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Intraspecific divergence in floral-tube length promotes asymmetric pollen movement and reproductive isolation

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Summary

- The causative link between phenotypic divergence and reproductive isolation is an important but poorly understood part of ecological speciation. We studied the effects of floral-tube length variation on pollen placement/receipt positions and reproductive isolation.
- In a population of *Lapeirousia anceps* (Iridaceae) with bimodal floral-tube lengths, we labelled pollen of short- and long-tubed flowers with different colour fluorescent nanoparticles (quantum dots). This enabled us to map pollen placement by long- and short-tubed flowers on the only floral visitor, a long-proboscid fly. Furthermore, it allowed us to quantify pollen movement within and between short- and long-tubed flowers.
- Short- and long-tubed flowers placed pollen on different parts of the pollinator, and long-tubed flowers placed more pollen per visit than short-tubed flowers. This resulted in assortative pollen receipt (most pollen received comes from the same phenotype) and strong but asymmetric reproductive isolation, where short-tubed plants are more reproductively isolated than long-tubed plants.
- These results suggest that floral-tube length divergence can promote mechanical isolation in plants through divergence in pollen placement sites on pollinators. Consequently, in concert with other reproductive isolation mechanisms, selection for differences in floral-tube length can play an important role in ecological speciation of plants.

Introduction

Modern treatments of speciation recognize natural selection as an important driver of phenotypic divergence and reproductive isolation (RI) (Schluter, 2000; Rundle & Nosil, 2005). Ecological speciation (*sensu* Schluter, 1996; Funk, 1998) posits that adaptation to different ecological environments can lead to phenotypic divergence, which may in turn promote RI and speciation (Nosil, 2012; Van der Niet *et al.*, 2014). Ecological divergence is expected to drive speciation at a faster and more consistent pace than neutral divergence because, unlike neutral divergence, it is directed through selection (Gavrilets, 2004; Feulner *et al.*, 2015).

The ecological speciation process thus consists of two important but closely linked steps: (1) phenotypic divergence between populations driven by ecological differences, followed by (2) RI resulting from phenotypic divergence. Flowering plant populations frequently experience geographic mosaics in pollinator composition across their range, and many studies have demonstrated that these mosaics can lead to phenotypic divergence in allopatry through local pollinator-mediated selection (Anderson *et al.*, 2010a,b, 2014; Boberg *et al.*, 2014; for review see Van der Niet *et al.*, 2014).

For example, Paudel *et al.* (2016) demonstrated that the geographic match between pollinator proboscis length and floral-tube length is underpinned by strong directional selection on floral-tube length, probably mediated by pollinators. Although many studies have investigated pollinator-driven divergence (e.g. Newman *et al.*, 2015; Sletvold *et al.*, 2016) or RI (e.g. Ramsey *et al.*, 2003; Kay, 2006), the direct effect of ecological divergence on RI within species has seldom been studied experimentally. This link is one of the most poorly documented parts of the ecological speciation process (Nosil, 2012; Van der Niet *et al.*, 2014).

Despite the paucity of empirical support for this link, Grant (1994) detailed some plausible mechanisms (termed floral isolation) by which pollinator-driven divergence can produce RI when divergent floral forms make secondary contact. For example, he described several instances where species appear reproductively isolated because their divergent floral morphology attracts different suites of pollinators in sympatry (ethological isolation) (also see Fulton & Hodges, 1999; Kay & Sargent, 2009; Whitehead & Peakall, 2014). Grant (1994) also proposed the possibility of mechanical isolation, which does not rely on shifts in suites of pollinators visiting divergent floral forms. Instead, he proposed that divergence in floral morphology may lead to divergence in pollen placement and receipt sites on a shared pollinator's body, resulting in restricted pollen movement between phenotypes and RI. When mechanical or ethological isolation develops, these early-acting barriers to gene flow often have an inordinately large effect on RI (relative to other reproductive barriers) because they

prevent gene flow before other barriers have a chance to act (Ramsey *et al.*, 2003).

Though putative examples of mechanical isolation in flowers are common (see Grant, 1994, Table 1), they are seldom studied directly, perhaps because it is difficult to differentiate and track the movement of pollen grains from the same, or closely related, species (but see Minnaar & Anderson, 2019). One comprehensive study of RI barriers in two closely related *Costus* species used array experiments and coloured dye powder as a pollen analogue to determine that mechanical isolation is the most important reproductive barrier in this system (Kay, 2006). Strong assortative dye movement (more movement within species than between) was found despite flowers of both species being visited exclusively by the same hummingbird pollinator that lacks floral constancy. Though the mechanics behind mechanical isolation were not investigated in depth, video footage revealed that tube-length differences between the two species resulted in different pollen placement positions on hummingbirds (Kay & Schemske 2003). Kay (2006) postulated that this may cause mechanical isolation; however, it is unclear why floral tubes diverged in the first place.

Plants bearing tubular flowers often display extensive geographic variation in floral-tube length, and this variation has frequently been linked to geographic variation or mosaics in pollinator morphology (e.g. Whittall & Hodges, 2007; Anderson & Johnson, 2008; Anderson *et al.*, 2010a,b, 2014). Furthermore, several studies, including translocation experiments (e.g. Boberg *et al.*, 2014), manipulative experiments (e.g. Nilsson, 1988), and selection studies (e.g. Muchhala & Thomson, 2009; Pauw *et al.*, 2009; Anderson *et al.*, 2010a,b), have demonstrated that pollinators select strongly on floral-tube length. Together, these lines of evidence suggest that geographic mosaics of pollinators have played an important role in generating diversification of floral-tube length within and among species. However, it is generally less clear whether, and how, tube length variation affects floral isolation (but see Kay, 2006; Wolf *et al.*, 2001).

Tube length is known to act as a filter, often excluding pollinators with short proboscides from visiting long-tubed flowers (Haber & Frankie, 1989). In addition, lower nectar rewards associated with short-tubed flowers may make it less profitable for nectar foragers with long proboscides to visit short-tubed flowers (Haber & Frankie, 1989; Klumpers *et al.*, 2019). Consequently, variation in floral-tube lengths can affect the likelihood of visitation by certain pollinators (Klumpers *et al.*, 2019) and potentially lead to RI resulting from distinct pollinator preferences. Indeed, Hodges & Arnold (1995) suggested ethological isolation as the reason for high diversification rates in lineages with nectar spurs. However, it is also possible that floral-tube length variation may further increase diversification rates because it leads to different pollen placement and receipt positions on a pollinator (e.g. short-tubed plants place and receive pollen near the tip of a pollinator's proboscis whereas long-tubed plants place and receive pollen near the head of the pollinator). Consequently, allopatric divergence in tube length could result in mechanical RI when populations with divergent tube lengths make secondary contact.

