=28727$, $DF=3$, $p<0.0001$) may explain this (Fig. 3.5): Long-tubed plants received eight times more pollen from other long-tube plants than from short plants (Tukey HSD: $p<0.0001$). Consequently, long-tubed plants were reproductively isolated from short-tubed plants. Long-tubed plants almost never exported pollen to short-tubed plants and thus short-tubed plants were also strongly reproductively isolated.

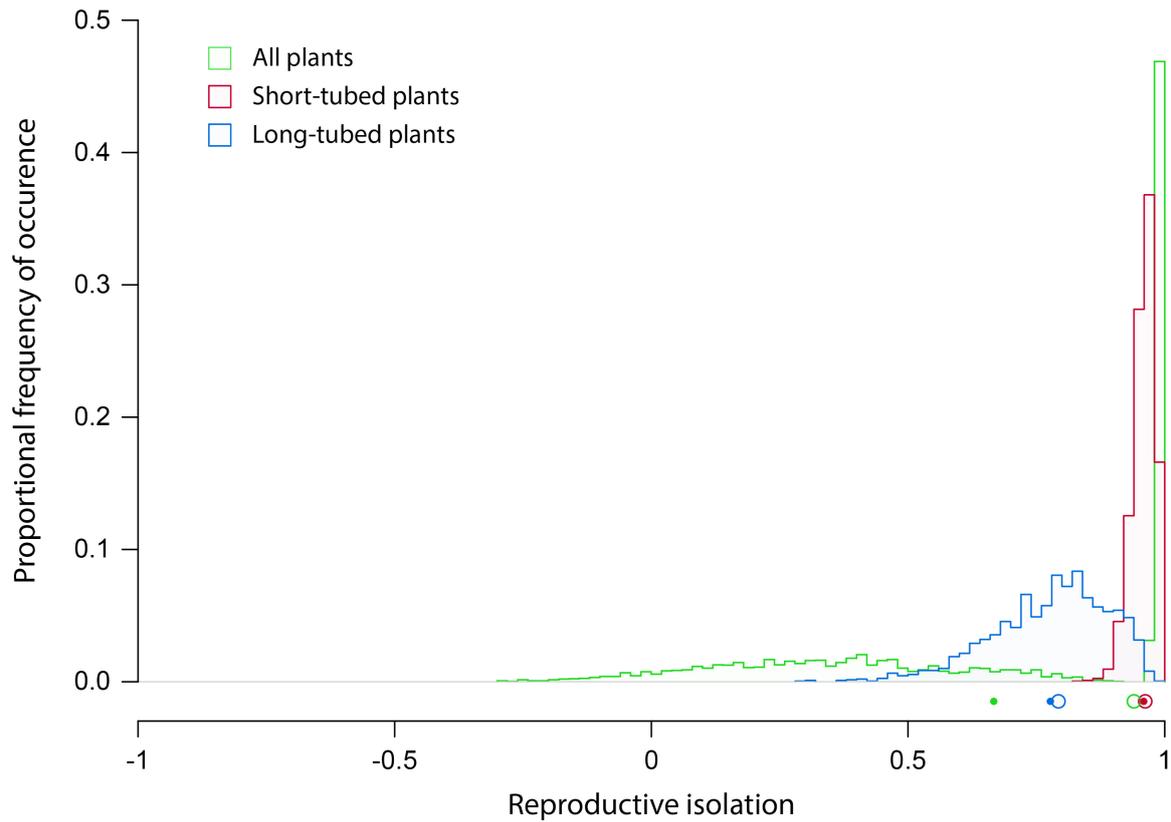


Figure 3.4. Proportional frequency distributions of estimated reproductive isolation (RI) calculated from a random bootstrap function. Dots underneath histograms indicate the mean RI for each category, while larger filled circles indicate the median RI value for each category.

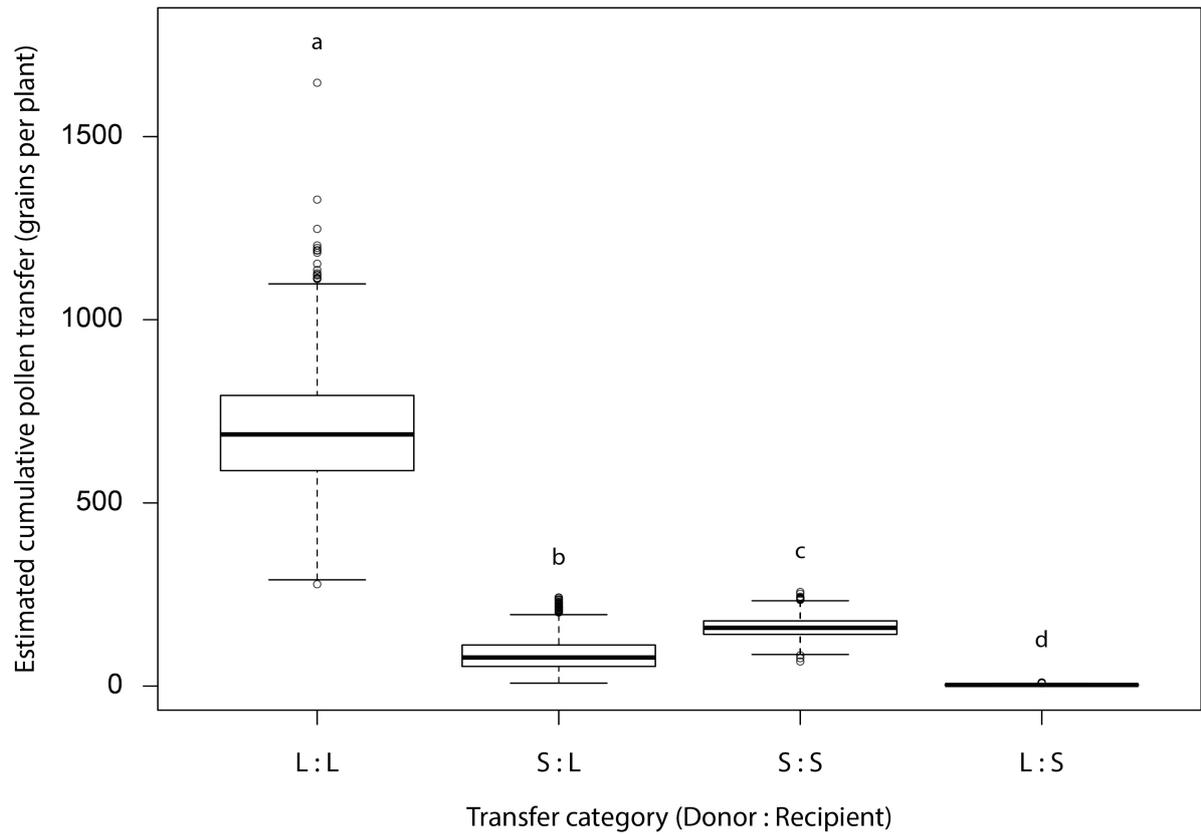


Figure 3.5. Boxplot showing the estimated total cumulative pollen transfer calculated from the bootstrap modelling procedure for each possible transfer category (long to long = L:L; short to long = S:L; short to short = S:S; long to short = L:S).

Is tube-length a magic trait in this population?

The first criterion for a magic trait is that the trait is under divergent selection (Servedio *et al.*, 2011). Pauw *et al.* (2009) showed that the match between pollinator proboscis length and floral-tube length has a significant effect on pollen receipt (a component of female fitness) for *L. anceps*. This, coupled with consistent spatial covariation between pollinator tongue and floral-tube length among populations (Pauw *et al.*, 2009), shows that variation in pollinator proboscis length has likely contributed to geographic divergence in floral-tube length, satisfying the first criterion for a magic trait.

Although a previous study revealed reproductive isolation barriers associated with tube length (incompatibilities between long and short-tubed flowers and non-random foraging - Anderson *et al.* 2016), neither of these barriers were a direct consequence of tube length itself. The experimental evidence presented in this chapter supports a direct effect of divergent floral-tube length on assortative mating, satisfying the second criterion for a magic trait (Servedio *et al.*, 2011).

While differences in floral-tube length may contribute significantly to reproductive isolation between flowers in this population, this reproductive isolation is not a direct result of mechanical isolation of pollen movement. I found no evidence of mechanical isolation in pollen export from short- to long-tubed flowers. Therefore, it could be argued that reproductive isolation between long- and short-tubed flowers is simply a consequence of relatively poor pollen export from short-tubed flowers to both long- and short-tubed recipients. Since total pollen receipt by short-tubed flowers was also relatively low, selection may indeed favour plants with longer floral tubes, leading to a decline in short-tubed plants over time. Rather than leading to speciation, this scenario may simply reflect poor mechanical fit by short-tubed flowers to the local pollen vector. However, both short- and long-tubed *L. anceps* are capable of facultative self-pollination (Anderson *et al.*, 2016); therefore, despite poor pollen receipt and

export determined for short-tubed flowers in this chapter, short-tubed plants may persist in this population, potentially allowing eventual speciation to occur given strong pre-mating isolation observed in this population.

CH. 4. POLLEN MOVEMENT IN A HANDED PLANT REVEALED BY QUANTUM-DOT-LABELLED POLLEN

INTRODUCTION

Darwin's seminal work on polymorphisms in stigma and anther arrangement exemplified the importance of pollen movement efficiency as a selective agent on floral form (Darwin, 1877). Five years after the publication of this work, his interest was piqued by another intriguing stylar polymorphism in *Solanum rostratum* described by J.E. Todd (1882) where plants display flowers with stigmas deflected to either the right or left of the central floral axis, and anthers deflected to the opposite side (i.e., flowers are left- or right-handed depending on which side their stigmas are). In his final scientific correspondence, Darwin wrote to Todd, requesting a box of *S. rostratum* seeds so that he could "...have the pleasure of seeing the flowers & experimenting on them.". Sadly, Darwin died only a few days later (Darwin, 1887), and more than a century would pass before the first experiments directly addressed the function of handedness in plants (Jesson and Barrett, 2002a; Jesson and Barrett, 2005).

The lateral separation between anthers and stigmas in handed plants likely reduces pollen movement within flowers and the likelihood of selfing (Webb and Lloyd, 1986; Karron *et al.*, 1997; Jesson and Barrett, 2002a, 2005). However, because anther–stigma separation results in different pollen placement and receipt sites on pollinator bodies, it carries the cost of reduced efficiency in between-flower pollen movement (Barrett, 2002a; Jesson *et al.*, 2003). Handed plants elegantly resolve this conundrum—a left-handed flower places pollen on the side of the pollinator where a right-handed flower's stigma would make contact with the pollinator. (Jesson *et al.*, 2003) (Fig. 4.1A). Therefore, although pollen transfer within flower morphs is made inefficient by lateral separation of anthers and stigmas, pollen may be readily transferred between morphs due to the reciprocity of anthers and stigmas of right- and left-handed flowers. Handedness thus maintains pollen transfer efficiency despite stigma–anther separation (Barrett *et al.*, 2000; Jesson *et al.*, 2003).

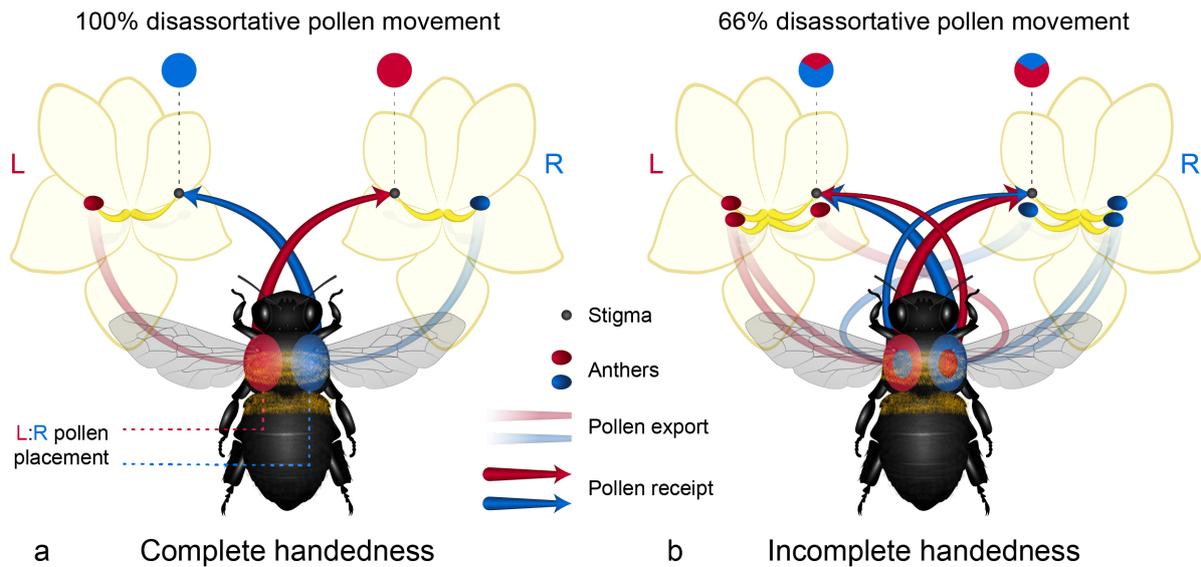


Figure 4.1. Comparing the theoretical efficiency of pollen movement in completely handed versus partially handed flowers, assuming perfect left–right segregation in pollen placement and receipt. (a) Complete handedness: pollen from the left and right morph is placed on the right and left sides of the pollinator’s body, respectively. Stigmas from left and right morphs make contact with separate sides of pollinator bodies and therefore only capture pollen from opposite morphs resulting in disassortative pollen movement. (b) Incomplete handedness: disassortative pollen movement is reduced because flowers place some of their pollen on the same side as stigmas thereby causing assortative pollen movement. In this example (as in *Wachendorfia paniculata*), two anthers are opposite to the stigma while one anther remains on the stigma-side resulting in maximum disassortative pollen movement of 66%.

We can therefore expect most pollen movement to occur between morphs (disassortative, Fig. 4.1A), while pollen movement within morphs (assortative) should be comparatively low (Jesson and Barrett, 2002a; Barrett, 2002a; Jesson and Barrett, 2005). The likelihood of disassortative pollen movement increases if pollen from the two morphs segregates either to the left or right of the pollinator's body when all anthers face a different direction to the stigma, as one may predict from complete lateral anther–stigma separation (Fig. 4.1A). However, many handed plants display incomplete handedness (Jesson and Barrett, 2003), where anthers occur opposite to, and on the same side as, the stigma [e.g., all handed Haemodoraceae, the family in which handedness is most prevalent (Simpson, 1990), and some Commelinaceae (Jesson and Barrett, 2003)]. For example, *Wachendorfia paniculata* Burm. (Haemodoraceae) has two anthers opposing the stigma and one that occurs on the same side as the stigma (Fig. 4.1B). All three anthers of *W. paniculata* have the same number of pollen grains (Ornduff and Dulberger, 1978); thus perfect left–right segregation in pollen placement by this 2:1 anther arrangement is expected to result in a maximum of 66.6% disassortative pollen movement (Fig. 4.1B). Since perfect left–right segregation in pollen placement is unlikely, we might expect even lower rates of disassortative pollen movement. Thus, by positioning anthers and stigmas on the same side of the flower, incomplete handedness should result in reduced disassortative pollen movement and increased pollen movement within flowers.

Nevertheless, populations of *W. paniculata* consistently display higher rates of outcrossing (range: 0.78–0.98) (Jesson and Barrett, 2002b) than predicted by anther arrangement. In fact, *W. paniculata* appears to outcross as effectively as plants displaying complete handedness (Jesson and Barrett, 2002a, 2005). However, *W. paniculata* shows reduced seed set from controlled self-pollination relative to outcross pollination (Jesson and Barrett, 2002b); since outcrossing rates were determined from seeds, it is unclear if high outcrossing rates reflect an effective disassortative pollen-transfer mechanism, or if post-pollination processes reduce the probability of self-fertilisation. Direct assessments of pollen

movement have never been done for this species, or any other handed species. The aim of this chapter was to determine if, and how, incomplete handedness functions in *W. paniculata* to promote outcrossing through disassortative mating between left- and right-handed plants. Using quantum dots as pollen labels, I first determined whether incomplete handedness resulted in lateral segregation of pollen on pollinators, a prerequisite for enantiostyly to generate disassortative pollen movement. Next, I tracked anther-level pollen movement among morphs of *W. paniculata*, thereby linking pollen placement patterns to pollen transfer *in situ*.

