Functional Ecology

British Ecological Society

Functional Ecology 2014 doi: 10.1111/1365-2435.12274

Heterostyly promotes disassortative pollination and reduces sexual interference in Darwin's primroses: evidence from experimental studies

Barbara Keller*[,1], **James D. Thomson**[2] **and Elena Conti**[1]

[1] *Institute of Systematic Botany, University of Zürich, Zollikerstrasse 107, 8008 Zürich, Switzerland; and* [2] *Ecology and Evolutionary Biology Department, University of Toronto, 25 Harbord St., Toronto, Ontario M5S 3G5, Canada*

Summary

1. Different strategies to reduce selfing and promote outcrossing have evolved in hermaphroditic flowers. Heterostyly, a complex floral polymorphism that occurs in at least 27 families of angiosperms, is hypothesized to achieve both goals by optimizing cross-pollination (*via* disassortative pollen transfer) and restricting gamete wastage to autogamy (*via* the reduction in sexual interference between male and female organs).

2. In heterostylous flowers, the reciprocal positioning of sexual organs in different morphs and the pollen incompatibility system within flower or between flowers of the same morph are thought to optimize both male and female functions, reducing the conflicts inherent to the occurrence of both sexual organs in the same reproductive unit. Specific elements of the disassortative-pollination and sexual-interference hypotheses have been tested individually before. However, despite the long-standing interest in heterostyly – ever since Darwin's seminal work on primroses – the predictions derived from these two hypotheses have never been experimentally and systematically examined in the same system.

3. Using distylous primroses (*Primula elatior, P. vulgaris*), we compare pollen transfer (i) between reciprocal and non-reciprocal flowers; (ii) from anthers onto different parts of the pollinator's body; and (iii) within flower and between flowers of the same morph. We further test whether (iv) anther–stigma distance correlates with self-pollen transfer and whether (v) seed set differs after pollinations with compatible, incompatible and both pollen types.

4. Reciprocal herkogamy promotes differential placement of pollen onto different parts of the pollinator's body, thus effecting transfer of more pollen to reciprocal than to non-reciprocal stigmas and realizing the key predictions of the disassortative-pollination hypothesis. However, short-styled flowers transfer pollen more disassortatively than long-styled flowers in both species, whereas long-styled flowers export more pollen to non-reciprocal than to reciprocal stigmas in *P. vulgaris*, thus compromising male function in this species. Furthermore, larger distance between sexual organs lowers self- and intra-morph pollination and the pollen incompatibility system decreases seed production after self-pollination, thus diminishing sexual interference.

5. Our results help us understand how the morphological and physiological components of heterostyly contribute to optimizing pollen transfer and minimizing self- and intra-morph pollination, thus promoting more efficient outcrossing in species with this floral polymorphism.

Key-words: breeding system, distyly, floral polymorphism, gamete wastage, male and female functions, outcrossing, plant–insect interaction, *Primula*, reciprocal herkogamy, selfing

Introduction

The mainly animal-pollinated angiosperms are considerably more diverse than their sister clade, the gymnosperms (Crane, Friis & Pedersen 1995). The greater species diversity of the angiosperms has been repeatedly linked to the extraordinary variety of their reproductive units, the flowers (e.g. Barrett 2002a). Most angiosperms are hermaphroditic, having flowers that contain both female and male sexual organs, a condition that enables pollen

---

*Correspondence author. E-mail: barbara.keller@systbot.uzh.ch produced by one flower to fertilize the ovules of the same flower (i.e. selfing). However, selfing is often associated with inbreeding depression, which tends to lower offspring fitness (Charlesworth & Charlesworth 1987). Consequently, different strategies aimed at reducing selfing have evolved in flowering plants, including the spatial separation of sexual organs within a flower, that is, herkogamy (Lloyd & Webb 1986; Webb & Lloyd 1986; Barrett 2002b, 2003).