We examine this possibility in *Lapeirousia anceps* (Iridaceae), a plant with large floral-tube length variation among populations and sometimes within populations (Pauw *et al.*, 2009; Anderson *et al.*, 2016). Pauw *et al.* (2009) demonstrated substantial variation in *L. anceps* floral-tube length among populations (range of population means: 27.5–77.0 mm), and this variation is strongly correlated with geographic variation in pollinator proboscis length. In addition, Pauw *et al.* (2009) provided a selective mechanism behind the close phenotypic matching by demonstrating that the effectiveness of pollen transfer was dependent on how closely floral tubes matched pollinator proboscis length. Taken together, these results suggest that tube lengths likely diverged allopatrically in response to pollinators with different proboscis lengths. However, some populations display a bimodal distribution in tube length (Zhang *et al.*, 2013), and Anderson *et al.* (2016) argued that these populations represent secondary contact zones between populations adapted to different pollinators.

Anderson *et al.* (2016) studied one of these populations where tube length was strongly bimodal, the two modes corresponding to 28 mm and 54 mm (Fig. 1). Over 7 years of study, a single *L. anceps* pollinator (*Moegistorhynchus longirostris*) has been observed in this population and it readily moves between long- and short-tubed plants (Anderson *et al.*, 2016; Supporting Information Video S1), although its proboscis length only matches long-tubed plants (Fig. 2). Despite being visited by a single pollinator species, marker genes indicated strong RI between long- and short-tubed plants (Anderson *et al.*, 2016). Experimental evidence from this study suggests that the restricted gene flow between long- and short-tubed plants may be a result of partial incompatibilities, nonrandom pollinator foraging patterns (flies are more likely to move within than between patches of long- and short-tubed flowers), and differences in competitive abilities of pollen when transferred to stigmas of the opposite morph (Anderson *et al.*, 2016).

However, an intriguing photograph (Fig. 1) suggesting two discrete pollen placement sites on the pollinator (one on the proboscis and one on the head) led to the hypothesis that differences in pollen placement and receipt positions may cause additional floral isolation between short- and long-tubed plants. This hypothesis was never tested, because accurate methods to track intraspecific and visually similar pollen grains were unavailable until recently.

Here, we use a promising new technique – outlined in Minnaar & Anderson (2019) – to label pollen grains using fluorescent nanocrystals (quantum dots), which enabled us to follow the fates of individual pollen grains instead of using pollen proxies like dyes (Waser & Price, 1982; Waser, 1988; Adler & Irwin, 2006), which may be unreliable for quantitative estimates of pollen dispersal (Thomson *et al.*, 1986; Campbell, 1991). Using this technique, we examine the possibility that tube-length variation results in assortative pollen movement between sympatric short- and long-tubed *L. anceps* plants. More specifically, we hypothesize that short-tubed flowers place pollen on the proboscis of the fly, whereas long-tubed flowers place pollen closer to the head. This results in assortative pollen movement where long-tubed flowers receive most of their pollen from other long-tubed flowers and short-tubed flowers receive most of their pollen from other short-tubed flowers.

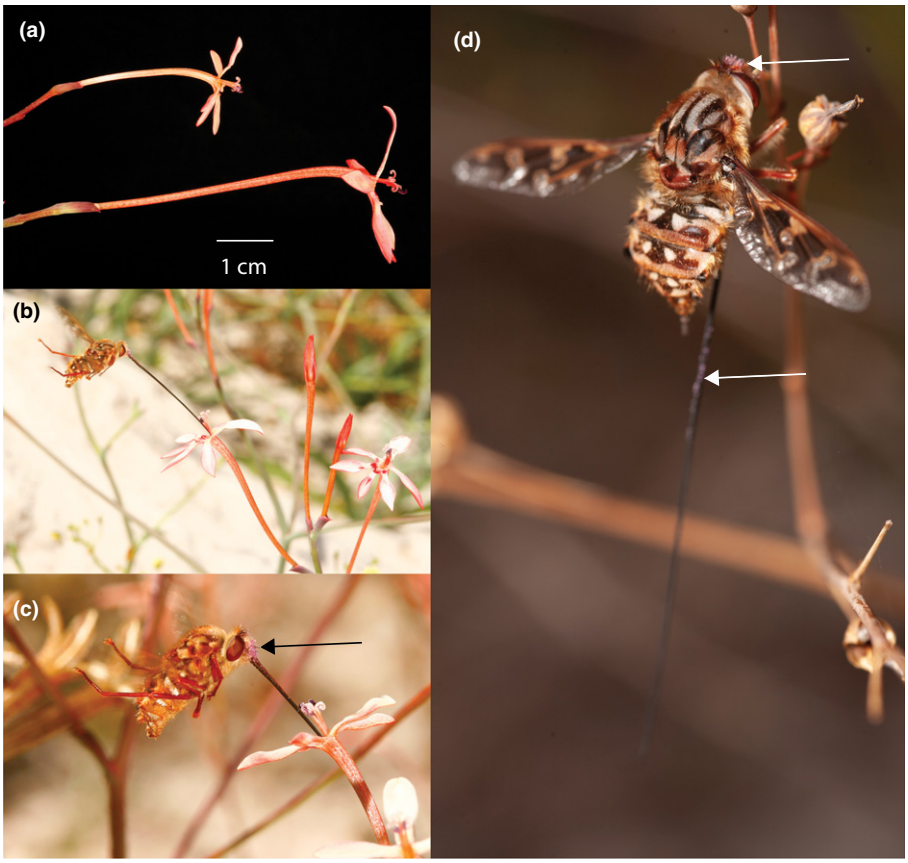

Fig. 1 Floral-tube length variation, pollination, and pollen placement of *Lapeirousia anceps* interacting with its pollinator *Moegistorhynchus longirostris* in a bimodal population. (a) Tube-length variation in the bimodal *L. anceps* population showing the short- and a long-tubed floral phenotype. (b) *Moegistorhynchus longirostris* visiting the long-tubed *L. anceps* floral phenotype. (c) Close-up of *M. longirostris* visiting the long-tubed *L. anceps* floral phenotype. Black arrow shows where the purple pollen is placed when the anthers make contact with the frons of the fly after the proboscis is fully inserted into the floral tube. (d) A perching *M. longirostris* fly with white arrows pointing to purple pollen on its frons, presumably deposited by long-tubed *L. anceps* flowers, and on the mid-proboscis, presumably placed by short-tubed flowers.