METHODS

I conducted all experiments on Stellenbosch mountain (33°56'47.3"S 18°52'50.0"E), Western Cape, South Africa between October and December 2016. I labelled pollen grains with quantum dots following procedures described in Chapter 2. Quantum-dot-labelling experiments in Chapter 2 revealed that most pollen inside *W. paniculata* anthers (ca. 97%) are successfully labelled with quantum dots. To visualise quantum dots on bees and stigmas, I used the quantum dot excitation box described in Chapter 2.

Pollen placement

I labelled pollen grains from each of the three anthers of a left- or right-handed *W. paniculata* flower a different colour (colours were randomly assigned to anthers) and presented it to foraging honey bees (*Apis mellifera capensis* Eschscholtz, 1822) and carpenter bees [*Xylocopa caffra* (Linnaeus, 1767)] at the end of a 1.5 m wooden. Once a bee visited the quantum-dot labelled flower, I captured and killed them using the same custom-net procedure described in Chapter 3. Bees were immediately pinned with a single needle through the centre of the thorax onto a foam board and stored at -20°C to preserve them for pollen mapping. I captured 19 carpenter bees (8 L; 11 R) and 19 honey bees (11 L; 8 R).

To map pollen on bee bodies, I created master body plan for carpenter and honey bees from photos of several individuals. Then, for each individual bee, I scaled the master body plan to match that particular bee by taking measurements of the length and height of the main parts of the body: wings, legs, abdomen, thorax, and head. Because bees died in various positions, I standardised wing and leg orientation as shown in Fig. 4.3. I then viewed each bee under UV excitation as described using the quantum dot excitation box. Each individual quantum-dot labelled pollen grain was mapped as accurately as possible onto its corresponding position on the individually scaled bee-body map. Pollen grains from different anther positions were mapped in different colours. Individual body maps were anchored at the centre of the thorax

which represented the origins of the x-y axes allowing standardised extraction of the x-y coordinates of each individual pollen grain. I used this mapping approach, because it required the least amount of handling, mitigating post-capture pollen movement and loss of quantum dot labelled pollen grains.

I created body maps and mapped pollen to scale in Adobe Illustrator CC (Adobe Systems, 2017) and subsequently extracted coordinates for each individual pollen grain for analysis. I then reversed the sign of the x coordinates of all pollen grains from left-handed morphs so that pollen placement could be analysed according to anther (i.e., upper, lower-opposite, and lower-stigma-side). I tested for differences in pollen placement of each anther on honey bees and carpenter bees on the x and y axis separately using linear mixed-effects models with bee individual as a random factor. I compared pollen placement among anther types using Tukey post-hoc comparisons.

Pollen placement heat maps

I reclassified pollen in pollen-placement maps as disassortative or assortative based on the flower origin and the side of the pollinator that the pollen grain was found. For example, a pollen grain on the left side of a pollinator facing the flower was classified as an assortative pollen grain if it originated from a left-handed flower (i.e., the pollen grain is on the same side as its donor's stigma) and disassortative if it originated from a right-handed flower (i.e., the pollen grain is on the side opposite to its donor's stigma). Because I had unequal sample sizes for left- and right-morph pollen placement for both bee species, I changed the sign of x coordinates of pollen grains so that disassortative–assortative pollen placement could be combined for both morphs and mapped on one side of bee bodies. I computed two-dimensional kernel density estimates of disassortative and assortative pollen on carpenter bee and honey bee bodies at a 0.1 mm² grid resolution. Density values in these maps were rescaled so that maximum density estimates matched actual pollen counts sampled from 0.5 mm² grid cells

overlaying pollen placement maps. I removed rescaled density values <1 and classified these cells as having no pollen. After computing disassortative and assortative pollen density estimates for carpenter bees and honey bees, I calculated a pollen quality–quantity index for each grid cell as the ratio of disassortative to assortative pollen multiplied by the total number of grains found in each grid cell. This created an overall heatmap of pollen quantity and quality across pollinator bodies. Kernel density estimations and heatmaps were computed in R [packages: MASS (Venables and Ripley, 2002), raster (Hijmans, 2015), png (Urbanek, 2015), RSAGA (Brenning, 2008)].

Pollen movement

I used quantum dots to label pollen of three focal plants of the same morph, each with three flowers, within a 50 m² plot. As before, I labelled pollen from each anther a different colour. Pollen grains were labelled in the morning and all stigmas within the plot were collected at sunset and frozen for subsequent pollen counting as described in chapter 2. A single pollen movement replicate therefore consisted of three donor plants (9 flowers) of either L or R morphs and all recipient flowers within a 50 m² plot. The position of the plot was randomised for each replicate. Since flowers of *W. paniculata* last for one day only (Jesson and Barrett, 2002b) (except during cold and rainy weather), it is unlikely that labelled pollen remaining in donor flowers from a previous day's experiments will be transferred to flowers in an experimental plot. Nevertheless, I removed all donor flowers from plots after collecting recipient stigmas. I also allowed at least one day's break between pollen movement experiments to reduce the probability that pollen remaining on plants or bee bodies from previous experiments could be transferred to flowers in subsequent experiments. I repeated pollen movement experiments four times for each morph. I standardised overall anther-level pollen receipt for each morph across replicates by adjusting receipt proportions to reflect a 50:50 ratio of left and right recipient stigmas.

To test whether disassortative pollen transfer was greater than the 66% predicted from the 2:1 anther arrangement, I manually computed a chi-square test on all pollen movement data pooled with 66% disassortative pollen movement as my expected contingency table frequencies.

I determined ratios of self- to outcross-pollen transfer by placing three plants with five flowers each within the population for a day. All pollen on a single plant was labelled using one of three colours so that I could distinguish self-pollen from outcrossed pollen and thus determine relative contributions of pollen movement within plants. I repeated these experiments twice for each morph resulting in self-pollen transfer rates for 12 plants and 60 flowers. Rates of self-pollen transfer were similar between days so I pooled data from different days and calculated self-pollen transfer rates at the level of the plant.

Stigma and anther positions

I picked flowers during peak pollinator activity (9:00–12:00) and measured distances between anthers, stigmas and the centre point of the two nectar apertures in horizontal (x) and vertical (y) planes. To do this, I positioned the anthers and stigma of a flower to make contact with a small piece of glass and marked their contact positions, as well as the centre point of the two nectar apertures when viewed directly from above on the piece of glass. I converted x and y distances to coordinates by using the centre point of the two nectar apertures as the origin and making the x-axis parallel to the intersection between the two lower anthers. I determined the positions for anthers and stigmas for 20 flowers of each morph. I tested for differences in the positions of each anther and stigma using linear mixed-effect models with plant identity as a random factor. I compared stigma anther positions using Tukey post-hoc comparisons.

Statistical analyses

All analyses were performed using R (R Core Team, 2017) version 3.4.1 (R Development Core Team 2017) and the packages nlme (Pinheiro *et al.*, 2017) and multcomp (Hothorn *et al.*, 2008).



Figure 4.2. Photograph of quantum-dot-labelled pollen grains on a carpenter bee (*Xylocopa caffra*) wing and body as well as a magnified view of an individual q-dot-labelled pollen grain. In this example, pollen grains labelled with green quantum dots were from the upper anther of a left-handed morph and pollen grains labelled with red quantum dots were from the lower-opposite anther.

RESULTS AND DISCUSSION

Pollen was predominantly placed on beating wings of bees as they approached and landed on flowers (Fig. 4.2; Supplementary video 4.1). Lateral separation in pollen placement was remarkably consistent, with no anther placing more than 1% of pollen on the opposing side of pollinators (Fig. 4.3). Field measurements of anther positions indicated that upper anthers were positioned significantly wider and higher than lower anthers relative to the flower midline (Fig. 4.3; Table 4.1). This resulted in significantly different pollen placement sites for upper anthers compared to lower anthers on the same side of the flower (Fig. 4.3; Table 4.2). While lower anthers placed pollen primarily on either flank of carpenter bees, upper-anther pollen made up the majority of wing pollen (69.3%), and was placed closer to wing tips (Fig. 4.3). Wing pollen on honey bees originated mostly from lower anthers of both morphs, as honey bee wings were too short to make consistent contact with the upper anthers (Fig. 4.3).

Differences in pollen placement by different anthers (Fig. 4.3) are expected to result in pollen quality variation across pollinator bodies: since pollen placement of each lower anther is always matched by an anther from the opposite morph, stigmatic contact with lower anther placement sites is likely to result in an equal mixture of assortative and disassortative pollen. Assortative pollen movement can include pollen movement within a plant (self-pollen). Since self-pollination by *W. paniculata* results in reduced seed set (Ornduff and Dulberger, 1978; Jesson and Barrett, 2002b), pollen mixtures containing self-pollen (i.e., assortative pollen) are of lower quality than pollen mixtures without (i.e., disassortative pollen). In contrast, upper-anther pollen placement is not matched by an anther from the opposite morph; contact in these areas by reciprocal stigmas should thus result in a greater proportion of disassortative pollen receipt. Accordingly, pollen quality should depend on the distribution and degree of overlap between upper-anther pollen placement (mostly disassortative—no selfing risk) and lower anther pollen placement (mixture of disassortative and assortative pollen—potential selfing risk). To quantify pollen quality variation across pollinator bodies, I created pollen quality heat maps (Fig. 4.4),

combining pollen placement data from all anthers of both morphs. These maps show large quantities of mostly high quality-disassortative pollen in the middle of carpenter bee wings but no clear disassortative pollen ‘hotspots’ on honey bees.

Consequently, I hypothesised that stigmas should be positioned to capture pollen from disassortative pollen hotspots on carpenter bee wings, thus maximizing outcrossed pollen receipt. I measured stigma positions during peak pollinator activity (9:00–12:00); stigmas were positioned significantly higher and wider than upper anthers (Fig. 4.3; Table 4.1), suggesting their position is not simply a reciprocal match to upper anthers. Instead, their positions precisely matched areas where pollen quantity and the ratio of high quality disassortative to lower quality assortative pollen were highest (Fig. 4.4).

If stigmas are indeed so-positioned, I expected disassortative pollen movement to be more prevalent than the 66% predicted by the 2:1 anther arrangement. Indeed, upper anthers contributed most pollen to stigmas (76.34%-left, 76.70%-right), predominantly through disassortative pollen movement (100.00%-left, 99.01%-right). Although lower-opposite anthers contributed mostly to the opposite morph (disassortative 90.46%), their overall contribution was far less than that of upper anthers. Stigma-side anthers contributed most pollen to their own morph (assortative 89.40%), but the overall contribution was small. The slight mismatch in stigma–anther position skews pollen receipt towards upper anthers (Fig. 4.4), resulting in very strong disassortative pollen movement (86.01%-left and 87.31%-right)—significantly higher than the 66% initially predicted ($\chi^2=178.82$, $df=1$, $p<0.005$). This also translated to low proportional receipt of self-pollen ($8.08\pm 4.81\%$ SD, $n=12$), explaining high rates of outcrossing previously measured in populations of *W. paniculata* (range: 0.78–0.98) (Jesson and Barrett, 2002b).

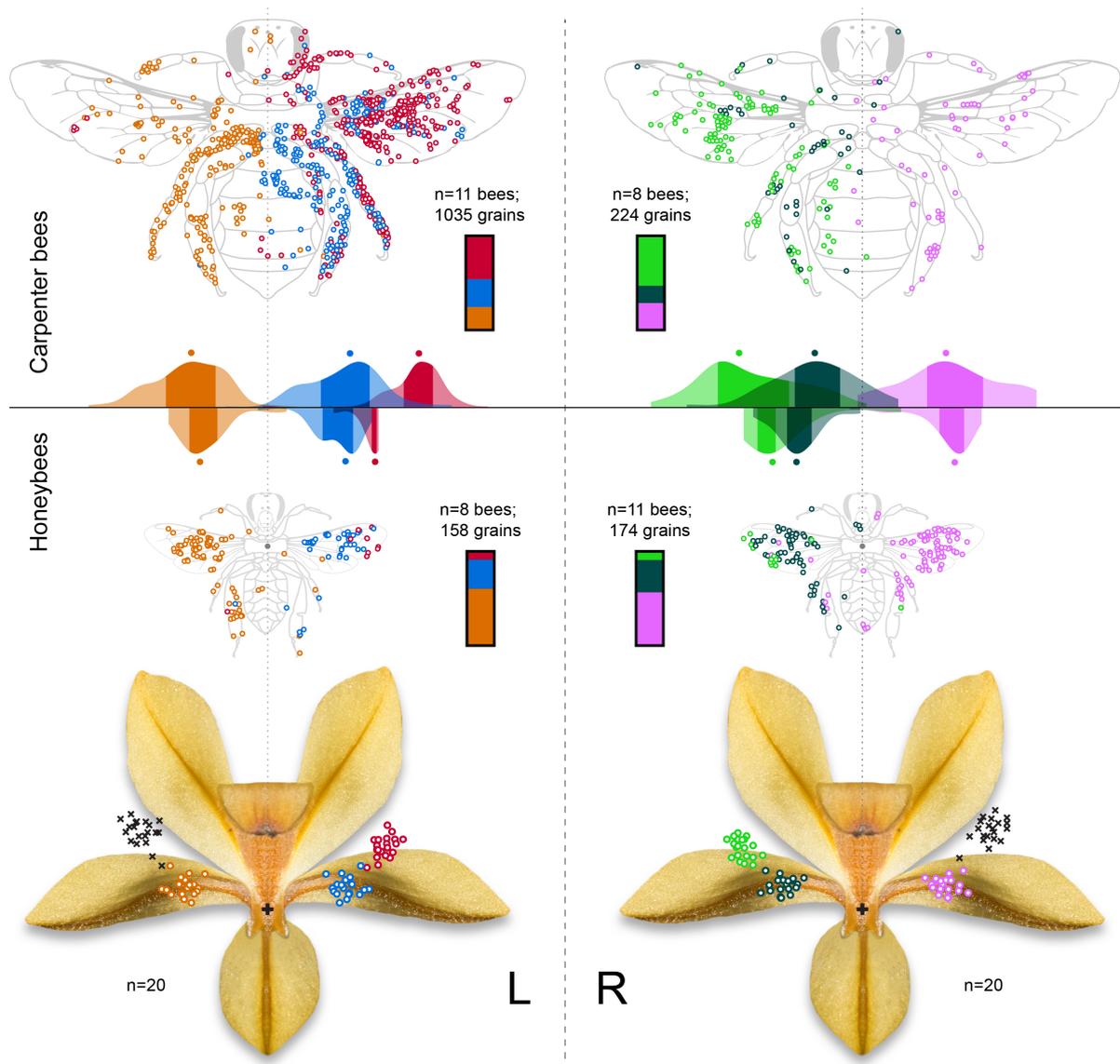


Figure 4.3. Anther-specific pollen placement on carpenter bees and honey bees. The position of anthers and stigmas are indicated by coloured dots plotted on left- and right-handed flowers shown from behind. The colours for each anther correspond to dots on pollinators which represent the positions of individual pollen grains determined for captured pollinators. Half-violin plots show the pollen placement distribution along the x-axis (L-R) on carpenter bees and honey bees for both morphs. Darker portions of violin plots represent the interquartile range and dots above distributions indicate the median. Bars show the proportion of pollen placed on bees from each anther.