A special type of herkogamy occurs in heterostylous species, in which male and female sexual organs are placed reciprocally in distinct types of flowers (Barrett 2002a). Populations of heterostylous species are usually composed of two (distyly) or three (tristyly) genetically determined morphs (Ganders 1979). The two floral types of distylous populations are referred to as the long- and short-styled morphs (hereafter L-morph and S-morph, respectively), also known as pins and thrums (Darwin 1877). Anthers are placed low in the corolla of the L-morph and high in the corolla of the S-morph, whereas stigmas are located high and low, respectively, in the two kinds of flowers. This arrangement of sexual organs, known as reciprocal herkogamy, is often associated with differences between the two morphs in pollen grain size and number, size of the stigmatic papillae and stigma shape (Richards 1997). In addition, heterostylous species are frequently characterized by a sporophytic incompatibility system that reduces self- and intra-morph pollen germination and pollen-tube growth, so that pollen movement between flowers of the two morphs (i.e. transfer of compatible pollen between reciprocal flowers) will result in optimal seed set, while pollen deposition within the same flower or between flowers of the same morph (i.e. transfer of incompatible pollen) will yield little or no seed.

Distyly is thought to optimize both male and female components of a plant's reproductive fitness. In particular, reciprocal herkogamy improves the efficiency of pollen movement between L- and S-flowers *via* the transfer of pollen grains onto distinct positions of the pollinator's body that correspond to the heights of the receiving stigmas in the reciprocal morphs, a process known as *disassortative pollination* (Darwin 1862, 1877; Ganders 1979; Barrett 1992, 2002a,b; Richards 1997; Barrett & Shore 2008). At the same time, herkogamy in each morph decreases the probability of pollen transfer within the same flower or between flowers of the same morph, thus decreasing the number of incompatible pollen grains wasted on non-reciprocal stigmas (Fig. 1a). Incompatible pollen might effect stigma or stylar clogging and/or self-pollination, if the incompatibility reaction is weak, thus diminishing the probability of ovules to be fertilized by compatible pollen. Consequently, such interactions between male and female functions reduce the number of both male and female gametes available for outcrossing, a phenomenon known as *sexual interference*. It is thus thought that reciprocal herkogamy improves primarily male function by reducing the wastage of male gametes (i.e. pollen discounting) and increasing effective pollen transfer, while the physiological incompatibility system mainly enhances female function by decreasing the wastage of female gametes (i.e. ovule discounting; Barrett 2002a,b). Despite the general predictions about the adaptive benefits of heterostyly explained above, the actual dynamics of disassortative pollination and sexual interference may deviate from theoretical expectations. Below, we review current knowledge on the two key functional benefits of heterostyly gained from experiments on different species, thus setting the stage for our own study.

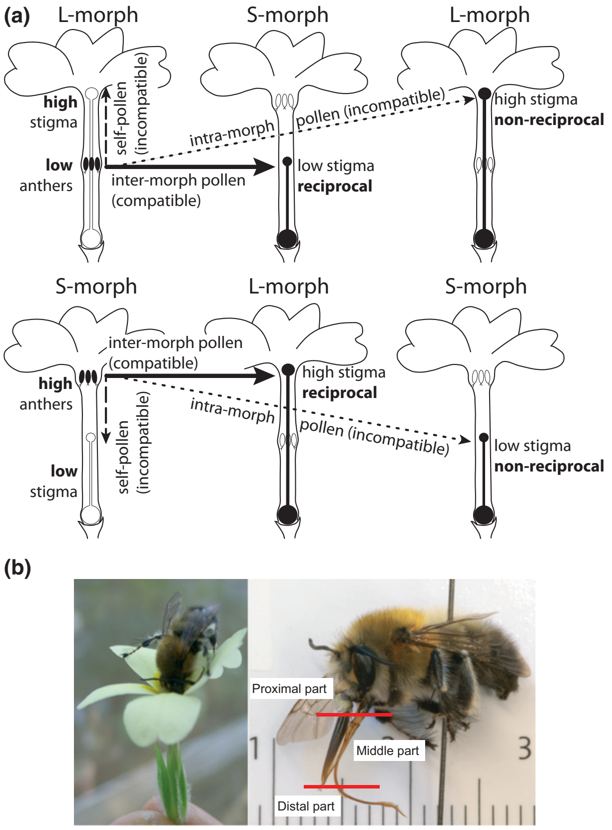

Fig. 1. Pollen transfer in distylous primroses: (a) pathways of intra- and inter-floral pollen movement from long-styled (L; upper panel) and short-styled (S; lower panel) pollen donors, illustrated with schematic drawings of *Primula vulgaris* flowers. Solid lines indicate transfer of compatible pollen between reciprocal organs (inter-morph pollen; low organs, above; high organs, below); dashed and dotted lines indicate transfer of incompatible pollen within flowers (self-pollen) and between non-reciprocal organs of the same morph (intra-morph pollen), respectively. (b) Photographs of an *Anthophora plumipes* male probing a *Primula vulgaris* flower (left; b. Keller photo credit), and with extended glossa (right; b. Weigelt and M. Rey photo credit); pollen-carrying structures are labelled as proximal, middle and distal.