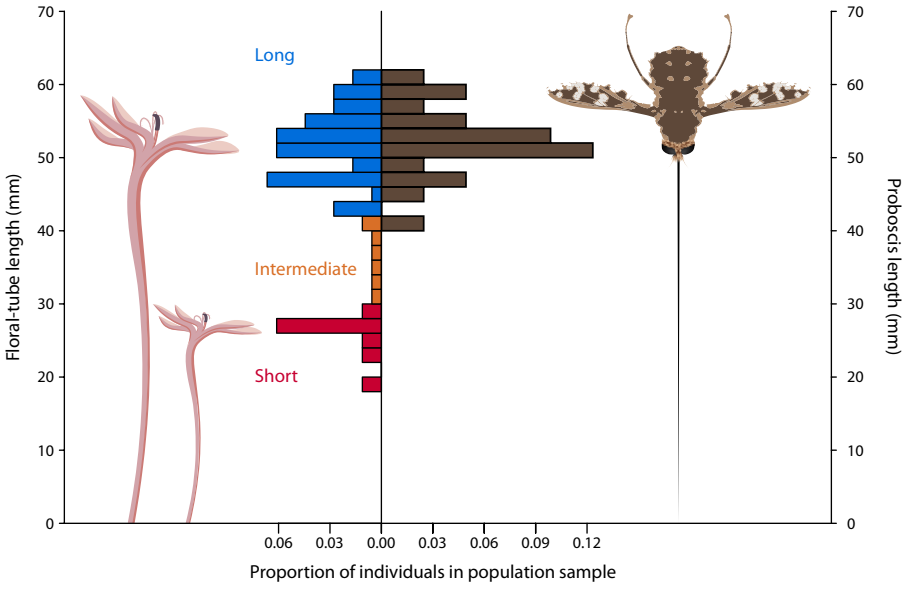

Fig. 2 Distribution of floral-tube length (*Lapeirousia anceps*) and fly proboscis length (*Moegistorhynchus longirostris*) from individuals within the Mamre population. Left: histogram of floral-tube length ($n = 90$) for short-tubed (< 32 mm; red bars), intermediate-tubed (> 32 mm < 42 mm; orange bars), and long-tubed individuals (> 42 mm; blue bars). Right: histogram of fly proboscis length ($n = 19$).

Materials and 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) and is part of the West Coast sand-plain fynbos community (Anderson *et al.*, 2016). Though some plants in the population bear flowers with intermediate tube lengths (32–42 mm), these plants are comparatively rare. The population is primarily composed of individuals with flowers on either side of the 32–42 mm range (Anderson *et al.*, 2016; also see Fig. 1). We therefore classified any flower with a tube length < 32 mm as short-tubed, and any flower with a tube length > 42 mm as long-tubed. We

conducted experiments in November 2015 and 2016, during which time we randomly measured single flowers from 90 individual plants in the population to characterize the current distribution of floral-tube length. We also characterized fly proboscis-length distribution from 19 flies caught during the experimental period.

Pollen transfer experiments

To capture ecologically realistic pollen-transfer sequences among flowers of varying tube lengths, we randomly selected virgin flowers from the population in the morning before flies were active. Flowers were placed inside a mesh cage to prevent pollinator visits. We then took eight flowers (four short-tubed and four long-tubed) from the pollinator-exclusion cage and using water-filled centrifuge tubes as receptacles, we secured flowers at 10 cm intervals along the end of a 1.5 m wooden dowel in random order. Using methods outlined in Minnaar & Anderson (2019), we labelled pollen of the first, fourth, and seventh flowers (i.e. every third flower) in the sequence with unique colours by applying either green, yellow, or red quantum dots (colours chosen at random). Quantum dots were dissolved in hexane (10 mg quantum dots per millilitre of hexane) and applied to anthers in 0.35 µl doses per anther following methods presented in Minnaar & Anderson (2019).

Flowers with labelled pollen acted as potential pollen donors among the eight flowers. All eight flowers could act as recipients since each donor was labelled with a unique colour. We walked through the population until we saw a foraging fly. We then placed the wooden dowel with flowers on the ground in the fly's foraging path to encourage visitation and recorded the sequence of visits to experimental flowers, if any. After a single visit sequence, we 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 Minnaar & Anderson, 2019). Completed microscope slides were stored at −20°C. Visited flowers were replaced with virgin flowers from the pollinator-exclusion cage. If a visited flower was a labelled donor, quantum dots were applied to the replacement flower.

For each stigma slide, we counted all labelled pollen and recorded the colour of these grains to link them to their donor in the transfer sequence. We excluded any self-transferred pollen by labelled donors from pollen counts. We counted pollen using a quantum dot excitation box placed under a dissection microscope (Minnaar & Anderson, 2019).

Pollen placement

When possible, we caught flies after they visited experimental flowers to determine the relationship between floral-tube length and pollen placement on flies. To catch and kill flies, we used a modified butterfly net (see Methods S1 for details) that allowed us to capture flies while avoiding excessive contact between the fly and the net (which may otherwise have resulted in pollen being dislodged from the fly).

To determine placement positions of pollen from donor flowers, we viewed captured flies under a custom-built excitation box that made any labelled pollen placed on flies fluoresce (see details in Minnaar & Anderson, 2019). We recorded the colour and distance of each quantum-dot-labelled pollen grain from the proboscis tip (to the nearest millimetre; see Methods S1 for details) so that pollen grains and their positions could be assigned to respective donor flowers visited by flies.