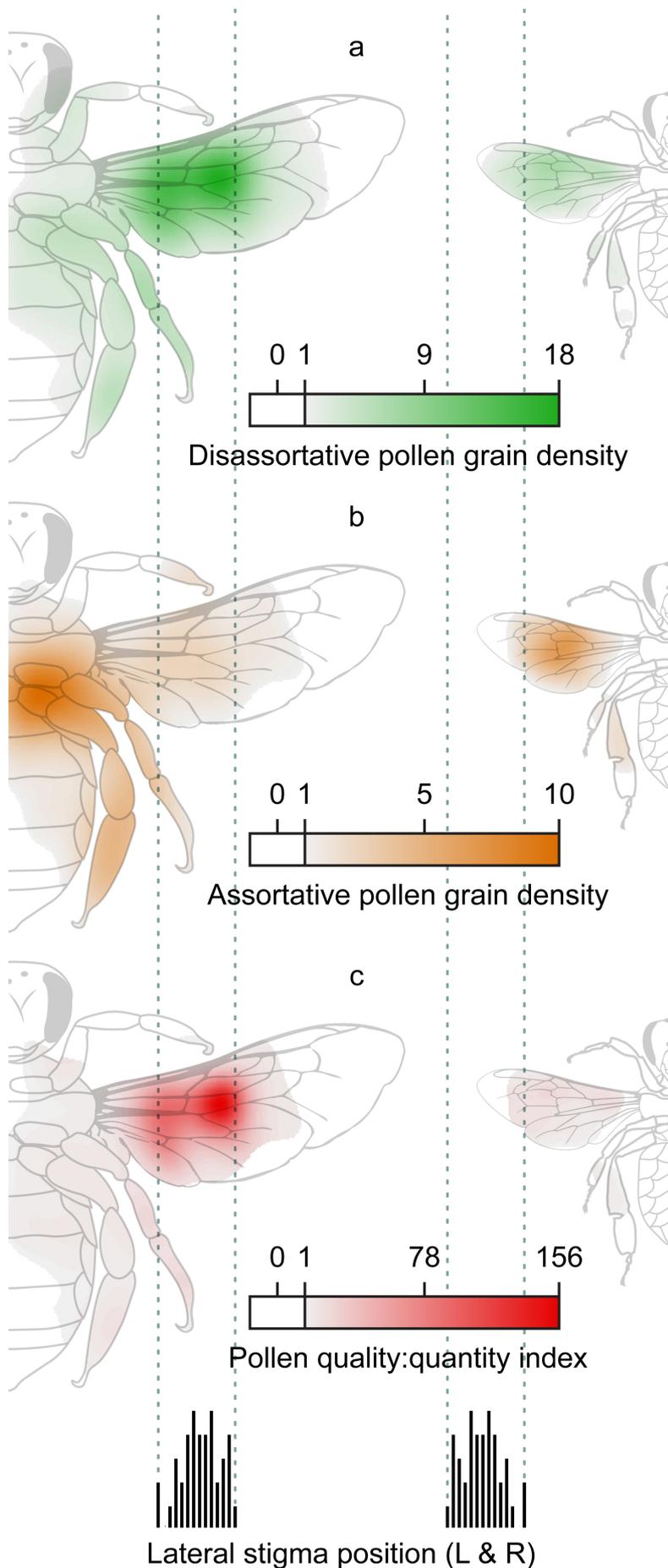


Figure 4.4. Heat maps combining pollen placement from left- and right-handed flower morphs for carpenter bees (left) and honey bees (right). Heat maps show (a) disassortative pollen density (pollen grains per 0.1 mm² grid cell), (b) assortative pollen density (pollen grains per 0.1 mm² grid cell), and (c) pollen quality–quantity (disassortative:assortative pollen grain ratio x total number of grains). White areas in heat maps represent areas where pollen density was estimated as zero. Dotted grey lines show the combined lateral range of left and right stigmas relative to flower central axes and plotted relative to bee body central axis. Black bars show the frequency distribution of lateral stigma positions within the total stigma range.

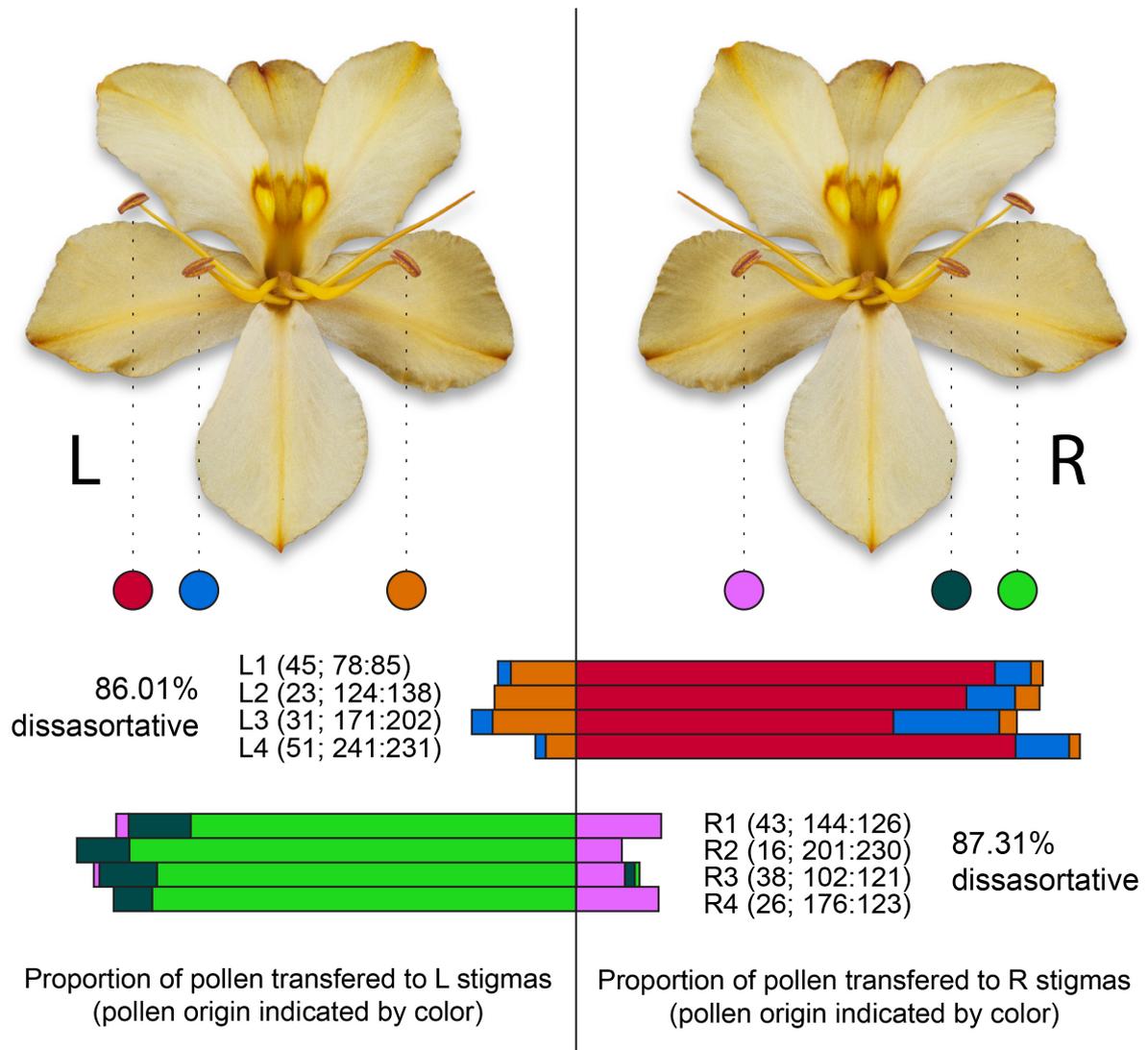


Figure 4.5. Quantum-dot-labelled pollen transfer and mating patterns between left- and right-handed morphs in a natural *Wachendorfia paniculata* population. Abundance-corrected proportions of pollen transferred from different anthers to L and R stigmas of recipients are indicated by horizontal coloured bars corresponding each anther. Each horizontal bar represents one replicate (L1–L4; R1–R4) of labelled-pollen transfer experiments. Mean disassortative mating percentages are shown for L and R morphs. The total number of labelled grains found on recipient stigmas and the number of L:R morph stigmas collected are shown for each replicate in brackets.

Table 4.1 Linear mixed-effect model post-hoc comparisons (Tukey HSD) of stigma and anther positions on x and y axes (n=20 per comparison group)

Comparison	X-axis (horizontal)			Y-axis (vertical)		
	Estimated difference (mm)	z-value	p	Estimated difference (mm)	z-value	p
Anthers within morphs						
L: stigma-side L: upper	15.162	63.268	<0.0001			
L: upper L: lower-opposite	2.889	12.054	<0.0001	-3.673	-13.403	<0.0001
L: lower-opposite L: stigma-side	-12.274	-51.214	<0.0001	NA	NA	NA
R: stigma-side R: upper	-15.422	-64.350	<0.0001	-3.427	-12.508	<0.0001
R: upper R: lower-opposite	-3.119	-13.013	<0.0001			
R: lower-opposite R: stigma-side	12.303	51.338	<0.0001	NA	NA	NA
Anthers between morphs						
L: upper R: upper	-17.977	-75.010	<0.0001	0.111	0.380	0.9999
L: upper R: stigma-side	-2.555	-10.660	<0.0001	3.539	12.091	<0.0001
L: upper R: lower-opposite	-14.858	-61.998	<0.0001			
R: upper L: lower-opposite	-15.088	-62.956	<0.0001	-3.562	-12.170	<0.0001
R: upper L: stigma-side	-2.814	-11.742	<0.0001			
L: stigma-side R: lower-opposite	0.304	1.270	0.9100			
L: stigma-side R: stigma-side	12.608	52.608	<0.0001	-0.134	-0.459	0.9998
R: stigma-side L: lower-opposite	0.334	1.394	0.8604			
L: lower-opposite R: lower-opposite	-11.969	-49.944	<0.0001			
Stigmas compared to anthers						
L L: lower-opposite	-16.002	-66.770	<0.0001	-5.005	-18.267	<0.0001
L L: stigma-side	-3.728	-15.556	<0.0001			
L L: upper	18.891	78.824	<0.0001	1.333	4.864	<0.0001
L R: lower-opposite	4.033	16.827	<0.0001	4.871	16.645	<0.0001
L R: stigma-side	16.336	68.164	<0.0001			
L R: upper	0.914	3.814	0.0035	1.444	4.933	<0.0001
R L: lower-opposite	3.891	16.238	<0.0001	-5.190	-17.735	<0.0001
R L: stigma-side	16.165	67.452	<0.0001			
R L: upper	1.003	4.184	0.0008	-1.517	-5.185	<0.0001
R R: lower-opposite	15.861	66.182	<0.0001	-5.056	-18.452	<0.0001
R R: stigma-side	3.557	14.844	<0.0001			
R R: upper	-18.979	-79.194	<0.0001	1.629	5.943	<0.0001

Table 4.2 Linear mixed-effect model post-hoc comparisons (Tukey HSD) of pollen placement on x and y axes for carpenter bees (Fig 4.3 for sample sizes) and honeybees (see Fig 4.3 for sample sizes)

Comparison		X-axis (horizontal)			Y-axis (vertical)		
		Estimated difference (mm)	z-value	p	Estimated difference (mm)	z-value	p
Carpenter bees							
Stigma-side	Lower-opposite	7.050	14.545	<0.0001	0.201	0.555	0.8435
Upper	Lower-opposite	-1.102	-2.615	0.0240	2.350	7.484	<0.0001
Upper	Stigma-side	-8.152	-17.711	<0.0001	2.150	6.307	<0.0001
Honeybees							
Stigma-side	Lower-opposite	-1.923	-2.596	0.0239	0.408	1.389	0.3366
Upper	Lower-opposite	-1.098	-0.837	0.6718	-0.141	-0.272	0.9588
Upper	Stigma-side	0.824	0.660	0.7806	-0.549	-1.109	0.4984

CONCLUSION

Handedness in *W. paniculata* clearly leads to disassortative pollen transfer and consequently low self-pollen movement. However, the stigma-side anther reduces the quality of pollen in the lower-anther deposition sites and may reduce male/female fitness through production of low-quality offspring. While this may seem counter-intuitive, pollen from the lower anthers may have an important role in seed fertilisation where/when carpenter bees are rare or absent—several cases of carpenter bee absence/rarity have been reported for *W. paniculata* (Ornduff and Dulberger, 1978; Jesson and Barrett, 2002b). Because of the reduced wingspan of honey bee wings, pollen transfer is unlikely to occur as frequently in populations without carpenter bees. But, when it does occur, most pollen transfer is likely to be from the lower anthers and plants may therefore trade-off slightly lower quality pollen for reproductive assurance when carpenter bees are absent. Moreover, although the stigma-side anther poses a risk of selfing, it can also contribute to outcrossing through intramorph pollen transfer. The arrangement of anthers and stigmas in *W. paniculata* may therefore represent a bet-hedging strategy to ensure that male and female function contributes to overall plants fitness in a variety of pollination environments: when carpenter bees visit flowers often, pollen movement will likely be dominated by upper anthers and result in predominant outcrossing; however, when carpenter bees visit flowers infrequently, or when morph ratios are highly skewed, pollen movement will likely be dominated by lower anthers, leading to reduced outcrossing, but with increased reproductive assurance relative to theoretical flowers only possessing upper anthers.

SUPPLEMENTARY INFORMATION

Supplementary video 4.1: Slow-motion video of honey bees visiting *W. paniculata*, showing wing contact with anthers and stigma

https://1drv.ms/v/s!Au0ibLocAa_sic9vpDGRwYQ8qunvbw

CH. 5. PLANT–POLLINATOR INTERACTIONS ALONG THE PATHWAY TO PATERNITY

INTRODUCTION

...plants are gene donors and gene receivers [...] these two activities are not necessarily complementary, compatible, or directed toward the same end.

Janzen, 1977

It has been more than 40 years since biologists began to fully appreciate the importance of male fitness in the evolution of plant reproductive traits (Horovitz and Harding, 1972; Willson and Rathcke, 1974; Gilbert, 1975; Janzen, 1977; Lloyd and Webb, 1977; Willson, 1979). In the preceding century, the view that seed production was a sufficient measure of reproductive fitness in hermaphroditic plants went largely unquestioned. However, the increasing realisation that some individuals in a population may achieve greater reproductive success through pollen export than pollen receipt (Horovitz and Harding, 1972) catalysed a major paradigm shift; by the end of the 1970s, male fitness could no longer be ignored.

The male-fitness awakening was followed by a burst of new theory, much of it highlighting the potential for sexual selection to act on male mating success in plants [as it does in animals (Bateman, 1948)], since large numbers of pollen grains often compete for a limited number of ovules (e.g., Willson, 1979, 1990, 1994; Queller, 1983; Stephenson and Bertin, 1983). To some, it was initially unclear if, or how, sexual selection could act in the early stages of the pollination process (i.e. before pollen deposition onto stigmas) when plants interact indirectly through pollination agents (e.g., Charlesworth *et al.*, 1987; Lyons *et al.*, 1989; Grant, 1995). However, the parallels between pollen-tube races for ovules in plants after pollination, and sperm competition for ova in animals after copulation, were immediately clear. Indeed, in the post-pollination phase, selection on pollen traits that enhance pollen competition and mating success have now been well-demonstrated. These traits include pollen size (McCallum and Chang, 2016), pollen provisioning (Delph *et al.*, 1997), and pollen-tube growth rate (Bertin,

1988; Spira *et al.*, 1996; Sorin *et al.*, 2016; Harder *et al.*, 2016a), providing clear functional links between pollen traits and male reproductive success.

The early controversy surrounding sexual selection in plants has since dissipated, with most plant-reproductive biologists today recognizing the importance of sexual selection in shaping male reproductive traits that affect both pollen-transport and post-deposition fertilisation success (Murphy, 1998; Skogsmyr and Lankinen, 2002; Delph and Ashman, 2006; Moore and Pannell, 2011). While aspects of post-deposition fertilisation success have been relatively well studied, fewer studies have directly addressed the link between various male reproductive traits and pollination success. Yet, a large proportion of male reproductive success may be determined by events that occur long before pollen germination on stigmas (Fig. 5.1). In most plants, the pathway to successful pollen export is highly complex: the combination of pollen transfer mediated by animals (that have no interest in pollinating flowers), and the simultaneous display of multiple flowers, introduces substantial variability into the male reproductive pathway (Barrett, 2003). As a result, male gametes can be lost to a dazzling array of fates before ovule fertilisation (Inouye *et al.*, 1994), with each avenue of pollen loss potentially acting as a unique selective force on plant reproductive traits. This review will focus on the complex journey undertaken by pollen, from anthers to stigmas, in animal-pollinated plants. Travelling along the different stages of this journey, I explore how male–male competition may drive the evolution of competitive pollen-export strategies. To give context to this journey, I start with a brief history on the study of pollen fates.