Disassortative pollination is expected to promote more efficient pollen transfer between the reciprocal sexual organs of different morphs than between non-reciprocal sexual organs of flowers of the same morph, a prediction first proposed by Darwin (1862, 1877) and later termed the 'disassortative-pollination hypothesis' (Lau & Bosque

2003). Consequently, the efficacy of disassortative pollination can be evaluated by comparing the number of pollen grains transferred to compatible stigmas (inter-morph pollination) with the number of pollen grains transferred to incompatible stigmas (intra-morph pollination). To date, most studies have quantified disassortative pollen movement by analysing pollen composition on the stigmas of intact flowers under natural conditions, an approach that cannot disentangle the relative contributions of intra-morph and intra-floral pollen transfer to total stigmatic loads (Levin 1968; Ornduff 1970, 1979, 1980; Ganders 1976; Olesen 1979; Weller 1980; Björkman 1995; Nishihiro *et al.* 2000; Ornelas *et al.* 2004; Massinga, Johnson & Harder 2005; Hernández & Ornelas 2007; Brys *et al.* 2008; Wolfe, Massinga & Johnson 2009; Valois-Cuesta, Soriano & Ornelas 2011). Pollen movement between vs. within morphs has also been investigated under natural conditions by either using pollen fluorescent dyes (Baena-Díaz *et al.* 2012) or comparing stigmatic pollen loads of intact and emasculated flowers (Ganders 1974; Nicholls 1986; Piper & Charlesworth 1986; Nishihiro & Washitani 1998). However, these studies could not control for variation in morph ratio, pollinator visitation sequence and pollen pickup probability from different morphs, thus preventing an accurate explanation of pollen transfer dynamics. Conversely, experiments with artificial flowers (Stone & Thomson 1994) or the quantification of pollen transfer from intact donor flowers to emasculated recipient flowers after single pollinator visits (Stone 1995, 1996; Lau & Bosque 2003; Ornelas *et al.* 2004) allow for more precise comparisons of pollen transfer between vs. within morphs. For example, if emasculated S- and L-flowers are alternatively presented to pollinators in controlled sequences, pollen export capacity from a single, intact pollen-donor flower can be directly compared between reciprocal and non-reciprocal stigmas and the effect of spatial matching between male and female organs of the flowers can be incorporated in the comparisons of transferred pollen (see experiments of Stone 1996; Lau & Bosque 2003).

Analyses of stigmatic pollen loads in natural populations have contributed to our understanding of sexual interference, revealing that incompatible pollen is often deposited on stigmas of distylous species, especially in L-flowers (Levin 1968; Ganders 1974; Schou 1983; Nicholls 1986; Piper & Charlesworth 1986; Björkman 1995; Nishihiro & Washitani 1998; Nishihiro *et al.* 2000; Ornelas *et al.* 2004; Massinga, Johnson & Harder 2005; Hernández & Ornelas 2007; Brys & Jacquemyn 2010; Valois-Cuesta, Soriano & Ornelas 2011). The proportion of incompatible pollen may range from as much as 99·3% on stigmas of L-flowers, as in *Lithospermum caroliniense* (Levin 1968), to as little as 7·7% on stigmas of S-flowers, as in *Pentanisia prunelloides* (Massinga, Johnson & Harder 2005), and may depend on corolla length and form (e.g. dish-shaped vs. tubular), as well as pollinator shape and species. Similarly, the relationship between stigma and/or stylar clogging and seed set varies among species. For example, the manual application of incompatible prior to compatible pollen onto stigmas caused a significant reduction in seed set in some distylous species (*Turnera ulmifolia*, Shore & Barrett 1984; *Lythrum salicaria*, Nicholls 1987), but not in others (*Pontederia cordata*, Barrett & Glover 1985; *Persicaria japonica*, Nishihiro & Washitani 1998).