Data analyses

A substantial proportion of flowers visited by flies in our experiments did not place any pollen on fly bodies (37.14% of labelled flowers visited; $n = 35$), whereas the 22 flowers that did place pollen on flies placed a total of 330 pollen grains. Pollen transfer between flowers was equally variable, with many visits resulting in zero labelled pollen transfer (79.72%; $n = 222$); and when labelled pollen was transferred, the amount varied from 1 to 98 pollen grains. Though this inefficiency in pollen placement and transfer is not a unique feature of plants in this population – pollination in general appears to be an inefficient and highly variable process (Minnaar *et al.*, 2019) – the stochasticity and zero inflation in these data need to be accounted for. To do this, we used hurdle-regression models (Cragg, 1971) to analyse pollen placement and transfer. Similar to zero-inflated models, hurdle models split the response into two latent variables, which allows the probability (on the logit scale) of a zero response (i.e. no pollen placed or transferred) to be modelled separately from the magnitude/count of the response (i.e. amount of pollen placed or transferred; Hadfield, 2010). We fit generalized linear mixed-effects hurdle models (MCMCglmm-hurdle) in R (R Core Team, 2017) using the Markov-chain Monte Carlo (MCMC) 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. We used standard procedures for specifying priors and determining the number of iterations for the burn-in period, sampling period, and the thinning interval of MCMC models (Hadfield, 2010; see Methods S2 for details).

Pollen placement on pollinators We calculated total pollen placement from a specific donor onto a fly as the sum of the number of pollen grains found on the fly's body and the number of pollen grains transferred to stigmas before capture. This procedure likely underestimates total pollen placement since we do not include pollen lost in flight or placed on other parts of flowers. We modelled (MCMCglmm-hurdle) 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.

Variation in placement position among individual pollen grains from a single donor flower was minimal (mean

range ± SE: 0.64 ± 0.29 mm; maximum range: 3 mm). We therefore used the mean position of pollen grains placed by a donor flower to represent that flower's placement position. We modelled placement position as a linear function of donor tube-length using ordinary least-squares regression in R (R Core Team, 2017).

Pollen transfer Our primary hypothesis was that large differences in tube length between donor and recipient flowers would result in poor pollen transfer and, therefore, in RI between short- and long-tubed plants. Therefore, the absolute difference in tube length between donor and recipient flowers for a given visit (hereafter the tube-length difference), should influence both the zero-transfer probability and the quantity of pollen transferred. Since we measured pollen transfer beyond the first visit, sequence number also needs to be considered – the proportion of a donor's pollen transferred is likely to decrease with each successive visit, since pollen may be transferred to stigmas, displaced, or lost in flight (Thomson *et al.*, 1986). We therefore included visit sequence number as a separate term in the model affecting both the zero-response probability and the count portion of the model. We ran separate models for short- and long-tubed recipients, to account for the possibility of a unique relationship between pollen receipt and tube-length difference for short- and long-tubed flowers.

Reproductive isolation The number of transfer events recorded during experiments was not equal across transfer categories (i.e. long–long, long–short, short–short, and short–long) or visit sequence number. Therefore, though raw data from our experiments are informative to assess broad patterns of RI, we need to verify whether the observed raw-data patterns represent an underlying cause or if they reflect an artefact of sampling.

To obtain an unbiased estimate of RI as a result of tube-length differences, we generated a balanced data set from which pollen transfer could be simulated for short- and long-tubed flowers using MCMCglmm-hurdle models computed for short and long recipients. The data set was populated with randomly drawn (with replacement) tube-length measurements for 431 individuals at the Mamre site, to create 1000 donor–recipient pairs. Recipients in the data set were either short- or long-tubed, since pollen receipt was simulated separately for each, with 500 short- and long-tubed donors, respectively. For each donor–recipient pair, we calculated the tube-length difference and assigned a random visit sequence number. The amount of pollen transferred for each pair was calculated by randomly drawing parameter values from the posterior distributions (with replacement) of MCMCglmm-hurdle models computed for short- and long-tubed flowers. By sampling the distribution instead of using the mean parameter estimate, we account for the variation/confidence around mean parameter estimates in our models. The total RI across all 1000 pairs was then calculated (see Eqn 1). This procedure was repeated 1000 times to obtain 1000 RI estimates for short- and long-tubed recipients, as well as 1000 RI estimates for total mechanical RI between short- and long-tubed flowers. Median RI estimates were considered significant if > 95% of estimates were above 0.50 (RI > 0.5 represents assortative pollen transfer). Mechanical RI was calculated using the following equation (Ramsey *et al.*, 2003):

$$RI_S = 1 - \frac{\sum L\ pollen_S}{\sum L\ pollen_S + \sum S\ pollen_S} \quad \text{Eqn 1}$$

where RI for short-tubed flowers (RI_S) is calculated from the sum of long-tubed pollen transferred to short-tubed flowers ($\Sigma L\ pollen_S$) as a proportion of the sum of both long and short pollen transferred to short-tubed flowers ($\Sigma L\ pollen_S + \Sigma S\ pollen_S$). RI for long-tubed flowers (RI_L) was calculated in the same manner. Total mechanical RI (RI_{Tm}) between short- and long-tubed plants was calculated similarly, with total pollen transfer between long- and short-tubed flowers taken as a proportion of all pollen transfer (between and within short- and long-tubed flowers).

Results

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 unimodal and matched to the distribution of long-tubed flowers (see Fig. 2, cf. Anderson *et al.*, 2016). This suggests that realized 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. In total, we recorded 52 foraging sequences that included 91 visits to quantum-dot-labelled donor flowers followed by 222 visits to recipient flowers. Foraging sequences ranged in length from one to eight recipients after visiting a donor flower, with a mean sequence length (± SD) of 2.90 (± 1.64).

Pollen placement on pollinators

Donor tube-length showed a significantly positive 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 (Fig. 3). By contrast, most incidences of pollen transfer from short-tubed flowers occurred as small numbers of pollen grains on the mid-proboscis. However, one short-tubed flower placed a large number of pollen grains near the head.

We were able to capture 19 flies which visited 35 quantum-dot-labelled flowers (15 short, 20 long). For each flower, we were able to sum the number of pollen grains found (if any) on the visiting fly's body and the pollen transfered to all subsequent flowers visited, to get a estimate of the initial amount of pollen placed on flies by each flower. This revealed that three times more pollen grains were placed on flies per visit by long-tubed flowers (mean ± SE: 13.64 ± 5.39) than by short-tubed flowers (mean ± SE: 3.80 ± 2.56). Correspondingly, the MCMCglmm-hurdle model for pollen placement found a significant effect of donor tube-length on the number of pollen grains placed on flies (posterior mean estimate (95% confidence interval, CI): 0.06 (0.00–0.12); effective sample size: 10 000; $P_{MCMC} = 0.02$). Although the percentage of flowers placing pollen on flies was