The study of pollen fates

For species with granular pollen, only a tiny fraction of pollen produced by flowers ever reaches conspecific stigmas. For example, 2.9% of pollen grains produced in a community of 26 flowering-plant species were deposited on conspecific stigmas (Gong and Huang, 2014).

Consequently, pollen export represents a significant challenge to male fertility, and a potentially

significant opportunity for selection to act on traits that optimize pollen production, transport, and delivery (Fig. 5.1).

Molecular paternity-assignment techniques, have allowed biologists to start linking variation in pollination-relevant traits [e.g., floral display size, (Harder and Barrett, 1995; Karron *et al.*, 2012); flowering phenology, (Austen and Weis, 2016); corolla-tube shape (Kulbaba and Worley, 2012, 2013); and petal area (Briscoe Runquist *et al.*, 2017)] to variation in siring success. However, because so few pollen grains ever sire seeds, it is difficult to make direct links between paternity and the multitude of possible non-reproductive pollen fates which may explain a large proportion of variation in male reproductive success (Fig. 5.1). To understand how male reproductive traits function in determining siring success during the pollination phase (Fig. 5.1) requires explicit tracking of pollen movement from individual plants.

Empirical studies of pollen movement have historically been limited to the few species for which pollen tracking has been possible [e.g., orchids, for which pollinia can be dyed (e.g., Peakall, 1989; Johnson and Harder, 2018), or species with pollen colour or size polymorphisms (e.g., Thomson and Plowright 1980; Nichols 1985; Holsinger and Thomson 1994; Stone 1995; Keller *et al.* 2014)]. Several important aspects of male reproductive function have been explored in orchids by direct tracking of massulae dispersed from dyed pollinaria (Johnson and Harder, 2018). These include trade-offs between pollination quantity and quality for rewarding and deceitful plants (Johnson *et al.*, 2004; Jersáková and Johnson, 2006; Walsh and Michaels, 2017), the detriment of self-pollination to male outcrossing success (i.e., pollen discounting) (Johnson *et al.*, 2005), and contrasting selection for floral morphology through male and female components of fitness (Ellis and Johnson, 2010).

Pollen movement studies exploring male function in plants with granular pollen dispersal are comparatively rare. Among these few studies, the landmark experiments conducted by Harder and Thomson (1989) and Thomson and Thomson (1989) highlight the importance of

tracking pollen movement: by exploiting a pollen-colour polymorphism, Harder and Thomson tracked dispersal of *Erythronium grandiflorum* pollen and generated detailed quantitative estimates of various components of the pollen export process. A surprising finding of these experiments was that total pollen export (from a single bumble bee visit) to multiple recipient stigmas did not increase linearly with the amount of pollen initially placed on bumble bees. Instead, flowers experienced diminishing returns on pollen export success: the larger a pollen load placed on a bee, the greater the proportional pollen loss during transport. This pattern may be common in animal-pollinated plants for several reasons. For example, pollen loss and displacement may increase with the amount of pollen placed on pollinators if large pollen loads increase the probability and intensity of grooming (as found by Harder 1990 for bumble bees). A greater proportion of large pollen loads may be more likely to fall off during transport since smaller proportions of pollen are in direct contact with the pollinator (Harder and Wilson, 1997; Johnson *et al.*, 2005; Harder and Johnson, 2008). The potential ubiquity of diminishing returns associated with large pollen loads should therefore broadly favour restricted pollen presentation, leading to small pollen loads placed on individual pollinators. However, the extent to which plants should restrict their rate of pollen presentation will depend on pollinator visit rates—restricting pollen presentation when pollinator visits are rare would result in lost mating opportunities and wasted pollen production (Harder and Thomson, 1989; Thomson and Thomson, 1992; Harder and Wilson, 1994, 1998a; Thomson, 2003). These two predictions form the core of pollen presentation theory.

Pollen presentation theory has provided much-needed insight into the evolution of pollen dispersal strategies and the role of male fitness in the evolution of plant-reproductive traits. However, several aspects of the pollination phase remain poorly explored. As new technology improves our ability to identify pollen donors and track movement of individual pollen grains [e.g., pollen grain sequencing (Matsuki *et al.*, 2007, 2008; Chen *et al.*, 2008; Hasegawa *et al.*, 2009, 2015) and pollen labelling (Minnaar and Anderson, 2018)], I envisage that

the empirical study of male reproductive function will become ever-more feasible, allowing biologists to fill some of the long-standing gaps between empirical studies and the 40+ years of theoretical work on male fitness in plants. In this review, I endeavour to identify some of these gaps and the progress made in addressing them, while also highlighting emerging lines of enquiry that promise to yield exciting results in the near future. Because post-pollination processes have been comparatively well-studied, and thoroughly reviewed elsewhere (Snow, 1994; Delph *et al.*, 1997; Delph and Havens, 1998; Harder *et al.*, 2016b; a; Williams and Mazer, 2016; Williams *et al.*, 2016), the scope of this review is limited to the pollination phase of the pathway to paternity. However, where applicable, I discuss how processes in the pollination phase of the pathway may alter post-pollination success.

The rest of the review will take the reader on a journey along the pathway from pollen production to pollen deposition onto stigmas. Along this pathway, I discuss multiple functional steps in the pollination process likely to influence eventual siring success, including mechanisms that divert or block pollen flow, resulting in an ever-narrowing pathway to paternity. In this review, I refer to the mechanisms that divert or block pollen flow as 'siring barriers' because they act as successive barriers that limit the siring potential of a donor's pollen grains. Through each barrier, fewer and fewer of the donor's pollen grains remain available for ovule fertilisation. Of course, in most cases, a plant's total pollen export does not occur in a single pollen-export event from a single flower, and therefore, the pathway to paternity consists of several sub-pathways from successive pollinator visits to multiple flowers on individual plants. I therefore also consider how plants should allocate pollen to these sub-pathways among visiting pollinators—the basic tenet of pollen presentation theory. I expand on prior reviews which highlight the pathway to paternity (Inouye *et al.*, 1994; Harder, 2000; Harder and Routley, 2006; Barrett and Harder, 2017), by discussing potential male reproductive strategies that may mitigate siring barriers, thereby increasing potential siring success. Furthermore, at each step along the pathway I present hypotheses, expose interesting questions, and suggest avenues for

future research. In particular, over-and-above competition for pollinator visits or pollen-tube competition in styles, I emphasize other mechanisms through which plants are likely to compete for male mating success. Specifically, I highlight the largely neglected possibility for pollinator bodies to act as competitive arenas where plants have the opportunity to displace, cover, and remove pollen grains of their competitors and increase their siring success. At the end of the journey, I hope that the reader will share the excitement about a research field filled with opportunities.

PATHWAY TO PATERNITY

The pathway to paternity can be divided into three phases: (1) pollen production and presentation; (2) pollen transfer (pollen placement, transport, and deposition); and (3) pollen germination and ovule fertilisation (Fig. 5.1). Each phase in the pathway can be further divided into sub-phases representing the sequential steps to siring success. This review only focuses on the first two phases of the pathway; however, to better illustrate the context of the first two phases, I have included pollen germination and ovule fertilisation in the depiction of the pathway to paternity (Fig. 5.1). From this point onwards, 'male' or 'males' refer to the male reproductive function of an individual hermaphroditic plant or the separate male functions of several hermaphroditic plants. For example, 'male reproductive success' refers to the male component of a hermaphroditic plant's reproductive success, while 'competition between males' refers to competition between the male reproductive functions of separate hermaphroditic individuals. References to 'female' and 'females' should be treated similarly. I also refer to pollinators as pollen vectors. Although the term 'pollinator' is more commonly used in pollination biology, the term 'pollen vector' was used instead as it places emphasis on plants as the agents manipulating floral visitors to transport pollen, instead of floral visitors themselves having agency in the pollination process.

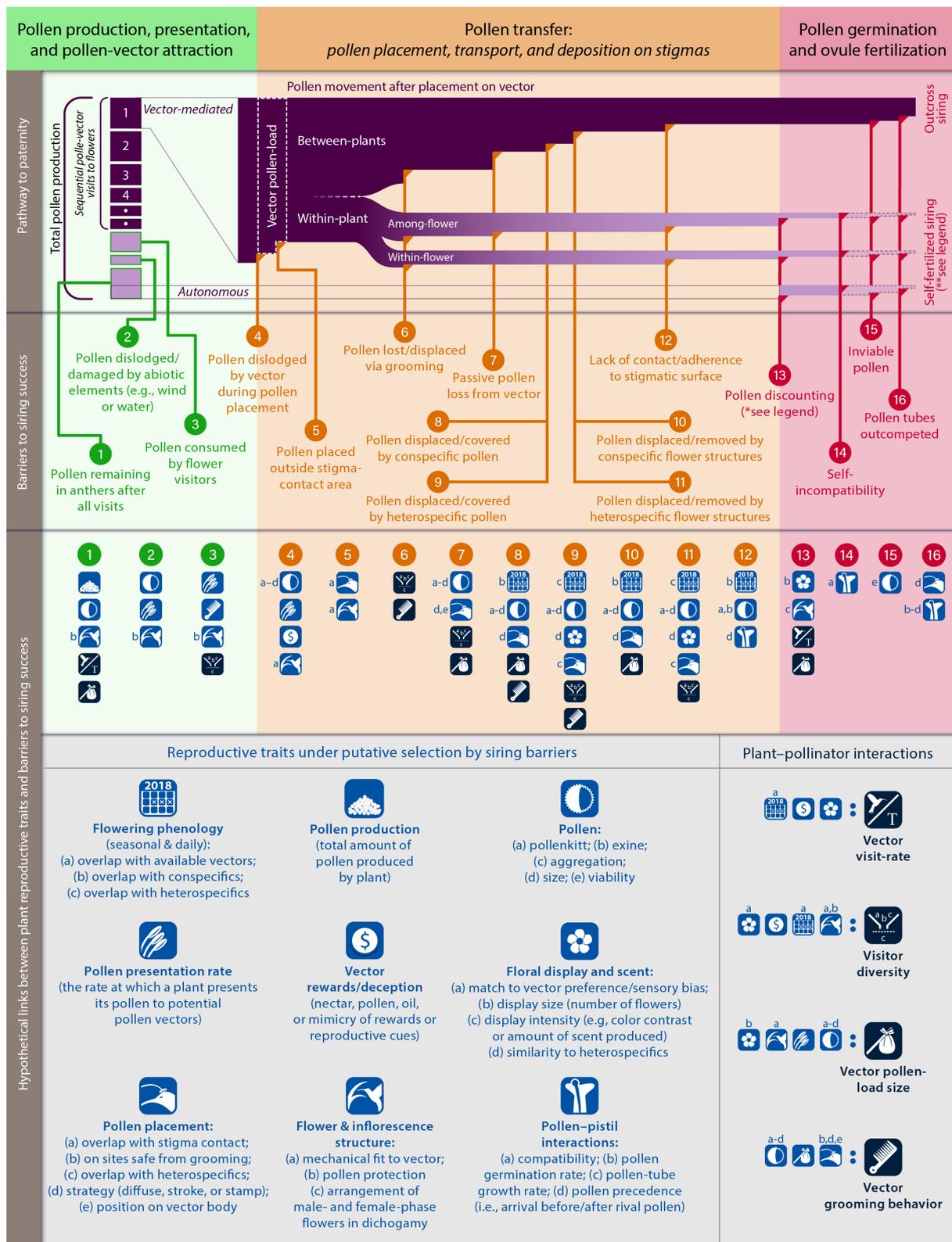


Figure 5.1. A conceptual diagram of the pathway to paternity (row 1). The pathway is divided into three phases: (1) pollen production, presentation and pollen vector attraction (green); (2) pollen transfer (orange); (3) pollen germination and ovule fertilization (pink). Along these three main phases, a sequence of 16 siring barriers potentially diminish the probability of siring success (row 2). Selection to increase male reproductive success through each siring barrier along the pathway is likely to act on certain suites of reproductive traits (blue icons, row 3) and the products of pollen vector interactions with combinations of these traits (dark blue icons, row 3);

the descriptions of these traits and the products of vector–trait interactions can be found in row 4. This review is primarily focused on the first two phases of this pathway (green and orange). *Pollen discounting represents the portion of pollen lost to self-stigmas that could otherwise have been exported to outcross mates (Harder and Wilson, 1998*b*). Autonomous self-pollination is not discounted if it is delayed and occurs once there is no chance of further visits by pollen vectors. **For self-incompatible plants, all pollen deposited on self-stigmas is lost. However, if self-compatible, the pathway to paternity may continue to self-fertilization (pale purple pathways with dotted-line borders) and may aid in ensuring reproduction when opportunities to export pollen are scarce. The extent to which self-fertilization may contribute to siring success depends on the combination of pollen discounting and inbreeding depression (Harder and Wilson, 1998*b*). Illustration: C. Minnaar.

Pollen production and presentation

Pollen production

Pollen production per flower and per plant varies widely within and among species of animal-pollinated plants (Stanton and Preston, 1986; Devlin, 1989; Young and Stanton, 1990a; Stanton *et al.*, 1991; Ashman, 1998; Gong and Huang, 2014). Since pollen production, in both quantity and quality, is often resource limited (Goldman and Willson, 1986; Rameau and Gouyon, 1991; Ashman, 1994; Delph *et al.*, 1997; Obeso, 2002), a significant proportion of this variation may be explained by environmental factors such as soil-nutrient availability (Young and Stanton, 1990b; Lau and Stephenson, 1993, 1994), extent of herbivory (Quesada *et al.*, 1995; Mutikainen and Delph, 1996), and environmental temperature (Johannsson *et al.*, 1994). Nevertheless, selection may act on the remaining heritable variation in pollen production (Young *et al.*, 1994; Queller, 1997) to increase male mating success, since total pollen production sets the upper limit of a plant's reproductive potential (Stephenson and Bertin, 1983).

In one of the earliest molecular paternity-assignment studies in plants, Schoen and Stewart (1986) found that increased cone production resulted in increased siring success for wind-pollinated white spruce trees. However, since then, very few studies have empirically tested the relationship between pollen production and realised siring success, especially in animal-pollinated plants; those that have, provide contrasting results. For example, Stanton *et al.* (1991) found that wild radish plants which produce more pollen also sired more seeds. However, the link between pollen production and siring success was indirect, since plants with high pollen production received more visits from small native bees relative to honey bees, which decreased siring success. Surprisingly, Ashman (1998) found no relationship between pollen production per plant (displaying a single flower) and realised paternity in wild strawberry plants. Instead, siring success was correlated with the total amount of pollen removed from anthers in a plant. These two studies highlight the importance of the fundamental functional link between pollen production and realised siring success—pollen removal and transport by pollen vectors—

which may limit the potential for pollen production to directly influence siring success in animal-pollinated plants. For example, a male producing more pollen than its rivals may simply end up with more pollen left in its anthers **1** (encircled numbers refer to siring barriers in Fig. 5.1) than rivals, if excess pollen production is not accompanied by an increased vector visit rate, or placement of larger vector pollen-loads than rivals. Still, increased vector attraction and placement of large vector pollen-loads may respectively aggravate diminishing returns on pollen production through increased self-pollination (Klinkhamer and de Jong, 1993) and increased proportional pollen loss during transport (Harder and Thomson, 1989). Evidently, the interaction between plants and biotic pollen vectors demands male reproductive strategies that go beyond simply increasing investment in gamete production.