In this study, we experimentally investigated intraspecific pollen transfer dynamics and its consequences on the seed set of distylous species using the phylogenetically close (possibly paraphyletic) *Primula elatior* and *P. vulgaris* as model organisms (Primulaceae; Mast, Kelso & Conti 2006; Schmidt-Lebuhn *et al.* 2012). Approximately 90% of the ca. 420 species of *Primula* are distylous and usually self- and intra-morph incompatible (Wedderburn & Richards 1992; Richards 1997, 2003). While *Primula* has been considered a classic model for heterostyly ever since Darwin (1862, 1877), the details of intraspecific pollen movement (see Fig. 1a) have not yet been quantified under rigorous experimental conditions. To our knowledge, disassortative pollination in *Primula* has been investigated exclusively under natural conditions by quantifying stigmatic pollen loads in intact and/or emasculated flowers (*P. elatior*, Schou 1983; *P. vulgaris,* Ornduff 1979; Piper & Charlesworth 1986; *P. veris*, Ornduff 1980; *P. sieboldii*, Nishihiro *et al.* 2000) or under semi-natural conditions by studying pollen carry-over (*P. sieboldii*, Matsumura & Washitani 2002), but quantitative comparisons of intra- vs. inter-morph pollen transfer directly linked with the degree of spatial matching between floral sexual organs are not available, thus precluding explicit tests of the functional significance of heterostyly.

We investigated both disassortative-pollination (Darwin 1862, 1877) and sexual-interference hypotheses (Barrett 2002a) in *P. elatior* and *P. vulgaris* by testing whether: (i) pollen transfer between reciprocal flowers exceeds pollen transfer between non-reciprocal flowers; (ii) more pollen is transferred within the same flower than between flowers of the same morph; (iii) more herkogamy decreases the deposition of self-pollen on a stigma; and (iv) stigma and/or stylar clogging decreases seed set. The results clarify how distyly promotes outcrossing by optimizing pollen transfer between compatible morphs, while simultaneously limiting pollen wastage on self- and/or non-reciprocal stigmas.

Materials and methods

STUDY ORGANISMS

The flowers

Primula elatior (L.) Hill (oxlip) and *P. vulgaris* Huds. (primrose) are perennial, rosette-forming diploid species ($2n = 22$) with distylous flowers that bloom in early spring. Both species have pale-yellow corollas with broad, overlapping, widely v-notched corolla lobes, but differ in the maximum diameter of the corolla (up to 25 mm in *P. elatior*; up to 40 mm in *P. vulgaris*); additionally, the corolla-limb shape is more flat-faced in *P. vulgaris* than in *P. elatior* (Richards 2003). The two species are mainly visited by long-tongued bees, bumblebees and bee flies, although butterflies and pollen-gathering bees also occasionally visit their flowers (Woodell 1960; Richards 2003; B. Keller pers. obs.).

The insects

We used males of the hairy-footed flower bee (*Anthophora plumipes* Pallas; Hymenoptera: Anthophoridae) as pollinators in our experiments. *Anthophora plumipes* is a common long-tongued, solitary bee, frequently foraging on early flowering, distylous spring flowers, including those of *Primula* (Meyer 2007; B. Keller pers. obs.) and *Pulmonaria* (e.g. Brys *et al*. 2008).

EXPERIMENTS

To investigate the disassortative-pollination hypothesis, we tested whether: (A) more pollen is delivered to reciprocal than to non-reciprocal stigmas and (B) pollen grains from L- and S-flowers are differentially placed on distinct parts of the pollinator's body. To investigate the sexual-interference hypothesis, we tested whether: (C) more pollen is transferred to self- than to intra-morph stigmas; (D) less herkogamy corresponds to more self-pollination; and (E) the presence of incompatible pollen on a stigma decreases total seed set. We tested the core element (A) of the disassortative-pollination hypothesis in both species (*P. elatior* and *P. vulgaris*), and specific aspects of the two hypotheses in either *P. elatior* (C, D) or *P. vulgaris* (B, E).

All pollination experiments were performed in a walk-in flight cage located in a greenhouse at the botanical garden of the University of Zurich (Switzerland). Experimental flowers were kept in a pollinator-free environment. Statistical analyses were performed in the generalized linear mixed effects model (GLMM) environment of SPSS (version 20.0.0; IBM Corp., Armonk, New York, USA). We used GLMMs with Poisson error distribution and log link function to analyse count data (i.e. pollen grains and seeds) and models with normal error distribution and identity link function to analyse anther–stigma distance data. We used the Satterthwaite's method to determine the approximate denominator degree of freedom when data sets were unbalanced and applied sequential Bonferroni correction to account for multiple tests.