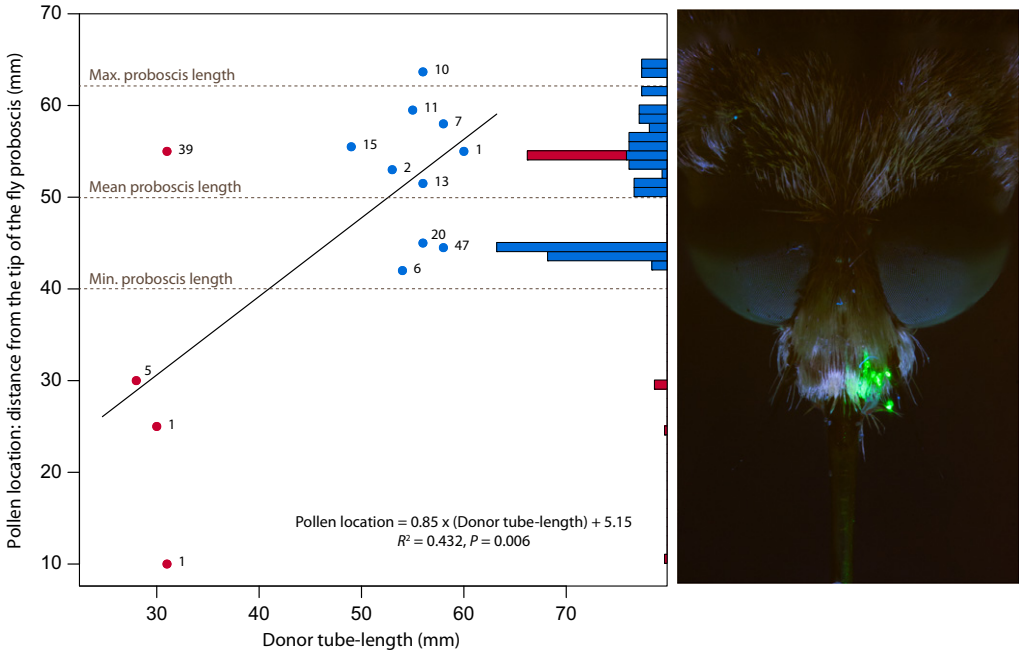

Fig. 3 Location of pollen from *Lapeirousia anceps* found on bodies of *Moegistorhynchus longirostris* flies. Pollen location (distance from the tip of the fly proboscis) is shown as a function of donor flower-tube length. Each dot represents the mean pollen position and floral-tube length of 14 individual flowers (red dots, short-tubed; blue dots, long-tubed). Numbers next to dots indicate the total number of pollen grains placed on flies (pollen found on the body of a fly + any pollen transferred to flowers before capture). Data presented in this figure excludes flowers for which all pollen initially placed on flies was transferred to recipient flowers, since we were unable to determine placement position for these flowers. The black line represents the linear regression model (bottom right) of pollen location as a function of donor tube-length. The histogram on the right shows pollen placement frequency from short-tubed (red bars) and long-tubed (blue bars) flowers. The minimum, maximum, and mean proboscis lengths for captured flies are indicated on the *y*-axis. A fly with quantum-dot-labelled pollen placed at the base of the proboscis is shown to the right of the graph.

higher for long-tubed (70.00%; $n = 20$) than for short-tubed flowers (53.33%; $n = 15$), the likelihood of pollen placement was not significantly influenced by donor tube length (posterior mean estimate (95% CI): −0.04 (−0.12–0.05); effective sample size: 10 000; $P_{MCMC} = 0.342$).

Pollen transfer

Our raw experimental data revealed that long-tubed flowers transferred most of their pollen to other long-tubed flowers (99.77%, $n = 441$), whereas short-tubed flowers transferred pollen to both long- (69.00%, $n = 95$) and short-tubed flowers (26.00%, $n = 95$) (Fig. 4); however, these patterns do not take visit sequence number into account, and long–long transfers are overrepresented relative to other transfer categories. To verify our experimental data, we assess the effect of tube length on pollen receipt from MCMCGLMM-hurdle models for long- and short-tubed flowers in the following sections.

Transfer to long-tubed flowers MCMCGLMM-hurdle models suggest that the likelihood of pollen transfer to stigmas of long-tubed recipients declines significantly with an increase in tube-length difference (posterior mean on the logit scale (95% CI): 0.06 (0.02–0.10); effective sample size: 10 000; $P_{MCMC} = 0.003$). Therefore, long-tubed flowers were more likely to receive pollen from other long-tubed flowers than from short-tubed flowers. For example, if the tube-length difference between a short-tubed donor and long-tubed recipient is 30 mm, the probability of pollen transfer occurring is 0.141, whereas a tube-length difference of 5 mm is three times more likely to result in pollen transfer (pollen transfer probability at 5 mm tube-length difference: 0.426). Nevertheless, though mismatch in tube length influenced pollen transfer probability, it did not have a significant influence on the amount of pollen transferred to long-tubed plants (posterior mean (95% CI): −0.04 (−0.09–0.02); effective sample size: 10 000; $P_{MCMC} = 0.165$) – see Table S1 for full model summary.

Transfer to short-tubed flowers As with long-tubed flowers, an increase in tube-length difference between donor flowers and short-tubed recipients significantly decreased the probability of pollen transfer (posterior mean on the logit scale (95% CI): 0.10 (0.00–0.22); effective sample size: 6340; $P_{MCMC} = 0.029$). However, the strength of this effect was much stronger for short-tubed recipients. For example, if the tube-length difference between a long-tubed donor and short-tubed recipient is 30 mm, the probability of pollen transfer occurring is 0.042 (an order of magnitude smaller than the same mismatch resulting in pollen transfer to long-tubed flowers). Moreover, tube-length difference also had a significant negative effect on the amount of pollen transferred to short-tubed recipients (posterior mean (95% CI): −0.16 (−0.37–0.00); effective sample size: 826; $P_{MCMC} = 0.030$) – see Table S2 for full model summary.