Pollen presentation

Animal-pollinated plants face a unique challenge in delivering pollen to mates. They cannot directly deliver gametes in measured doses through copulation, nor can they release their gametes into a surrounding medium for diffuse transport. Instead, available pollen needs to be presented and placed on individual pollen vectors in a way that maximizes siring success. Since plants cannot anticipate the arrival of vectors, they face strategic 'decisions' on how to present and place vector pollen-loads of the correct size, which may vary with time and vector-visit frequency (Harder and Wilson, 1994). Therefore, in most species, the total amount of pollen produced by a plant is not simply presented all at once: a limited number of flowers may be displayed simultaneously over a long flowering period, or anthers may release pollen gradually (Fig. 5.3) (Castellanos *et al.*, 2006). The degree to which plants restrict pollen presentation, depends on the frequency of visits over time (Harder and Wilson, 1994) and the severity of diminishing returns, which may vary widely between pollen-vector types (Thomson, 2003). Plants appear to dynamically vary vector pollen-load sizes in response to vector visit frequency (Harder and Barclay, 1994; Harder and Wilson, 1994), or dynamically alter floral display size (and therefore the amount of pollen presented) with pollination rate (Harder and Johnson, 2005). A

recent study also found geographic divergence in pollen presentation rate for *Claytonia virginica*, likely reflecting local adaptation to pollen vectors that differ in visitation and pollen depletion rate (Parker *et al.*, 2018). The generally accepted explanation for the evolution of restricted pollen presentation (while accounting for vector-visit frequency) is to mitigate diminishing returns associated with increasing vector pollen-load size (i.e., pollen presentation theory: Harder and Thomson, 1989; Thomson and Thomson, 1992; Harder and Wilson, 1994). However, plants do not always benefit from placing small vector pollen-loads (see **Pollen placement and deposition competition** below), and pollen presentation traits may also be under selection through processes that occur prior to pollen transport (e.g., abiotic pollen loss).

Abiotic pollen loss. By presenting pollen, plants face the risk of losing it through abiotic mechanisms ② (Fig. 5.1) such as wind dislodgement [e.g., in a single night, ca. 50% of pollen grains were lost from the anthers of *Silene* plants under pollen-vector exclusion (Reynolds *et al.*, 2009)] (Fig. 5.2A) and water damage (Mao and Huang, 2009). Consequently, floral structures thought to function in vector attraction and mechanical aspects of pollen presentation and placement may, in part, be under selection to protect pollen from wind and rain (Mao and Huang, 2009). Reductions in siring potential as a result of abiotic pollen loss or damage, and the traits putatively evolved to prevent such loss [e.g., sticky pollenkitt and large pollen grains (Pacini and Hesse, 2005) or floral orientation (Wang *et al.*, 2010)], have rarely been explored.

Consumptive emasculation. While pollen is frequently offered as a reward to vectors to promote pollen export, some animals consume large quantities of pollen without transferring appreciable amounts between flowers (Hargreaves *et al.*, 2009). These so-called pollen thieves or robbers lower male fitness by reducing the amount of pollen available for export ③ (Fig. 5.1) [e.g., (do Carmo and Franceschinelli, 2004; Koski *et al.*, 2018)]. Pollen presentation mechanisms control not only the amount of pollen presented to vectors per visit, but also the quantity of pollen exposed to potential theft (Hargreaves *et al.*, 2009). Thus, traits controlling pollen release could also have evolved to protect pollen from thieves. For example, buzz-pollinated anthers

could have evolved to increase pollen export efficiency by controlling the amount of pollen removed by pollen-collecting vectors per visit (Harder and Barclay, 1994). However, buzz-pollinated anthers may also have evolved to prevent pollen theft by insects incapable of buzzing at the correct frequency (Hargreaves *et al.*, 2009).

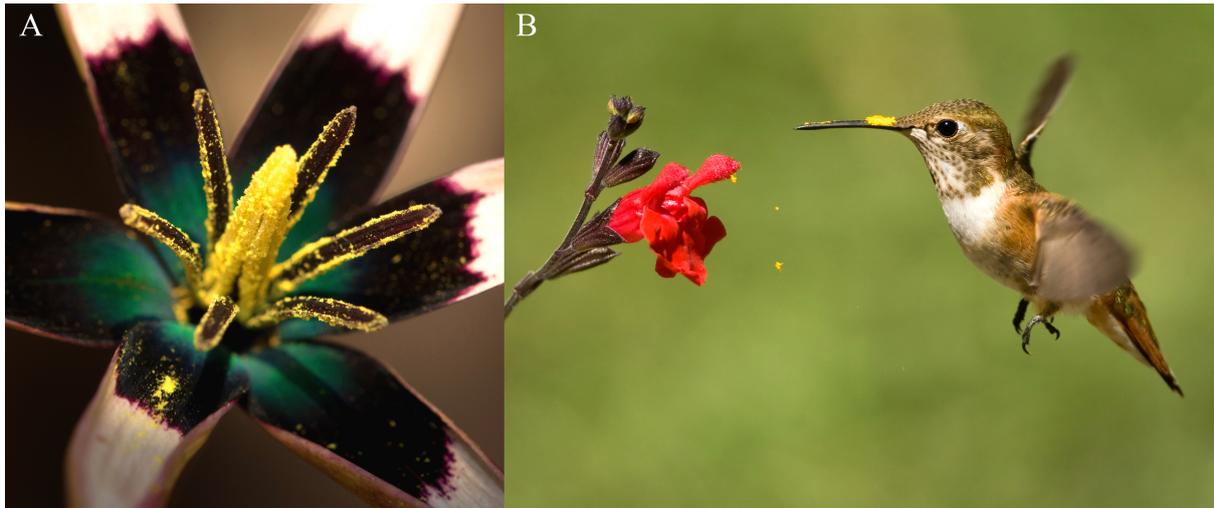


Figure 5.2. Pollen that is dislodged from the reproductive parts of flowers either by the abiotic environment or by pollinators has a much lower probability of ever reaching the stigma of another flower. (A) Pollen shows up yellow against the contrasting petal background of *Pauridia capensis* after a gust of wind dislodged it from the anther. Photo: M. de Jager. (B) Pollen from the anthers of *Salvia greggii* falls to the ground after being dislodged by a visiting rufous hummingbird. Photo: S. Tekiela – Nature Smart Images.



Figure 5.3. Plants have evolved various mechanisms to control the rate of pollen presentation to plants. Plants may stagger the opening of flowers or anthers within flowers to control the amount of pollen exposed to individual pollinators. (A) For example, the anthers of some species (e.g. *Lillium longiflorum*) expose pollen quickly so that large vector pollen loads are placed onto vectors with each visit. Other species expose their pollen over the course of many days so that pollen is placed onto vectors in small doses through multiple visits. Here, I show the slow release of *Gethyllis verticillata* pollen as the anthers roll up and dehisce over several days: (B) day 1, (C) day 3. Photos: B. Anderson. (D) Although pollen from *Asclepias verticillata* is aggregated in pollinia and presented to pollen vectors all at once, the rate of pollen removal may be controlled by the low probability of the pollinarium's corpusculum attaching to a pollinator. This can be influenced by floral features such as floral structure or nectar abundance which can impact on visitation duration. Each pollinarium comprises joined pollinaria from adjacent anthers (there are five anthers in *Asclepias*), and the attachment of a single pollinarium to the hairs or bristles of visiting pollinators removes one-fifth of the flower's pollen in a single visit. Note the paired pollinaria (indicated by an arrow) on the left front leg of the bee *Bombus griseocollis*, magnified in (E). Photos: J. Karron.

Pollen-vector attraction

Attractive floral traits are often thought to be largely under selection through male function because male reproductive success is more likely to be enhanced by frequent visitation, while female reproductive success is more likely to be limited by resources for provisioning fertilised ovules (Willson and Rathcke, 1974; Burd and Callahan, 2000). Initial studies found support for male-biased function in large floral displays (Willson and Rathcke, 1974; Queller, 1983). However, these two studies used pollen removal as a proxy for male fitness, which may not relate directly or linearly to eventual siring success. These studies also focused on milkweeds which disperse pollen in the form of pollinaria that take almost two minutes to dry and move into the orientation best suited for insertion into stigmatic slits (Queller, 1983). Since pollen vectors typically spend fewer than two minutes foraging on individual plants (Queller, 1983), the risk of geitonogamous pollen transfer may be low compared with species that disperse granular pollen. Subsequent studies on species with granular pollen add to a growing body of evidence that increased floral display size may increase seed production but decrease seed quality through increased pollen movement within plants (geitonogamy) (Harder and Barrett, 1995; Karron and Mitchell, 2012).

The argument that male fitness in plants is more dependent on vector-visit frequency than female fitness is based on Bateman's (1948) principle: selection for traits that increase mate acquisition should almost always be stronger in males since their gametes vastly outnumber those of females. However, this direct application of Bateman's principle fails to address two important aspects of reproduction unique to animal-pollinated plants. First, although plants rely on animal vectors to facilitate reproduction, they do not mate with their vectors. Consequently, visitation frequency cannot be equated with mating frequency. Second, most animal-pollinated plants have several independent, but simultaneously-functioning male and female reproductive organs. This creates opportunity for self-pollination and self-fertilisation within, and between a plant's flowers, which may counter selection for increased

attractiveness through large floral displays (Barrett, 2002b; Mitchell *et al.*, 2004). Therefore, when considering selection for increased attractiveness, we need to examine how traits influence pollen-vector foraging patterns at the plant- and flower-level. For instance, a rewarding plant with a relatively large number of simultaneously receptive flowers will likely attract more pollen vectors than a plant with fewer flowers (Conner and Rush, 1996; Mitchell *et al.*, 2004). However, pollen vectors are likely to visit several flowers on the same plant in succession (Mitchell *et al.*, 2004). Consequently, plants with larger floral displays increase their risk of geitonogamous pollen transfer (Harder and Barrett, 1996; Karron *et al.*, 2009). Therefore, production of smaller floral displays over a longer flowering period should increase reproductive success (Karron and Mitchell, 2012).

Much of the debate surrounding male-biased selection for attractive floral displays has been informed by studies on floral display size and the associated costs of geitonogamy. Yet, several other floral traits may influence pollen-vector visit rates without an increased risk of geitonogamy or pollen discounting. These traits include: flower size (e.g., Conner and Rush 1996), scent (e.g., Kessler *et al.* 2008; Larue *et al.* 2015), colour [e.g., overall colour (Stanton *et al.*, 1986), colour pattern (de Jager *et al.*, 2016; Kemp *et al.*, 2018), and colour brightness and contrast (Sletvold *et al.*, 2016)], shape and symmetry (e.g., Møller 1995; Gómez *et al.* 2006), and height above ground (e.g., Peakall and Handel 1993). Since these traits do not directly influence geitonogamy, they are more likely to show male-biased selection following Bateman's predictions, and therefore deserve more detailed study in the context of male fitness.

Plants may also increase floral reward quality and quantity to attract pollen vectors to increase visitation rates and siring success (Thomson, 1988). However, the potential male-fitness benefits of offering greater rewards to vectors may be limited: increased floral-nectar quantity may increase the number of flowers visited per plant, as well as the amount of time spent on a single flower (Zimmerman, 1983; Thomson, 1986; Klinkhamer *et al.*, 1991), leading to increased self-pollination and pollen discounting (Klinkhamer and de Jong, 1993; Hodges, 1995;

Jersáková and Johnson, 2006). Another way of increasing vector recruitment and, potentially, siring success, is to attract, reward, and place pollen on a greater variety of potential flower visitors (generalization). However, plants with relatively generalized pollination strategies may risk increased pollen loss during pollen transport 6 7 8 9 10 11. For example, plants with more diffuse pollen placement may place pollen on a larger subset of available flower visitors but may in turn suffer increased pollen wastage if more of their pollen is placed outside of grooming safe-sites 6 or if pollen is placed on novel pollinators that visit heterospecific flowers 9 11 more frequently. The various siring barriers in the pathway to paternity are thus closely linked to the evolution of specialization and generalization.

Pollen transfer

The process of pollen transfer is often depicted as sequential (pollen placement, transport, and deposition). However, the reality is far more complex. Once pollen is placed on a vector, the number of potential fates for individual pollen grains is immense. Pollen may move within plants, between plants, and between species, from anthers to vectors and back to other anthers again, onto stigmas only to be removed again and deposited onto other stigmas. Pollen grains might move across vector bodies through grooming and displacement, or coverage by other pollen grains, and most pollen grains never reach plants. In fact, nearly all pollen produced by a plant may be lost before reaching compatible stigmas, and many grains that do eventually reach stigmas may no longer be viable (Thomson *et al.*, 1994; Dafni and Firmage, 2000; Parker *et al.*, 2015). The movement of pollen is therefore highly stochastic, with successful pollen transfer being strongly dependent on chance (Richards *et al.*, 2009; Harder *et al.*, 2016b). Yet, in this complex chaos of pollen movement, males may still increase their siring success through various strategies that influence the fate of their pollen and the pollen of their rivals. In this section I examine these strategies. The subsections presented below address various important aspects

of the pollen transfer process which, in addition to siring success, may also influence the distance of pollen moved, mate diversity, and pollen carryover.

Mechanical fit of pollen-vectors

After producing and presenting pollen, and attracting a potential pollen vector, a plant still needs to physically manipulate the vector to place pollen on their bodies. This manipulation requires a reward (or the promise of it) (Pyke, 2016) which acts as a lure, drawing pollen vectors into the flower's morphological structure, where they are forced to make contact with anthers and pollen (Fig. 5.4). Thus, while the amount of pollen available for placement on a vector depends primarily on pollen presentation traits (e.g., pollen aggregation and anther dehiscence rate), every other aspect of pollen placement (realised vector pollen-load size and pollen placement position, direction, and accuracy) depends on the extent to which a flower's morphology and offered rewards manipulate and restrict the movement of a potential vector relative to the plant's sexual organs (Muchhala, 2007; Armbruster *et al.*, 2009a; b). The importance of mechanical fit in plant–pollinator interactions has been well demonstrated by several examples of plant species that show consistent geographic covariation in floral-tube length and local vector proboscis-length (Pauw *et al.*, 2009; Anderson *et al.*, 2014; Newman *et al.*, 2014, 2015) (Fig. 5.4A, B). However, most flowers do not show mechanical fit to one specific vector, but rather, a subset of available pollen vectors in their environment. Flower morphology may therefore reflect selection to exclude inefficient pollen-vectors in favour of a more efficient subset of vectors (Thomson, 2003), or selection to balance the fitness trade-offs between relatively efficient and inefficient pollen vectors (Aigner, 2001, 2004).

The amount of pollen placed on vectors (and lost due to dislodgement **4**) may further be affected by the duration of a flower visit (Harder and Thomson, 1989), since this is likely to influence the probability of contact between anther and pollen vector, as well as the area of the vector body available for pollen placement. Visit duration tends to increase with the amount of

reward offered (Zimmerman, 1983; Thomson, 1986; Klinkhamer and de Jong, 1993), and is further influenced by the ease of access to rewards (Harder, 1983; De Kock *et al.*, 2018).



Figure 5.4. The architecture and mechanics of flowers and inflorescences often manipulate foraging visitors in different ways to maximize contact between visitors and the anthers and/or stigma as they forage for nectar. (A) Representative of many interactions between long-tubed angiosperm species and their floral visitors; the proboscis of the long proboscid fly *Prosoeca longipennis* is closely matched to the floral tube length of many flowers from which it forages (e.g. *Tritoniopsis revoluta*, tube length approx. 65 mm). To obtain nectar at the base of the tube, the fly must insert its entire proboscis and crawl inside the gullet of the flower (B) where the stigma and/or anthers make contact with the thorax and abdomen of the fly. Photos: C. von Witt. (C) Similarly, the unique architecture of *Babiana ringens* depicts another of the varied ways in which plant morphology is able to manipulate the behaviour of floral visitors to enhance contact with reproductive structures. Sunbirds (*Nectarinia famosa*) visit *B. ringens* flowers while perching on a highly modified, naked inflorescence axis. This forces the birds to lean over the exerted stigmas and anthers in order to probe the tubular flowers on the ground. In doing so, the anthers place pollen on the chests of visiting birds (Anderson *et al.*, 2005). Photo: B. Anderson.

Pollen landscapes on pollen vector bodies

Pollen landscapes that vary in two- or three-dimensional structure and pollen donor composition may be generated on the bodies of pollen vectors due to sequential pollen placement by different males, sequential pollen capture by recipient females, and grooming by pollen vectors (Lertzman and Gass, 1983; Morris *et al.*, 1995; Harder and Wilson, 1998a). The concept of multi-donor pollen landscapes is a crucial prerequisite for understanding the potential for pollen-vector bodies to serve as platforms for pollen transfer, interfaces for competitive male–male interactions and interspecific competition. In particular, the interaction between vector pollen-load size and the distribution and position of pollen placement on vectors potentially influences a male's position and dominance within a pollen landscape, thus affecting almost every subsequent aspect of the pathway to paternity including siring success.