(A) IS MORE POLLEN DELIVERED TO RECIPROCAL THAN TO NON-RECIPROCAL STIGMAS?

Experimental set-up

We performed flight-cage experiments with flowers of *P. vulgaris* in spring 2011 and *P. elatior* in spring 2012. We obtained 10 plants of *P. vulgaris* (five L- and five S-plants) from a locality in Niederönz (Switzerland) and cultivated them individually in pots. For *P. elatior,* we collected entire inflorescences with young buds from 21 plants (11 L- and 10 S-plants) in a natural population at the botanical garden of the University of Zurich. Inflorescences were maintained in water until flowers were used in the experiment. Pollen-recipient flowers were emasculated in bud stage. Undehisced anthers were carefully removed with forceps from S-flowers by unfolding petals and from L-flowers by making a small longitudinal slit in the corolla. Bees were captured in the botanical garden, kept in individual containers and cooled for ease of handling. They were allowed to warm up and groom prior to experiments. We used bees multiple times, giving them time to groom and clean head and proboscis between experimental runs. We used 11 male bees of average body size in total, five with *P. vulgaris* and six with *P. elatior*.

As our basic experimental unit, we used a set of three flowers (triad) that consisted of one intact pollen donor (either S- or L-flower) and two emasculated pollen recipients (one S- and one L-flower). Each experiment consisted of four triads divided into two experimental runs: one run with two L-flowers from the same plant as pollen donors and the respective combination of reciprocal and non-reciprocal flowers as first and second recipients, and one run with two S-flowers from the same plant as pollen donors and the respective combination of reciprocal and non-reciprocal flowers as first and second recipients (Table 1). This design accounts for variation in number of deposited pollen grains dependent on pollinator visitation sequence (see Lau & Bosque 2003). We presented flowers so that bees could walk from one to the next and excluded all triads in which bees flew and/or groomed between flowers. Experimental runs within experiment as well as triads within experimental run were performed in random order, and experiments were replicated 10 times in *P. elatior* and 12 times in *P. vulgaris*.

Data collection

Flowers of each triad were dissected directly after use by longitudinally cutting the calyx and corolla tube. Stigmas were separated from the style with forceps and squashed in fuchsin jelly on microscope slides (Dafni, Kevan & Husband 2005). The rest of the flower was preserved in 70% ethanol and used for subsequent measurements of anther and stigma heights (see below). We took digital images of squashed stigmas under a slide scanning microscope (Axio Imager.Z1, Zeiss, Switzerland) and counted the pollen grains on the two recipient stigmas exhaustively using ImageJ 1.40 (http://rsbweb.nih.gov/ij/). Because both recipient flowers in each triad had been emasculated prior to experiment, no self-pollination occurred. Some of the pollen data from non-reciprocal transfers in *P. elatior* were also analysed to answer question D below.

We took digital images of ethanol-preserved flowers with a Nikon D70 (Nikon Corp., Tokyo, Japan) camera and measured distances from the base of the gynoecium to (i) the middle of the anthers (anther position) and (ii) the base of the stigma (stigma position) (for details see Keller, de Vos & Conti 2012). Floral measurements were taken to an accuracy of 0·01 mm using ImageJ 1·40. We calculated the anther–stigma distances between the anthers of the pollen donor and the stigmas of each of the two recipients by subtracting the height of the higher organ from the height of the lower organ.

Data analyses

First, we fitted a GLMM to compare the stigmatic pollen loads of the two recipient flowers in each triad and test whether pollen transfer differed between organs (reciprocal vs. non-reciprocal organs), morphs (L- vs. S-morph) and species (*P. elatior* vs. *P. vulgaris*). We used simple contrasts to test whether the number

Table 1. Experimental design to compare pollen transfer within vs. between morphs in *Primula elatior* and *P. vulgaris*. Pollen transfer was effected by males of *Anthophora plumipes*. Both recipient flowers were emasculated prior to experiment. Each experiment was repeated 10 times in *P. elatior* and 12 times in *P. vulgaris*, using different flowers for each triad

Experiment	Triad	Pollen donor	First recipient flower	Second recipient flower
L-run	I	L-flowers	S-flowers	L-flowers
	II	L-flowers	L-flowers	S-flowers
S-run	III	S-flowers	S-flowers	L-flowers
	IV	S-flowers	L-flowers	S-flowers

L, long styled; S, short styled.