These results suggest that, for short-tubed flowers, a mismatch in donor–recipient tube-length reduces the chances of pollen

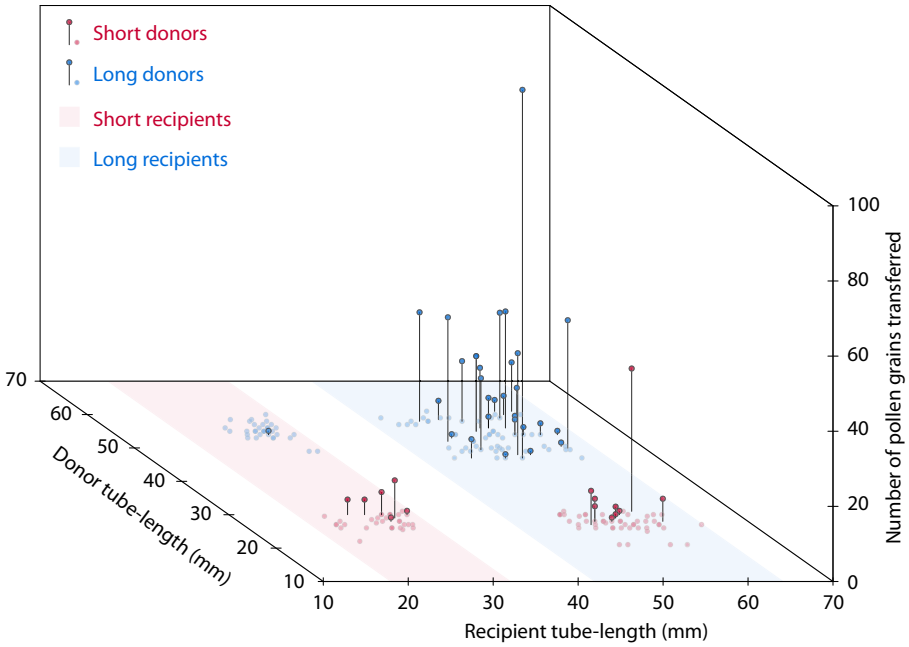

Fig. 4 Pollen transfer between *Lapeirousia anceps* flowers relative to donor and recipient tube length. Each dot represents a transfer event (i.e. a visit to a recipient flower after visitation to a donor), with translucent dots indicating zero pollen transferred and opaque dots indicating successful pollen transfer.

receipt and the amount of pollen received to a greater degree than for long-tubed recipients. Surprisingly, visit sequence number did not have a significant effect on the likelihood or magnitude of pollen receipt for either short- or long-tubed recipients (Tables S1 and S2).

Reproductive isolation

Both the raw pollen transfer data and MCMCGLMM-hurdle models suggest that large differences in tube-length between short- and long-tubed flowers reduce pollen transfer and may result in RI. To understand the effect of tube-length mismatch on RI without the influence of raw data sampling bias, we turn to simulated estimates from MCMCGLMM-hurdle models based on real tube-length data.

Pollen transfer estimates from simulations show that pollen received by short-tubed flowers is almost entirely from short-tubed donors (mean ± SD: 93.63 ± 0.07%), resulting in strong RI for short-tubed flowers as recipients (median $RI_s = 0.96$; $P = 0.001$). In comparison, pollen received by long-tubed recipients is more mixed (mean ± SD: 78.11 ± 0.10% from long donors) resulting in lower, but still significant RI for long-tubed flowers as recipients (median $RI_L = 0.80$; $P = 0.014$; Fig. 5). Despite the asymmetrical effect of tube-length differences on RI for short- and long-tubed flowers, RI_{Tm} remains high and significantly different from random pollen movement (median $RI_{Tm} = 0.82$; $P = 0.005$).

Discussion

This paper provides direct evidence that pollinators can facilitate RI through mechanical fit with floral structures, as opposed to ethological isolation involving the attraction of distinct pollinators. Our results suggest that floral-tube length divergence – often the result of divergent pollinator selection – may directly lead to partial RI. This provides evidence of the link between pollinator-driven divergence and RI in plants – as postulated by Grant (1994), Kay & Sargent (2009), and Van der Niet *et al.* (2014) – and clarifies how this link may facilitate the process of ecological speciation.

In the following, we discuss how variation in floral-tube length affects the placement and receipt positions of pollen on pollinators, which dictates pollen movement and potential mating patterns in *L. anceps*. Patterns of pollen movement are discussed under two subheadings (pollen placement and pollen transfer), representing key steps along the pathway to fertilization and gene flow (outlined by Minnaar *et al.*, 2019).

Pollen placement

The total number of recorded pollen placement events recorded was relatively low, especially for short-tubed donor plants, and any conclusions based on these data should be tentative. However, we think discussion of these pollen placement data provides useful mechanistic background to the pollen transfer patterns found in this study. We found an association between floral-tube length and the position of pollen placement along the bodies of pollinators. Our findings also suggest that different parts of pollinator bodies vary in their suitability as pollen deposition sites. This may generate differences in the number of pollen grains placed by short- and long-tubed flowers after a single visit.

Long-tubed flowers always placed pollen near the head of the fly, whereas pollen from short-tubed plants was placed along the mid-proboscis, with numerous grains from one flower also being found near the head. Long-tubed flowers were able to place approximately three times more pollen per visit on pollinators than short-tubed plants were able to place on pollinators. Similarly, Anderson *et al.* (2016) found an order of magnitude more