Theoretical studies of pollen landscapes on vector bodies are rare, and empirical studies are practically non-existent. The first studies to address pollen landscape structure (Lertzman and Gass, 1983; Morris *et al.*, 1995), predicted that a layered pollen landscape would extend pollen carryover because deeply buried layers may resurface and deposit pollen on recipients, long after pollen placement. Harder and Wilson (1998a) added more biological realism to these initial models by comparing pollen dispersal in vertically structured landscapes (layered) to horizontally heterogeneous pollen landscapes that result from pollen placement across pollinator bodies within sites exposed to, or safe from pollen grooming. While mean pollen dispersal characteristics were similar for both scenarios, they found that variation in female characters had a greater influence on pollen redistribution and carryover in vertically structured landscapes than in horizontally structured landscapes—pollen capture by stigmas determined the rate at which buried pollen-layers were subsequently exposed. However, male characteristics influenced the pollen landscape in both scenarios. These models clearly demonstrate that successive vector pollen-loads placed by different plants are the fundamental building blocks that form pollen landscapes, and that the structure of these landscapes is likely

to have a significant impact on an individual's pollen dispersal in space, time, and diversity of mates.

Thus, to understand how pollen landscapes might form and how they might influence siring success, we need to understand pollen placement mechanisms in plants. While detailed work has been done on the adaptive accuracy of pollen placement on vectors (Armbruster *et al.*, 2009a), the link between pollen-placement strategies, pollen landscapes, and how males might influence these landscapes to increase siring success has received little study. And almost nothing is known about the structure and donor composition of actual pollen landscapes.

Thus, I can only present tentative hypotheses about how different pollen placement strategies and their associated pollen-landscape structures may influence male reproductive success. I do so by limiting the discussion to three distinct pollen-placement strategies and the respective pollen landscapes they may produce. Similar to Armbruster *et al.*'s (2009) more comprehensive classification, these three pollen-placement strategies—diffuse placement, stroke placement, and stamp placement (Fig. 5.5)—differ with respect to two variables: pollen-placement area, and pollen-vector movement with respect to plant sexual organs. The three strategies roughly represent the mid- and end-points along continuums of these two characteristics. Although not inclusive of all pollen-placement systems (see Armbruster *et al.* 2009a for a detailed treatment), this simplified classification provides a useful functional basis from which to generate possible pollen-landscape scenarios and explore the potential fitness consequences of those landscapes. **Diffuse placement** includes any mechanisms that place pollen over large, undefined areas of vectors. This strategy is likely associated with actinomorphic flowers that diffusely place pollen using multiple, spatially separated anthers (Fig. 5.6A, B) [cf. floral class 1 in Armbruster *et al.* (2009a)]. In contrast, **stroke placement** will require the pollen vector to drag a part of its body across tightly-packed anthers in a consistent direction, leaving a streak of pollen (Fig. 5.6C, D) [cf. floral classes 2–4 in Armbruster *et al.* (2009a)]. Stroke placement is likely to be associated with zygomorphic flowers where plants can

more accurately control the relative body position and approach direction of pollen vectors (Macior, 1974; Muchhala, 2007; Westerkamp and Claßen-Bockhoff, 2007; Armbruster *et al.*, 2009a). **Stamp placement** includes any pollen-placement strategy where anthers are not dragged across vector bodies, but instead, stamp pollen onto vector bodies in a single contact event (Fig. 5.6E, F) [cf. floral class 5–7 in Armbruster *et al.* (2009a)].

The three pollen-placement strategies are likely to yield different pollen-landscape structures (Fig. 5.5). Diffuse-placement strategies are most likely to produce an unlayered pollen-landscape structure since pollen grains are distributed over a large area of vector bodies and unlikely to build up in layers. Stroke placement may result in a layered pollen-landscape structure; however, the layered structure may vary along the length of the stroke placement area. When anthers stroke against vector bodies to place pollen, they may also displace pollen from previous donors towards the back of the stroke (Fig. 5.5). Stigmas may similarly capture more pollen at the start of the stroke and push some of the previously placed pollen towards the back of the stroke. I therefore hypothesise that pollen landscapes resulting from stroke placement may be more layered towards the back of the stroke and consist of more recent pollen and less layering towards the front of the stroke. Stamp-placement strategies are most likely to result in symmetrically distributed and layered pollen-landscapes since pollen loads are deposited in succession on top of each other (Fig. 5.5).

The number of flowers visited per plant is also likely to affect the donor composition of pollen landscapes. For example, several stroke-layers from a single plant may combine to form a very thick layer, or in the case of diffuse placement strategies, multiple visits to the same donor may generate vertical structure. The following sections examine how these placement strategies are likely to influence siring success at different points along the paternity pathway. By the end of this review, it will be clear that understanding many aspects of male fitness may rely on accurate depictions of real pollen landscapes, providing an exciting new direction of study for plant-reproductive biologists.

Intra-floral pollen transfer and pollen placement strategies

Pollen-placement strategies influence the degree to which male reproductive success may be limited through sexual conflict arising from simultaneous male and female reproductive functions in hermaphroditic plants (Barrett, 2002b). The primary source of sexual conflict is vector-mediated self-pollination (Barrett, 2002b) which can reduce male and female fitness through inbreeding depression and pollen discounting (reduction in pollen available for export) (Harder and Wilson, 1998b). While I consider the consequences of geitonogamous pollen transfer and pollen discounting ¹³ for the evolution of male reproductive strategies (Fig. 5.1), I do not address the full complexities of the costs and potential benefits of selfing and the joint effects of inbreeding depression and pollen discounting—these have been reviewed extensively elsewhere (Holsinger and Thomson, 1994; Harder and Wilson, 1998b; Porcher and Lande, 2005; Devaux *et al.*, 2014). Here, I focus on differences between pollen-placement strategies in their likelihood to produce sexual conflict and male reproductive-traits selected to reduce the potential costs of sexual conflict. I divide sexual conflict into intra-floral processes (discussed here) and inter-floral processes (discussed below in *Geitonogamous pollen transfer*).

Within-flower sexual conflict is primarily determined by the proximity between male and female reproductive structures in flowers, which increases the probability of self-pollination and may reduce the likelihood of pollen export (Karron *et al.*, 1997; Fetscher, 2001; Barrett, 2002b). Evolutionary strategies thought to resolve this conflict, primarily involve spatial separation of male and female reproductive organs (herkogamy) (Webb and Lloyd, 1986), or separation in the timing of pollen presentation and stigma receptivity (dichogamy) (Lloyd and Webb, 1986). While sufficient herkogamy may completely eliminate autonomous selfing, the efficacy of herkogamy in preventing vector-mediated pollen transfer within flowers may vary with different pollen placement strategies.

Herkogamy is likely to be less effective in reducing vector-mediated pollen transfer within flowers with diffuse pollen-placement, because vectors move less predictably between

anthers and stigmas when compared to stroke and stamp placement (Fig. 5.5A). For example, the splayed anthers and stigmas of the flower in Fig. 5.6A are clearly herkogamous, which likely prevents autonomous pollen transfer within a flower. However, when a pollen vector visits the flower, it may mediate self-pollination if it contacts the stigma after crawling around the flower while foraging for pollen rewards. Temporal (dichogamy), rather than spatial separation may therefore be a more common mechanism of reducing pollen-vector-mediated sexual conflict in flowers with diffuse pollen-placement strategies (Fig. 5.5A).

Herkogamy might also introduce inefficiency in pollen transfer if anthers and stigmas do not place and receive pollen on the same parts of the pollen-vector's body (Lloyd and Webb, 1992). However, with stroke placement, spatial separation between anthers and stigmas may reduce within-flower sexual conflict without significantly reducing pollen-transfer efficiency: Stigmas that are exerted beyond anthers may make first contact with pollen vectors and drag along vector bodies to capture pollen from previous donors. Behind the stigma, anthers contact the vector secondarily and place pollen by dragging across the vector's body. In this way, stigmas and anthers are still able to largely overlap in pollen-vector contact-area, allowing spatial separation between anthers and stigma without a substantial cost to pollen-transfer efficiency. Consequently, herkogamy may frequently be associated with flowers characterized by strong stroke pollen-placement.

Stamp placement represents the least diffuse of all the placement strategies and, therefore, pollen-transfer success will depend on accurate matching of pollen placement and capture sites on vector bodies. As a result, the reduced pollen-transfer efficiency associated with herkogamy will be most acute for stamp placement relative to other pollen-placement strategies (Fig. 5.5A). Instead, Armbruster *et al.* (2009a) predicted that flowers with stamp pollen-placement are most likely to separate male and female functions in time, not space.

Geitonogamous pollen transfer

When pollen-vectors visit multiple flowers sequentially on a plant (Darwin, 1876; de Jong *et al.*, 1993; Snow *et al.*, 1996) they frequently cause within-plant pollen movement (Lloyd and Schoen, 1992; Harder and Barrett, 1996; Eckert, 2000). This process, known as geitonogamous pollen transfer, reduces the amount of pollen available for export (i.e., pollen discounting ¹³) (Harder and Barrett, 1995; Karron *et al.*, 2004) and the extent of pollen carryover (Mitchell *et al.*, 2013). For self-compatible plants, geitonogamy also increases the male selfing rate (Harder and Barrett, 1995; Karron and Mitchell, 2012) which can lower male fitness through inbreeding depression (Holsinger and Thomson, 1994; Harder and Wilson, 1998b; Devaux *et al.*, 2014). The risk of geitonogamy is likely to be highest in stroke and stamp pollination because these mechanisms layer pollen onto vectors, and most pollen transfer onto stigmas will be from the last few flowers visited (often from the same plant) (Karron *et al.*, 2009) (Fig. 5.5). In contrast, with diffuse pollen-placement, stigmas capture pollen from a more diffuse, mixed-donor pollen-landscape, thus reducing the risk of geitonogamy, as long as the proportion of self-pollen in the pollen landscape is not very high (Fig. 5.5B). When inbreeding depression is severe, selection on plants with multi-flowered displays should favour traits which reduce the extent of within-plant pollen transfer, such as longer flowering windows with small floral displays (Karron and Mitchell, 2012), reciprocal herkogamy (Jesson and Barrett, 2002a), dichogamy (Lloyd and Webb, 1986), or even rewardlessness (Johnson and Nilsson, 1999).

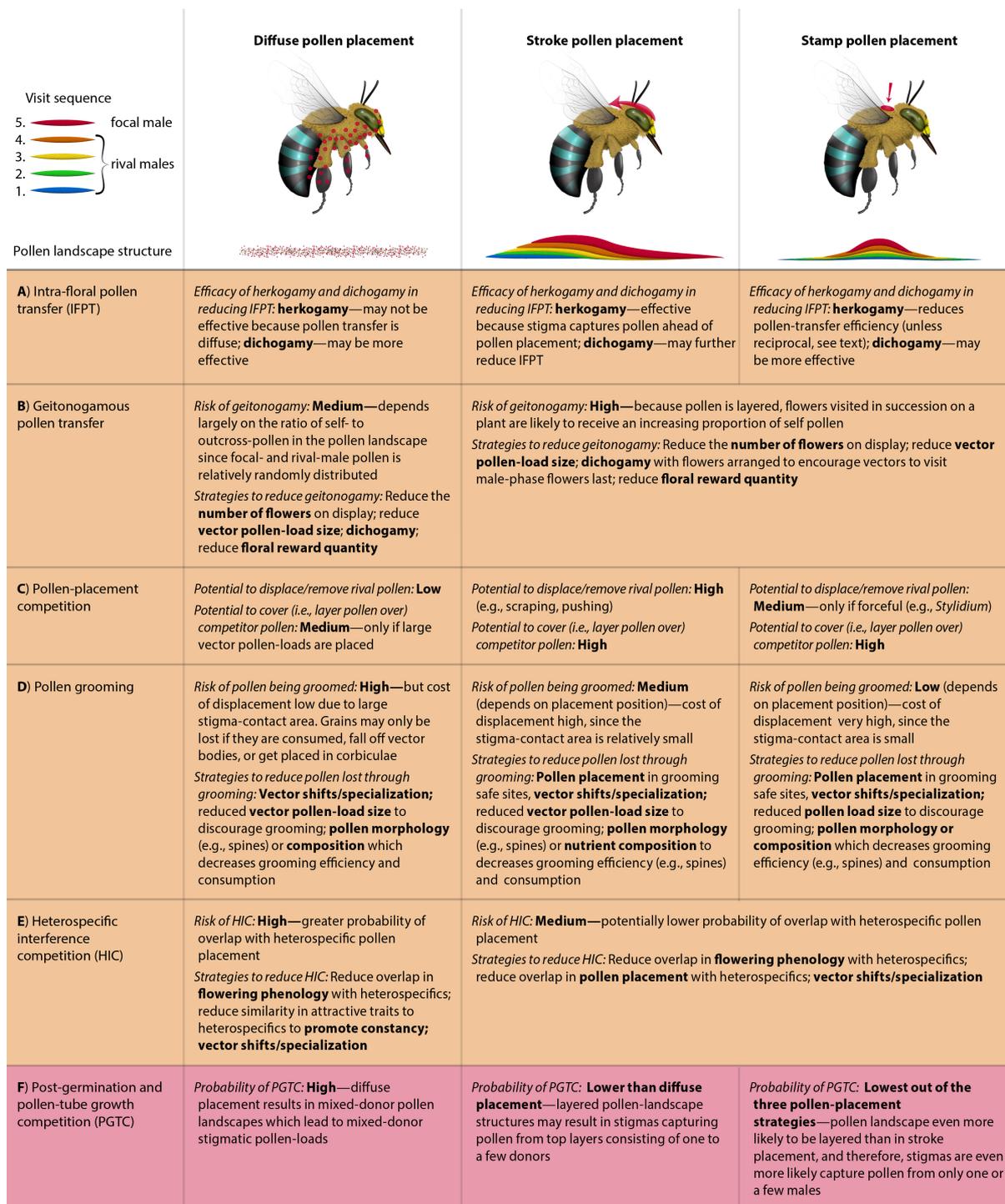


Figure 5.5. Graphic depiction of pollen landscapes (top row) that may form on a pollen vector’s body after visiting a sequence of five different plants. Each plant’s contribution to the pollen landscape is depicted in a different colour, ranging from blue (first plant visited) to red (the final plant visited – the focal male). Underneath these graphic depictions, I provide a comparative table of potential male fitness implications for each of the three, hypothetical pollen placement strategies. The rows of the table correspond to various potential plant–pollinator interactions that may influence siring success within the pollen transfer (orange) and pollen germination and ovule fertilization (red) phases of the paternity depicted in Fig. 5.1.



Figure 5.6. Three common strategies for placing pollen on floral visitors: diffuse (A, B), stroke (C, D) and stamp (E, F). (A) The open flowers and circular anther arrangement of species such as *Drosera cistiflora* place pollen diffusely all over the bodies of monkey beetle pollinators like this *Lepisia rupicola*. Photo: B. Anderson. (B) Alternatively, a central anther arrangement and wide floral tube (as depicted in *Roella ciliata*) can also result in diffuse pollen placement when visitors circle the anthers as they forage for rewards. The blue pollen can clearly be seen, diffusely covering the hairs on the bodies of two foraging monkey beetles. Photo: I. Minnaar. (C) The anthers of *Salvia chamaedryoides* (Lamiales) deposit pollen on the head and thorax of visiting honey-bees in a stroke-like motion. The stroke starts on the head and ends on the thorax, forming a distinct stripe of pollen. The stroking or pushing motion of the anthers appears to displace pollen towards the back of the stroke as predicted in Fig. 5.5 for stroke pollen placement. Photo: Christine Dimech. (D) Narrow, tubular flowers can also result in stroke placement. In this example, as the proboscis of *Moegistorhynchus longirostris* reaches into the depth of a *Lapeirousia anceps* floral tube, the anthers first make contact with the fly's head and then stroke towards the thorax as the fly tilts its abdomen in order to get the last remaining nectar at the very base of the deep tube. Photo: C. Minnaar. (E) The anthers of *Campsomeris plumipes* push against the head of a visiting *Hyptis alata* forming a very discrete, circular pollen signature characteristic of stamp placement. Photo: J. Lampkin. (F) Stamp placement in *Stylidium* occurs actively when the anthers and stigma forcibly slap floral visitors with surprising accuracy. Here, *Stylidium tenue* stamps *Urocolletes rhodurus* with pollen. Photo: F. Hort and J. Hort.