of pollen grains exported to reciprocal stigmas differed from that exported to non-reciprocal stigmas for each pollen-donor type. Similarly, we tested whether anther–stigma distances between donor and recipients differed significantly between organs, morphs and species. More specifically, we used simple contrasts to test whether anther–stigma distances differed (i) between reciprocal and non-reciprocal organs for each pollen-donor type and (ii) between L- and S-pollen donors for reciprocal organs and non-reciprocal organs, respectively. As fixed effects we used *species membership* (*P. elatior* and *P. vulgaris*), *donor-morph type* (L- and S-morph), *organ type* (reciprocal and non-reciprocal) and all two-way and three-way interactions in both models. We used *triad* nested in *donor-morph type* and *species membership* as random effect to account for hierarchical data structure in both models. Finally, we estimated the probability of an individual pollen grain to be exported to a certain stigma type using the equation $T_{ij} = r_{ij}/p_i$, which is a modification of the formula of Lloyd & Webb (1992) for our experiment. T_{ij} is the probability of a pollen grain of the morph i to land on the stigma j, r_{ij} is the average number of pollen grains calculated from our flight-cage experiments for this transfer type, and p_i is the pollen production per flower of the donor morph obtained from previously published data. In *P. elatior*, L- and S-flowers produce on average 138 710 and 86 645 pollen grains, respectively (Schou 1983). In *P. vulgaris*, two sets of data are available: Piper & Charlesworth (1986) report that L- and S-flowers produce on average 183 100 and 85 825 pollen grains per flower, respectively; Ornduff (1979) states that L- and S-flowers produce 283 000 and 89 000 pollen grains per flower, respectively. Thus, for *P. vulgaris,* we calculated the probability of pollen export for both sets of data.

(B) ARE POLLEN GRAINS FROM L- AND S-FLOWERS DIFFERENTIALLY PLACED ON POLLINATOR BODIES?

Experimental set-up

We performed a separate flight-cage experiment where we allowed bees of *A. plumipes* to visit a series of five to seven intact S- and/or L-flowers of *P. vulgaris.* We performed this experiment in spring 2011 using the same plants as in A (see above). Bees were caught and handled as described before. Three bees visited L- and S-flowers in alternating order, one bee visited only L-flowers and another bee only S-flowers. Bees were euthanized in the freezer directly after visiting the last flower.

Data collection

All the numerous *Anthophora* bees observed under natural and experimental conditions approached and probed primrose flowers by lowering the proboscis and rostral part of the head into the corolla-tube opening (B. Keller pers. obs.; Fig. 1b); therefore, we counted pollen grains only on these parts of the insect bodies. We dissected the proboscis into (i) prementum, (ii) labial palpi, (iii) galea, (iv) proximal part of the glossa, (v) part of the fully extended glossa sticking out over the galea and (vi) labial palpi and mounted these six parts individually on microscope slides with fuchsin jelly (Dafni, Kevan & Husband 2005). Small cubes of jelly were used to carefully remove all pollen from the head, labrum and stripes, as these structures are too big and/or thick to be directly mounted on microscope slides. We took digital images from mounted pollen-carrying parts and counted pollen exhaustively (see above). We used class-size differences to assign the pollen grains on the pollen-carrying structures of the three bees that successively visited different morphs to either L- or S-flowers. In *P. vulgaris,* the diameter of pollen produced by L-flowers ranges from 13·6 to 23·4 μm, with a mean of 19·3 μm, while pollen of S-flowers ranges from 21·5 to 37·1 μm, with a mean of 30·0 μm (Ornduff 1979).

Data analyses

Anthers of S-flowers are close to the corolla-tube opening, while anthers of L-flowers are located approximately half-way down the corolla tube (Fig. 1a). Given the general dimensions of the body of *A. plumipes*, we expected to find pollen from high anthers predominantly on the head and the part of the proboscis closest to it (i.e. proximal part of the proboscis), and pollen from low anthers on the middle part of the proboscis. To test this expectation, we divided the bee's pollen-carrying structures into *proximal* (dorsal and ventral head, labrum, stripes and prementum), *middle* (labial palpi, galea and proximal part of the glossa) and *distal* parts (part of the fully extended glossa sticking out over the galea and labial palpi; Fig. 1b). We used GLMMs to test whether (i) L-pollen is preferentially placed on the middle pollen-carrying structures and (ii) S-pollen is preferentially placed on the proximal structures. As fixed effects we used *pollen-carrying structures of the bee* (distal, middle and proximal) in both models. Finally, we performed a GLMM with *pollen type* (pollen from L- and S-flowers), *pollen-carrying structure* and their interaction as fixed effects and used contrasts to compare whether different body parts carried more pollen from low or high anthers, respectively. To account for hierarchical data structure, we included *bee identity* as a random effect in all models.