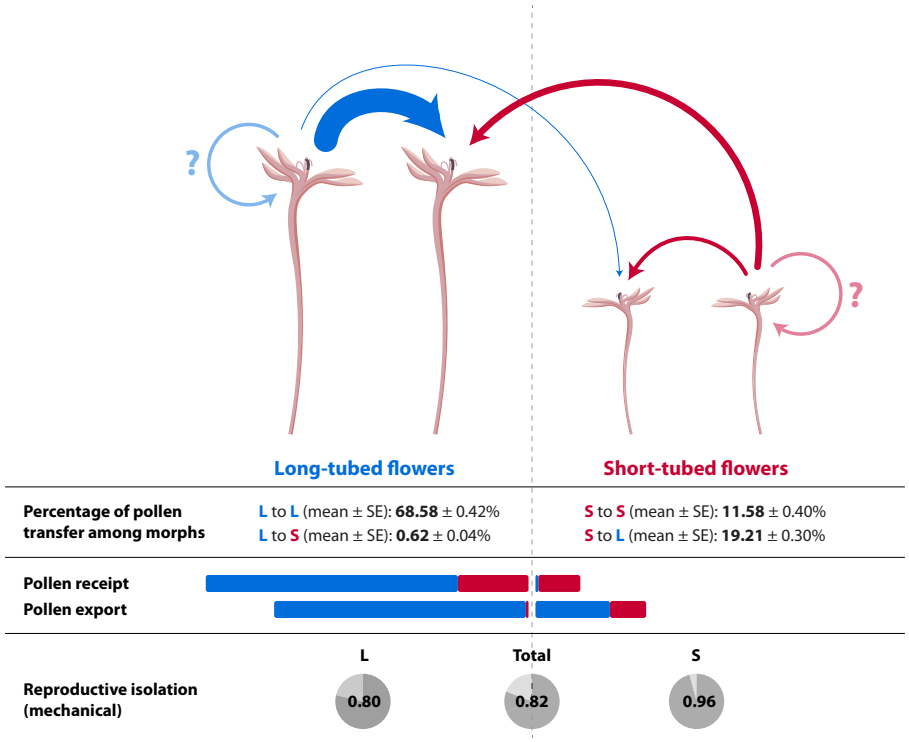

Fig. 5 Pollen transfer and reproductive isolation (RI) among short- and long-tubed flowers of *Lapeirousia anceps* estimated from MCMCglmm-hurdle models. Throughout the figure, red indicates short-tubed flowers and blue indicates long-tubed flowers. Relative arrow thicknesses depict differences in the amount of pollen transferred to and from long-tubed and short-tubed flowers by pollinators. The amount of self-pollen transfer was not measured and is indicated by pale arrows with adjacent question marks. Bars represent pollen received from and exported within and between morphs as a proportion of total pollen received and exported. Pie charts indicate RI resulting from mechanical isolation alone: for long- and short-tubed flowers, where dark grey section indicate pollen receipt from within phenotype and light grey sections indicate pollen receipt from a different phenotype. For total mechanical RI, the proportion of pollen movement within phenotypes is indicated in dark grey and the proportion of pollen movement between phenotypes is shown in light grey. Numbers on pie charts show calculated mechanical RI values.

pollen on the heads vs the proboscides of flies captured randomly in the population. This can partially be explained by a lower incidence of pollen placement on pollinators from short-tubed flowers (53%) than from long-tubed flowers (70%). In addition, pollen is also likely to adhere better to the large, hair-covered head of the fly than it is to the smooth, narrow proboscis.

One surprising result of this study was that pollen from short-tubed flowers was sometimes found near the head of the pollinator. This was unexpected because the large difference between pollinator proboscis length and the floral tubes of short-tubed flowers make head contact very unlikely (Video S1). Perhaps the most reasonable explanation is that the reproductive parts of long-tubed flowers occasionally scrape against the proboscis and sweep pollen from short-tubed plants up towards the head of the pollinator. In some species, this kind of pollen displacement has been hypothesized to play an important role in male–male competition, where structures on flowers may displace the pollen of rival males to areas on the pollinator where they are unlikely to be picked up by receptive stigmas (Minnaar *et al.*, 2019). Although this mechanism suggests that long-tubed flowers may displace the pollen of short-tubed flowers, displacing the pollen upwards in *L. anceps* may actually move it to an area where it has a greater likelihood of being picked up by stigmas (see 'Pollen transfer' below).

Despite some short-tubed pollen being placed near the heads of flies, the generally 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. Based on pollen placement patterns alone, long-tubed flowers are unlikely to transfer pollen to short-tubed flowers; however, short-tubed flowers should transfer limited amounts of pollen to long-tubed flowers (see the 'Pollen transfer' subsection below).

Pollen transfer

The export of pollen grains to stigmas partially mirrors what one may expect given pollen deposition patterns on the bodies of pollinators: in our experiment, long-tubed flowers transferred almost all of their pollen to other long-tubed flowers (99%). However, one surprising result was that short-tubed flowers transferred a larger portion of their pollen to long-tubed flowers (73%) than would be expected from pollen placement patterns. This may suggest that stigmas (but not anthers) on long-tubed flowers make contact with fly proboscides as they enter floral tubes. If stigmas do graze past fly proboscides in this way, it could explain the short-tubed pollen we found on one of the captured fly's heads – long-tubed stigmas may push short-tubed pollen up fly proboscides, further promoting short–long pollen transfer. The unexpectedly high prevalence of short to long pollen transfer remained after accounting for unequal sample sizes across tube-length phenotypes using model predictions. Nevertheless, models predicted that short-tubed flowers exported far fewer pollen grains (31% of total export) than long-tubed flowers do (69% of total export). The low proportion of pollen exported from short-tubed flowers (raw and model predicted) is likely the result of the small amounts of pollen that short-tubed flowers were able to place on pollinators and export compared with long-tubed flowers. Therefore, while two-thirds of short-tubed pollen export was predicted to end up on long-tubed flowers, short-tubed pollen only represented 22% of total pollen receipt for long-tubed flowers. Another potential explanation for the relatively low pollen export efficiency from short-tubed donors is that short-tubed flowers produce fewer pollen grains than long-tubed flowers.

The small amounts of pollen received by short-tubed flowers were almost all from other short-tubed flowers (proportion of total receipt, raw: 96%; model-predicted: 94%), which we attribute to the observation that long-tubed flowers seldom place pollen on the lower part of the proboscis where it could be picked up by short-tubed stigmas. Differences in pollen placement and receipt consequently result in very high, but asymmetric, RI, where short-tubed plants are more reproductively isolated than long-tubed plants are.

In reality, total RI is often dependent on multiple, sequential barriers (e.g. Ramsey *et al.*, 2003; Kay, 2006). When the additional reproductive barriers (assortative foraging by flies + assortative siring success, calculated in Anderson *et al.*, 2016) are included, total RI amounts to 0.98 for long-tubed plants and 1.00 for short-tubed plants. Since nonrandom pollinator foraging precedes pollen transfer and results in RI of 0.77 for both long- and short-tubed flowers, the absolute contribution of mechanical isolation to gene-flow restriction is 0.18 for long-tubed plants and 0.22 for short-tubed plants. This paper has not considered the potential effects of self-pollination on RI, despite *L. anceps* being capable of autonomous self-pollination (Anderson *et al.*, 2016). However, seeds produced through self-pollination may further promote the maintenance of tube-length differences in sympatry by reducing introgression between short- and long-tubed flowers.