Pollen placement and deposition competition

Pollen competition is a post-pollination phenomenon just as sperm competition is a post-copulation phenomenon. The process of fertilization in plants is most closely related to that which occurs in animals that have internal fertilization.

Delph and Havens, 1998

Until recently, all research on pollen competition was narrowly focused upon competition between pollen grains germinating on stigmas and growing pollen tubes within styles of flowers (Stephenson and Bertin, 1983; Snow, 1994; Willson, 1994; Delph and Havens, 1998; Skogsmyr and Lankinen, 2002; Moore and Pannell, 2011). This limited scope for competitive pollen interactions comes from the notion that pollen competition is similar to sperm competition in internally-fertilising animals—i.e., it only occurs after pollination or copulation (see quote above, Delph and Havens, 1998). As a result, only two phases of male–male competition in animal-pollinated plants are typically recognised. The first is competition between plants for pollen-vector visits, with strong parallels to competition for access to mates in animals (Stephenson and Bertin, 1983). Pollen competition, the next phase of male–male competition, is typically thought to occur after pollen deposition onto stigmas, where races between rival pollen grains to germinate and grow pollen tubes inside the stylar tissue of recipient flowers may ensue (Harder *et al.*, 2016b), just as sperm race to ova in reproductive tracts of animals.

Here, I identify a newly emerging realisation that pollen competition is not just a “post-pollination phenomenon”, and that the body of a pollen vector represents the first opportunity for males to interact directly with pollen of other male competitors (Cocucci *et al.*, 2014), allowing them to potentially alter pollen-landscapes to their advantage. In this respect, pollination is less similar to the internal fertilisation process of animals (see quote above, Delph and Havens, 1998) than it is to the sperm-casting of marine invertebrates which also use a

vector (water) for gamete transport. In sperm-casting, sperm competition is thought to occur from the time of sperm production and release until the time of fertilisation (Parker, 1984; Bode and Marshall, 2007; Beekman *et al.*, 2016). I suggest that if sperm can compete in a shared vector (water) for access to eggs, pollen grains could compete on a shared vector (pollinators). In fact, it could be argued that competition between pollen grains should be more intense in animal-pollinated plants, as the total amount of vector space for pollen transport is far more limited than the vast amounts of water available to sperm-casting marine animals. Moreover, pollen placement on vector bodies requires physical contact between plant and vector, and therefore physical contact between the plant and pollen previously placed by rivals, providing ample opportunity for physical interactions between plants, their pollen, and their rival's pollen prior to pollen germination.

The potential for pollen competition prior to germination has historically been neglected—I know of only one explicit, although brief, statement considering competitive interactions between pollen grains on pollen vectors by Lertzman and Gass (1983) (pg. 488): "For instance, flooding a pollen pool [i.e., pollen landscape] with one's own pollen may increase success as a male...". However, two recent studies have since provided the first evidence of physical competition between pollinaria of different plants for space on pollen-vector bodies (Cocucci *et al.*, 2014; Duffy and Johnson, 2014). Importantly, Cocucci *et al.* (2014) revealed that such "physical struggles" between pollinaria may have led to the evolution of pollinaria horns that function in preventing unwanted attachment to pollinaria from rival males (because it interferes with deposition on stigmas). This finding provides the first evidence of sexually-selected male weaponry in plants—once considered an exclusively animal phenomenon—contradicting the widely held notion of pollen competition as a post-pollination phenomenon (e.g., Lloyd and Webb, 1977; Stanton, 1994; Grant, 1995; Murphy, 1998; Delph and Ashman, 2006; Moore and Pannell, 2011).

There seems to be no sound theoretical reason to continue the historic restriction of pollen competition to interactions that involve germination and pollen-tube growth only—the scope of pollen competition should include the pollen transfer process, where pollen-placement and pollen-deposition competition may occur. While evidence for pollen-placement competition on vector bodies is currently limited to two studies on species that disperse pollen in the form of pollinaria, I suggest this form of pollen competition should also occur in plants dispersing granular pollen. In this review, I would like to expand on the idea of pollen competition on vector bodies by exploring hypothetical mechanisms of pollen-placement and pollen-deposition competition using the three different granular pollen-placement strategies as a foundation. However, since the composition and structure of multi-donor pollen landscapes on vectors have never been studied, I make the following predictions with caution and encourage their future empirical exploration.

The potential for males to alter the pollen landscape is likely to vary among the three pollen-placement strategies (Fig. 5.5). In diffuse pollen-placement, a male's ability to alter the structure of the pollen landscape is limited, due to the diffuse distribution of competitor pollen grains across the vector's body. Plants with diffuse pollen-placement may change the pollen landscape by contributing more pollen than their competitors. However, this may increase the risk of geitonogamous pollination because it increases the proportion of potential self-pollen on a pollen vector (see *Geitonogamous pollen transfer* above).

In contrast to diffuse pollen-placement strategies, stroke- and stamp-placement provide males more opportunity to alter pollen-landscape composition and structure to their advantage. With stroke placement, anthers can physically displace competitor pollen (or have their own pollen displaced 8) towards the back of the stroke (Fig. 5.5, 5.6C) as a result of the dragging motion of anthers and pollen placement in a single direction. By displacing rival pollen, males may increase their relative contribution to the pollen landscape without increasing pollen-load size. Intense pollen-placement competition may drive selection for traits that amplify the

relatively passive displacement-effect inherent in stroke-placement systems. While speculative, it is not difficult to conceive of simple, secondary anther structures or floral appendages that could potentially scrape, sweep, or scoop rival pollen from vector bodies prior to pollen placement. This would be analogous to ancillary structures on the penises of male animals that remove rival sperm from female reproductive tracts (Waage, 1979; Hosken and Stockley, 2004).

A possible example of a flowering plant structure that may potentially scrape or remove rival pollen from vector bodies are the hairs surrounding anther tubes in *Lobelia tomentosa* (Fig 5.7A). In *Lobelia*, pollen is extruded through the end of an anther tube by where pollen is placed on vectors, usually in an accurate stroke-fashion along the head and thorax of visiting pollen vectors (Macior, 1967; Johnston, 1991; Yeo, 1992; Howell *et al.*, 1993). Many *Lobelia* have hairs surrounding the anther-tube opening which appear to control pollen extrusion (Ladd, 1994). In *L. tomentosa*, pollen is stroked onto the head of visiting bees as the anther tube is levered upwards and pushed from the front, causing the undeveloped stigma to push pollen through the anther-tube opening (Fig. 5.7B, C). While doing so, the hairs surrounding the anther-tube opening, especially the elongated front-facing hairs (Fig. 5.7A), may also sweep away rival pollen on vectors before the donor's pollen is laid down (Fig. 5.7D). I performed a preliminary test of this hypothesis by placing quantum-dot labelled *L. tomentosa* pollen (Minnaar and Anderson, 2018) on an *Amegilla* bee's head and pushing it into a virgin flower to simulate a visit. I found q-dot labelled pollen grains from the bee's head on the front-facing hairs of the anther tube and a stroke of unlabelled pollen on the bee's head where the q-dot labelled pollen appeared to be partially removed. While this may represent an intriguing example of rival-pollen removal, I stress that there is enough data to support this hypothesis, and alternative functions of the elongated front-facing hairs need to be considered (e.g., concealment of pollen from pollen thieves or protection of pollen from rain). I only present this as one of many putative examples of structures that may function in rival-pollen removal that deserve further exploration.

The evolution of competitive pollen removal in plants ¹⁰ may be limited in species with large floral displays because the risk of removing the focal donor's pollen likely increases with the number of flowers visited on the same plant. I therefore expect that, if present, pollen scrapers may be most developed in species with small floral displays, or in dichogamous plants where the risk of focal donor pollen removal is reduced.

Stamp pollen-placement strategies may also displace rival pollen concentrically outward if the force of the stamp action is great enough. Forceful stamp-displacement of competitor pollen may have contributed to the evolution of triggered-hammer pollination mechanisms (Scott Armbruster, pers. comm.). For example, *Stylidium* species swing their anthers (or stigmas in the female phase) forcefully onto vectors during visits (Fig. 5.6F) (Armbruster *et al.*, 1994).

In addition to displacement and removal, pollen may also be covered/buried by large loads of rival pollen ⁸, denying rivals access to stigmas until the pollen covering is sufficiently depleted. Large vector pollen-loads may also saturate a stigmatic surface, limiting access to subsequent rival pollen ¹² (Ashman *et al.*, 1993). This form of pollen-deposition competition is analogous to mating plugs in animals (Alcock, 1994). Large vector pollen-loads may also facilitate rapid pollen export allowing males to gain siring priority in species where ovules become available relatively synchronously [e.g., species with single-day flowers or short vector-activity periods (Harder and Johnson, 2008)]. However, if pollen vectors visit multiple flowers on an individual plant, large-vector-pollen-load strategies may be costly, since self-stigma saturation could lead to a reduction in outcross pollen export ¹³ and receipt. Therefore, this strategy is most likely in plants with small floral displays, dichogamous plants with daily flowers, or dichogamous plants that manipulate flower visitor behaviour through inflorescence architecture so that flowers in male phase are visited last.

Placing large vector pollen-loads may also increase pollen-germination and tube-growth competition among a plant's own pollen grains on outcross stigmas. This may amplify

diminishing returns on pollen production, limiting selection for placing large numbers of pollen grains in vector pollen-loads (Charnov, 1982). However, plants do not only compete for ovules in a population—they first compete for access to pollen vectors, then access to stigmas, and only then do they potentially compete for access to ovules. If access to vectors or stigmas is limited in a population, selection may still favour males that place large vector pollen-loads in terms of volume and proportion of total pollen production, as they are most likely to capitalize, and potentially monopolize, available mating opportunities. For example, Orchids, which are typically poorly visited by pollen vectors, place vector pollen-loads that are both large in volume and proportion of total production, but with low pollen grain numbers relative to ovules (Harder and Johnson, 2008).

Different pollen placement strategies and their associated pollen landscape structures may also influence the donor composition of stigmatic pollen-loads and, therefore, the likelihood of pollen-germination and tube-growth competition. For example, diffuse placement is more likely to generate multi-male stigmatic pollen-loads and therefore strong pollen-tube competition than stroke placement, where stigmas likely capture pollen from top layers in the pollen landscape that consist of pollen from one or a few donors (Fig. 5.5F).

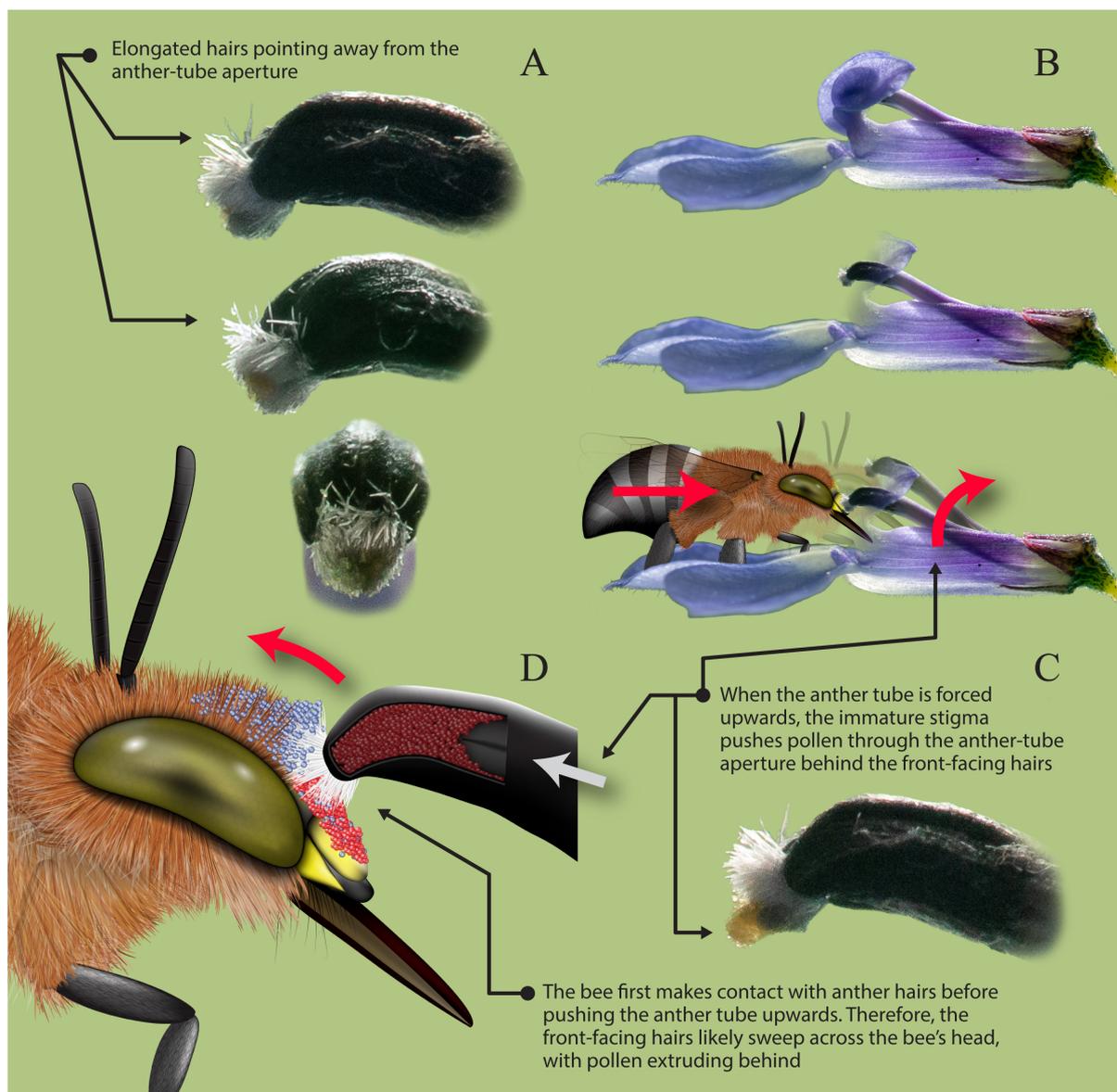


Figure 5.7. In this review, I hypothesise that plants may have evolved secondary anther structures or floral appendages that function in displacing or removing rival pollen from pollen vector bodies. Here, I demonstrate a putative example of competitive pollen scraping. (A) The hairs surrounding the anther tube aperture in *Lobelia tomentosa* are elongated towards the front. These elongated hairs also appear to point away from the anther tube aperture, in the same direction that pollen is placed on bees. (B) To reach the nectar in *L. tomentosa* flowers, bees are forced to push against the anther tube and, in doing so, force the anther tube upwards. This causes the immature stigma to push pollen, like a piston, through the anther tube aperture. (C) Since pollen is extruded behind the forward-facing hairs (D), I hypothesise that bees entering *L. tomentosa* flowers will make contact with the anther hairs first, potentially allowing the forward-facing hairs to scrape away rival pollen, while the plant's own pollen is extruded onto the bee behind these sweeping hairs. Preliminary experiments suggest that this hypothetical scenario may be likely (see text); however, sufficient empirical data to confirm this mechanism is lacking, and caution readers to view this example as speculative. Illustration and photos: C. Minnaar.

Pollen grooming and passive pollen loss

A major source of pollen loss is the displacement or removal of pollen from the original placement site due to pollen-vector grooming **6** (Thomson, 1986; Harder, 1990; Holmquist *et al.*, 2012). However, pollen vectors may not be able to effectively groom all areas of their bodies and with equal ease (Macior, 1967, 1974; Kimsey, 1984; Thorp, 2000). Many bees for example, seem to struggle to reach and groom the mid-line along the dorsal and ventral surfaces of their thorax and abdomen (Koch *et al.* 2017; Tong and Huang 2018, Fig 5.8B). Pollen placed within these hard-to-reach areas is relatively safe from grooming (pollen "safe sites") and selection may therefore favour pollen placement within these areas (Macior, 1974; Westerkamp and Claßen-Bockhoff, 2007). The widespread occurrence of bilabiate flowers among angiosperms, and especially Lamiales (Fig. 5.6C), may reflect a convergent pollen protection and placement strategy: bilabiate flowers are able to protect pollen from pollen thieves by hiding anthers under the top lip of the flowers (away from the nectar source), while simultaneously stroking pollen onto the dorsal midline of pollen vectors, thereby limiting pollen lost to grooming (Macior, 1967, 1974; Westerkamp and Claßen-Bockhoff, 2007).