(C) IS MORE POLLEN TRANSFERRED WITHIN FLOWERS THAN BETWEEN FLOWERS OF THE SAME MORPH?

Experimental set-up and data collection

We reanalysed stigmatic pollen loads of pollen donors and non-reciprocal recipient flowers of the experiment performed with *P. elatior* in spring 2012 (see questions A and D). We only analysed experimental runs with non-reciprocal flowers as first recipients.

Data analyses

We fitted a GLMM to compare the stigmatic pollen loads of pollen donors and first recipients to test whether pollen transfer differed within flower and between flowers of the same morph (intra-floral vs. intra-morph pollination) and morphs (L- vs. S-morph). We used simple contrasts to test whether transfer of pollen differed between self- and non-reciprocal stigmas for L- and S-morphs. We used *pollination type*, *morph type* and their interaction as fixed effects. We used *experimental run* as random effect to account for hierarchical data structure.

(D) ARE HERKOGAMY AND SELF-POLLINATION INVERSELY CORRELATED?

Experimental set-up and data collection

To investigate how herkogamy affects self-pollination, we performed flight-cage experiments with *P. elatior* in 2009 and 2012. In 2009, we used 20 plants (10 L-plants and 10 S-plants) obtained from a local wild-plant nursery (Vogt Stauden, Erlenbach; EL09); in 2012, we analysed stigmas of pollen-donor flowers of experiment A above (EL12). Bees were caught and handled as described before. We offered single, recently opened, intact flowers to one bee at a time. We used 24 bees of average body size in total, 18 in 2009 and six in 2012. Experiments with L- and S-flowers were performed in random order and replicated 10 (EL09) or 11 (EL12) times. We dissected flowers, collected stigmas, counted pollen and measured anther and stigma heights as described before. We calculated the distance between anthers and stigma within each flower by subtracting the height of the higher organ from the height of the lower organ (i.e. herkogamy). Some of the pollen data from EL12 were reanalysed to answer question C above.

Data analyses

We used a Poisson regression to analyse correlations between self-pollination and anther–stigma distance, with *number of pollen grains* as the response variable and *anther–stigma distance* (quantitative) and *morph type* (categorical) and their interaction as predictors. Furthermore, we tested whether mean anther–stigma distance generally differed between morphs by fitting a GLMM with *morph type* (L- vs. S-morph) as main effect. We used *flower provenance* and *plant identity* nested within *flower provenance* as random effects to account for variation between flower origins and hierarchical data structure in both models.

(E) DOES INCOMPATIBLE POLLEN ON A STIGMA DECREASE TOTAL SEED SET?

Experimental set-up and data collection

In spring 2011, we performed hand-pollination experiments on the same plants of *P. vulgaris* as in A and B (see above). We used freshly opened flowers from each plant to perform the following manual pollination treatments: stigma pollinated with (i) compatible pollen of the reciprocal morph; (ii) incompatible pollen of the same morph; (iii) incompatible pollen from the same flower; and (iv) a mixture of pollen from the same and the reciprocal morphs. Mixed pollen loads were obtained by applying (i) both pollen types simultaneously, (ii) compatible pollen first and incompatible pollen two hours later and (iii) incompatible pollen first and compatible pollen two hours later. Every pollination treatment was repeated one to three times, for a total of 18 manually pollinated flowers per plant and 180 hand pollinations (divided between 5 L- and 5 S-plants). For the self-pollination treatment, we used intact flowers; all other flowers were emasculated as described earlier. On each experimental day, newly harvested pollen from at least five flowers per morph (i.e. one flower per plant) was collected and applied with fine kolinsky-hair brushes on receptive stigmas. Self-pollen was applied by gently dabbing anthers on the stigma of the same flower. To check for accidental cross-pollinations, we included one negative control (no manual pollination) per plant. To avoid seed loss, we counted seeds from well-developed capsules that were collected before they opened.

Data analyses

We used a GLMM to test whether total seed set differed between morphs and treatments. Differences in seed set between inter-morph pollinations (reference category) and intra-morph pollinations, self-pollinations and each of the three mixed pollinations were assessed with simple contrasts. As fixed effects we used *pollination treatment*, *morph type* and their interaction. We used *plant identity* as a random effect to account for hierarchical data structure.