Although few other studies have examined the possibility of RI resulting from differences in pollen placement and receipt sites of long- and short-tubed plants, we were able to find several studies suggesting that tube-length differences can limit pollen movement between flowers in sympatry (Table 1). Like ours, all suggest that, when pollen movement occurs between plants with different tube lengths, pollen movement is asymmetric and often occurs more readily from short-tubed to long-tubed plants than the other way around. For example, Wolf *et al.* (2001) demonstrated that captive hummingbirds (*Selasphorus platycercus*) transferred short-tubed *Ipomopsis arizonica* pollen to long-tubed *Ipomopsis aggregata* stigmas at a much higher rate than in the reverse direction. Like us, they hypothesized that short-tubed flowers place pollen near the tips of hummingbird bills that brush past stigmas of the longer tubed species. However, the longer tubed species places pollen on the heads and faces of hummingbirds, which seldom make contact with stigmas of the short-tubed species. Together, these studies suggest that divergent selection on floral-tube length is likely to have important implications for RI (as hypothesized by Grant, 1994), providing a seldom-explored link in the process of ecological speciation (Nosil, 2012; Van der Niet *et al.*, 2014). In the case of *L. anceps*, tubes are thought to have diverged allopatrically in response to pollinators with different proboscis lengths (Pauw *et al.*, 2009); consequently, tube length may function in a similar way to so-called 'magic traits', where ecotypic divergence leads directly to RI upon secondary contact. In a classic example of a magic trait, wing-colour patterns of mimetic *Heliconius* butterflies are under divergent selection to match existing, but geographically variable, mimicry rings (Mallet & Barton, 1989; Merrill *et al.*, 2012). The divergence in wing-colour patterns also results in nonrandom 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. However, unlike wing coloration in *Heliconius* butterflies, the 'magic' of floral-tube length divergence as an RI mechanism appears to be asymmetrical in many cases studied so far. The generality of this asymmetry in mechanical isolation, and how it influences speciation in plants, deserves further exploration.

Tube length evolution via the male vs the female fitness pathway

Whereas the primary intention of this paper was to examine RI, some of the results do potentially give us insights into putative

Table 1 Other studies suggesting asymmetry in pollen movement or gene flow associated with different floral tube/spur/style lengths.

Study genus and family	Type of pollen	Inter-/ intraspecific	Measurement method	Natural variation/ manipulated	Direction of asymmetry in pollen movement or effects on pollen export/receipt	Reference
Platanthera (Orchidaceae)	Pollinaria	Intraspecific	Pollinia removal and receipt	Manipulated	Spur-shortening reduced pollinia receipt more than removal	Nilsson (1988)
Ipomopsis (Polemoniaceae)	Granular	Interspecific	Pollen counts	Natural variation	Short to long	Wolf *et al*. (2001)
Costus (Costaceae)	Granular	Interspecific	Dye surrogates	Natural variation	Long to short	Kay (2006)
Burmeistera (Campanulaceae)	Granular	Interspecific	Pollen counts	Natural variation	Long to short[1]	Muchhala & Potts (2007)
Centropogon (Campanulaceae)	Granular	Intraspecific	Pollen counts	Manipulated	Short to long (marginal)	Muchhala & Thomson (2009)
Satyrium (Orchidaceae)	Pollinaria	Intraspecific	Dyed massulae	Manipulated	Short to long	Ellis & Johnson (2010)
Gladiolus (Iridaceae)	Granular	Intraspecific	Paternity analyses	Natural variation	Longer tubes increased maternal fitness, but not paternal[2]	Rymer *et al*. (2010)

[1]Long and short refer to staminal column exertion distance from constriction of the corolla tube.
[2]This effect was only detected in one of the sampling seasons.

patterns of pollinator-driven selection and the evolution of floral-tube length. These results are important because they provide mechanistic context for why floral-tube length may diverge in the first place (the first step in the process of ecological speciation—see Introduction section). In particular, modelled pollen export and import (which accounts for sampling bias in our experiments) suggests that long-tubed flowers export 69% of all pollen and receive 88% of all pollen. Greater pollen export and import is likely to enhance outcrossing and potentially select for longer tubes and a match with pollinator proboscides under conditions of pollinator limitation (however, extreme pollinator limitation may favour the evolution of selfing and smaller flowers). Consequently, pollinator selection on floral-tube length via both the male and female fitness pathways can provide complementary explanations for the positive association between pollinator proboscis lengths and floral-tube lengths in *L. anceps*. Anderson *et al.* (2010b) documented that floral-tube–proboscis length associations are almost ubiquitous among long-tubed plants; however, most studies have explained them through the female fitness pathway (e.g. Alexandersson & Johnson, 2002; Paudel *et al.*, 2016 – but see Muchhala & Thomson, 2009; Ellis & Johnson, 2010), whereby seed set or pollen receipt is higher when tubes and proboscis lengths are matching. Similarly, Pauw *et al.* (2009) highlighted the female fitness pathway for *L. anceps* by demonstrating that pollen receipt increased with floral-tube length and the resultant match with pollinator proboscis length. Whereas our study suggests similarities in the direction of selection imposed on tube length through the male vs the female fitness pathways (also see Muchhala & Thomson, 2009), Ellis & Johnson (2010) demonstrated contrasting selection on orchid spur length via the male vs the female fitness pathway. They proposed that short-spurred orchids may be better exporters of pollen than the long-spurred individuals because proboscis placement of pollinaria allows contact with stigmas from long- and short-tubed flowers, whereas head placement only allows contact with long-tubed flowers. They also proposed that long-spurred orchids would be better receivers of pollen for the same reason, setting up the possibility of contrasting selection patterns through the male and female fitness pathway. As in our study, they found that short-spurred orchids were better able to export pollen to long-spurred individuals than vice versa. However, unlike our study, they found that short-spurred orchids were good pollen exporters, and this gave rise to the contrasting patterns of selection through the male and female fitness pathways (see also Nilsson, 1988; Rymer *et al.*, 2010). Differences in the relative pollen export ability of short-tubed *L. anceps* vs orchids highlights the possibility that the sticky attachment of orchid pollinia may be better suited for placement on the mid-proboscis of pollinators than the granular pollen grains of *L. anceps*.

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Author contributions

CM, MLdJ and BA jointly designed the experiments and collected data in the field; CM processed and analysed all data; CM created data figures and illustrations; CM, MLdJ and BA jointly interpreted data analyses; CM and BA co-wrote the first draft of the paper; CM, MLdJ and BA contributed critically to subsequent drafts and gave final approval for submission.

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Supporting Information

Additional Supporting Information may be found online in the Supporting Information section at the end of the article.

Methods S1 Description of fly capture and pollen placement determination methods.

Methods S2 Description of MCMCglmm-hurdle model procedures.

Table S1 Model summary table for MCMCglmm-hurdle model of pollen transferred to long-tubed recipients.

Table S2 Model summary table for MCMCglmm-hurdle model of pollen transferred to short-tubed recipients.

Video S1 Compilation of videos showing flies foraging from long- and short-tubed flowers.

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