Pollen loss as a result of grooming **6** may also be ameliorated by reducing vector pollen-load sizes so that stimulation of grooming behaviour is reduced (Harder, 1990). Pollen morphology, nutrient content, and pollenkitt composition could reduce the ease or incentive to groom. For example, spines and pollenkitt on pollen grains inhibit the ability of corbiculate bees to package pollen and reduce the incentive to collect it (Lunau *et al.*, 2015). Specialization or pollinator shifts towards non-grooming (less wasteful) vectors may ameliorate grooming related pollen loss (Stebbins, 1970; Thomson, 2003). For example, directional trait evolution such as tube-length elongation or colour shifts to red are frequently associated with shifts from grooming vectors like bees to less-frequently-grooming vectors like flies (Anderson *et al.*, 2014) (Fig. 5.8A) or birds (Castellanos *et al.*, 2004; Wilson *et al.*, 2006).

The three pollen placement strategies potentially differ in their susceptibility to pollen loss through grooming (Fig. 5.5D). Diffuse pollen-placement is most susceptible to grooming, as there is a high probability of pollen placement on areas that vectors are able to groom. However, because the area of stigma pollen-capture is also large, the detrimental effect of pollen being displaced from one area of the vector body to another by grooming may be small. Diffusely-placed pollen may mainly be lost when it is groomed off of vector bodies, consumed, or packed into corbiculae where pollen grains may be rendered inviable (Parker *et al.*, 2015) and unlikely to be captured by stigmas (Thomson, 1986).

In contrast, pollen placed by stroking or stamping would likely be lost as soon as it is groomed from the relatively small stigma-contact area (Fig. 5.5D). The potential costs of grooming-related pollen loss in stroke and, especially, stamp pollen-placement should therefore select for placement on pollen-grooming safe sites (Koch *et al.*, 2017; Tong and Huang, 2018) (Fig. 5.8B), or traits that increase recruitment and pollen placement on non-grooming pollen vectors.

Even if pollen vectors do not groom regularly, pollen may still be lost passively from vectors during transport ⁷. As with grooming, the extent of passive pollen loss may vary among pollen vectors. For example, bat fur may hold more pollen for a longer period during transport than feathers on birds (Muchhala and Thomson, 2010), and selection to decrease passive pollen loss during transport may therefore drive specialization on, or shifts to different pollen vectors. Selection may also favour stickier pollen grains, smaller pollen-load sizes, or less exposed pollen placement sites.

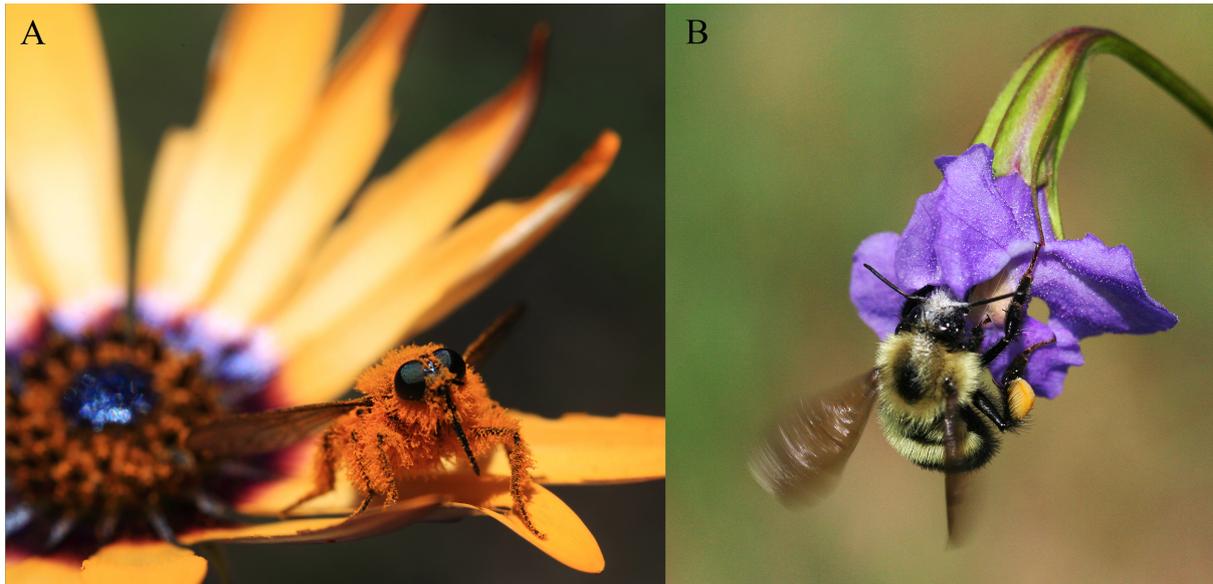


Figure 5.8. The accumulation of pollen on pollinators can be strongly affected by variation in the grooming patterns of different pollinators. (A) *Rhigioglossa nitens* grooms infrequently and its entire body is densely covered with *Dimorphotheca sinuata* pollen. Photo: B. Anderson. (B) Other pollinators such as *Bombus vagans* groom regularly but are only able to access pollen from certain parts of their bodies. Pollen 'safe sites' (see stripe of *Mimulus ringens* pollen on the head and thorax) often lie along the midlines of bees' bodies because they are unable to reach those areas with their legs (Macior, 1974; Westerkamp and Claßen-Bockhoff, 2007; Koch *et al.*, 2017). Photo: J. Karron.

Interspecific interactions: reproductive interference and isolation

Most flowering plants share pollen vectors with other co-flowering species, potentially lowering male and female fitness through heterospecific pollen transfer (Flanagan *et al.*, 2009; Mitchell *et al.*, 2009). Receipt of heterospecific pollen on stigmas may reduce female fitness by preventing conspecific pollen attachment or by interfering with pollen germination and pollen tube growth (Waser, 1978; Ashman and Arceo-Gómez, 2013; Briggs *et al.*, 2016). However, interactions between co-flowering species may have even greater consequences for male function; for example, pollen wastage (pollen loss) 11 can occur if pollen is deposited on stigmas or other floral parts of co-flowering species (Bell *et al.*, 2005; Morales and Traveset, 2008). Furthermore, every visit to a heterospecific flower could result in a male's vector pollen-load being covered or displaced by heterospecific pollen 9. Muchhala and Thomson (2012) elegantly measured both pollen loss to heterospecific stigmas and the additional decrease in pollen transfer associated with pollen displacement and covering, following a visit to a single flower of a co-flowering species in male phase. They found that pollen loss to heterospecific stigmas reduced pollen export to the next conspecific flower by 43.1% 11, while pollen displacement/covering by heterospecific pollen resulted in a 66.1% reduction in pollen export to the next conspecific flower 9. This study highlights the substantial costs to pollen export potential when pollen vectors visit co-flowering species. Moreover, every visit to a heterospecific flower increases the time spent away from potential conspecific recipients, and therefore the amount of pollen lost passively or through grooming (Flanagan *et al.*, 2009). This study also suggests that if different species can compete through pollen displacement or covering on the pollinator, then pollen displacement/covering may lead to even more intense intraspecific male–male competition because pollen placement sites of intraspecific rival males are expected to have greater overlap.

To avoid interspecific reproductive interference 9 11, selection may act on several traits (Fig. 5.1). First, selection may reduce the overlap in flowering time between heterospecifics that share a common pollen vector (Rathcke, 1983). Second, interspecific

reproductive interference may drive specialization on, or shifts to, vectors that visit fewer heterospecifics (Muchhala *et al.*, 2010). Interspecific reproductive interference can also promote character displacement to reduce the overlap in sites of pollen placement and receipt (Armbruster *et al.*, 1994; Muchhala and Potts, 2007; Muchhala and Thomson, 2012) (Fig. 5.9). However, plants that place pollen diffusely are unlikely to successfully reduce overlap in pollen placement with heterospecifics. Instead, I predict that selection to avoid interspecific reproductive-interference ⁹ ¹¹ in diffuse pollen-placement systems would act on traits that promote constancy (*sensu* Waser, 1986) in vector foraging sequences (Fig. 5.5E). Bees are often described as generalist flower visitors in pollination network studies (e.g., Alarcón *et al.*, 2008). However, individual bees often forage exclusively from a single flowering species during a foraging bout (Grant, 1950; Waser, 1986), limiting the probability of interspecific reproductive interference. Consequently, selection may favour dissimilarity in floral display colour, colour pattern, or scent amongst co-flowering species to promote individual pollen-vector constancy in bee pollen-vectors (Jones, 1978; Grant, 1994; Ellis and Johnson, 2012; Kemp *et al.*, 2018) (Fig. 5.1).

Autonomous pathway to paternity

Although this review has focused on vector-mediated male reproductive strategies, it is important to note that not all reproduction by animal-pollinated plants is facilitated by vectors. Plants may transfer pollen autonomously within flowers, leading to a vectorless portion of the pathway to male reproductive success (Lloyd and Schoen, 1992; Kalisz *et al.*, 2004). The proportion of offspring sired through this pathway varies widely among species (Busch and Delph, 2012) and the evolution of autonomous selfing from outcrossing ancestors is one of the most frequent evolutionary transitions in plants (Wright *et al.*, 2013). Although many factors [e.g., the automatic selection advantage of selfing (Fisher, 1941)] are thought to contribute to an increase in the rate of autonomous selfing, considerable evidence suggests that poor

pollinator service often plays a major role (Kalisz *et al.*, 2004; Bodbyl Roels and Kelly, 2011; Yin *et al.*, 2016).



Figure 5.9. Pollen competition between species may affect the evolution of where flowers place pollen on pollinators. Here, the yellow pollen of *Burmeistera ceratocarpa* is placed mostly between the eyes of a bat (*Anoura geoffroyi*), while the white pollen of *Burmeistera borjensis* is placed between the ears. This is thought to be the product of character displacement and, in sympatry, it minimizes interspecific pollen interference (Muchhala and Potts, 2007). Photos: N. Muchhala.

CONCLUSION

[C]ompetition among plants to pollinate other plants does not involve struggles, or even contact, between competitors...

Murphy, 1998

The use of biotic gamete-vectors is unique to plants (Bishop and Pemberton, 2006; Beekman *et al.*, 2016) and so, comparisons to fertilisation processes in animals need to be made with care. In particular, the notion that pollen competition only occurs after deposition onto stigmas, as with post-copulation sperm competition in animals, is a false equivalence. Pollen competition is likely to occur along most of the pathway to paternity, starting at the time of pollen production and placement, continuing all the way through to pollen deposition on stigmas, pollen germination and pollen-tube growth, and ovule fertilisation. Every time a plant places pollen on a pollinator, it has the opportunity to displace, cover, and remove pollen grains of its competitors and increase its siring success. When access to stigmas or pollen vectors is limited, plants may benefit from gaining exclusive access to stigmas by placing large vector pollen-loads that saturate recipient stigmas upon deposition. This form of pollen-deposition competition may be similar to mate guarding in animals. Pollen-placement and deposition competition on vectors may be as important as pollen-germination and tube-growth competition, and selection for increased competitiveness could influence pollen placement strategies and pollen presentation, as well as several siring barriers further along the pathway. Unlike internally fertilising animals, the pathway to paternity in animal pollinated plants is a complicated obstacle course along which pollen can be lost at multiple stages before the final stylar race to fertilise ovules. Nevertheless, after considering the potential ramifications of pollen-placement and deposition competition, it is perhaps possible to make some comparisons with animal reproductive strategies and Janzen's jarring parallels between plant and animal reproduction appear to hold more than ever:

...plants are not trying to maximize outcrossing but rather to optimize it. In doing so they perform courtship displays, [...] promiscuity, and fickleness just as do animals.

Janzen, 1977

GLOSSARY

Fitness: the lifetime reproductive output of an individual. In hermaphroditic plants that produce both male and female gametes, this is the sum of the individual's male fitness (number of viable seeds sired) and female fitness (number of viable seeds produced).

Geitonogamous pollen transfer: the dispersal of pollen between flowers of a hermaphroditic plant.

Pathway to paternity: the voyage of a pollen grain from production and release in an anther, to fertilization of an ovule.

Pollen competition: the competitive interactions between pollen of rival individuals to fertilize a limited set of ovules. Different forms of competition can occur at several phases along the pathway to paternity, ***pollen-placement competition*** on biotic pollen-vector bodies, ***pollen-deposition competition*** on stigmas, and ***pollen-germination and tube-growth competition*** (progammic pollen competition) within styles.

Pollen discounting: the reduction of pollen available for export following self-pollination.

Pollen landscape: the two or three-dimensional structure and composition of multi-donor pollen on the bodies of biotic pollen-vectors. Pollen landscapes arise as a result of successive pollen placement by different rival individuals on pollen-vector bodies and provides an interface for competitive male–male interactions that can include the displacement, covering, or removal of competing pollen grains.

Pollen presentation: the process through which plants make pollen available for placement on pollen vectors.

Pollen presentation rate: the rate at which pollen is presented at the level of the plant. Various pollen presentation traits can influence pollen presentation rates; for example, the number of flowers simultaneously on display, or the rate of anther dehiscence.

Siring barriers: the mechanisms along the pathway to paternity that reduce the likelihood of an individual's pollen siring seeds.

Siring success: the relative success of an individual's pollen at fertilizing ovules.

Stigmatic pollen-load: the quantity of pollen deposited on a stigma.

Strategies: Adaptive solutions to increase siring success by mitigating siring barriers along the pathway to paternity.

Vector pollen-load: the quantity of pollen placed on a biotic vector.

GENERAL CONCLUSION

Floral biology has contributed significantly to our understanding of evolution for more than three centuries² through studies of trait inheritance (Mendel, 1866), sexual reproduction (e.g., Grew 1682; Camerarius 1694), the effects of inbreeding and outcrossing (e.g., Knight 1799; Darwin 1876; Levin 1984), mating systems (e.g., Darwin 1877; Barrett 1977), local adaptation (e.g., Robertson and Wyatt 1990; Newman *et al.* 2015), and coevolution (Nilsson, 1988; Alexandersson and Johnson, 2002; Anderson and Johnson, 2008; Pauw *et al.*, 2009). Remarkably, many of these studies, spanning 333 years, used the same basic techniques, highlighting the suitability of flowering plants as subjects in low-tech evolutionary experiments: their seeds are easy to store, transport, and grow; controlled crosses can easily be achieved through hand pollination; variations in floral and pollinator traits are easy to quantify; floral interactions with pollinators are easy to observe; and perhaps most important to evolutionary biologists, reproductive fitness can be quantified by simply counting the number of seeds produced by in flowers of a plant. However, this low-tech approach has its limits and modern pollination biologists have increasingly adopted modern tools to better understand plant reproductive biology. With this thesis, I hope that I have added another useful tool to the plant biologist's toolbox.

In concluding my thesis, I realise that are many questions left to explore: (1) How far does pollen disperse, and how do micro-spatial barriers to pollen movement affect gene flow? In other words, what constitutes a geographic barrier to gene flow in the context of pollen movement? (2) Is the asymmetry in pollen transfer between long- and short-tubed flowers a general phenomenon? If so, what are the implications for speciation through mechanical isolation? (3) If pollen landscapes on pollinators vary predictably in pollen quality, can plants

² Although many of the studies listed here were performed prior to the development of evolutionary theory, they all contributed in some way to our understanding of evolutionary floral biology. For an historical review on floral biology up until 1979, see Baker (1979).

selectively capture good quality pollen from specific sites through changes in stigma position?

Would this constitute female mate choice? (4) How important is pollen transfer efficiency?

Pollination biology seems somewhat preoccupied with the notion that plants are under constant selection through male fitness to reduce pollen wastage, whereas animal reproductive biologists place far more emphasis on competitive mating strategies. While I explore some male reproductive strategies that may be more competitive than efficient, a more explicit exploration seems warranted. (5) Are competitive pollen removal and displacement mechanisms common in plants?

I look forward to exploring these questions, and more, and I hope that the findings presented and questions raised in this thesis will generate further research as well as opposing views and evidence.

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