Results

(A) IS MORE POLLEN DELIVERED TO RECIPROCAL THAN TO NON-RECIPROCAL STIGMAS?

A few runs were excluded from the analyses because recipient flowers had shrivelled styles. The final number of analysed runs was thus 18 in *P. elatior* (10 runs with L-pollen donors and eight runs with S-pollen donors) and 23 in *P. vulgaris* (12 runs with L-pollen donors and 11 runs with S-pollen donors). Statistical models for pollen-count data (i.e. pollen transfer) and anther–stigma distances had significant three-way interactions (see Table S1 in Supporting Information), justifying the execution of subsequent contrast tests.

Overall, anthers of the two species exported similar numbers of pollen grains to recipient flowers (41 109 vs. 50 902 in *P. elatior* and *P. vulgaris*, respectively; $F_{1,37} = 1{\cdot}3$, $P = 0{\cdot}262$; see Table S1A, for all GLMM results). Low anthers, on average, exported more pollen grains (23 487 vs. 28 020 for *P. elatior* and *P. vulgaris*, respectively) than high anthers (17 622 vs. 22 882 for *P. elatior* and *P. vulgaris*, respectively; $F_{1,37} = 0{\cdot}78$, $P = 0{\cdot}002$; Fig. 2a). Stigmas of L-flowers captured 1·7 and 3·9 times more pollen per visit than stigmas of S-flowers in *P. elatior* and in

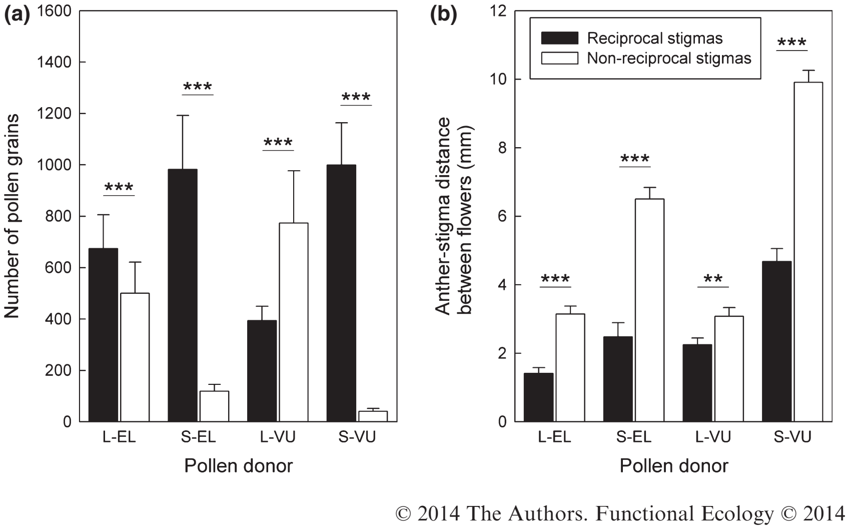

Fig. 2. Mean values and standard errors of (a) number of pollen grains transferred by *Anthophora plumipes* bees to reciprocal (black) and non-reciprocal stigmas (white) and (b) anther–stigma distances between reciprocal and non-reciprocal sexual organs (absolute values) for long-styled (L) pollen donors and short-styled (S) pollen donors of *Primula elatior* (EL) and *P. vulgaris* (VU). Results from comparisons between reciprocal and non-reciprocal sexual organs: $^{***}P \le 0{\cdot}001$ and $^{**}P \le 0{\cdot}01$. Sequential Bonferroni correction was used to account for multiple tests.

P. vulgaris, respectively. As expected (Fig. 1a), more pollen was transferred between reciprocal than between non-reciprocal organs, with the following exception: in *P. vulgaris*, anthers of L-flowers exported significantly more pollen to non-reciprocal than to reciprocal stigmas (Fig. 2a). High anthers exported almost all pollen to compatible stigmas and only a small fraction of pollen to incompatible stigmas (*P. elatior*: 11%; *P. vulgaris*: 4%; Fig. 2a). Low anthers, however, exported a large fraction of pollen to incompatible stigmas (*P. elatior*: 43%; *P. vulgaris*: 66%). Thus, pollen of S-flowers had a 2·3–8·1 times higher probability of reaching a reciprocal stigma than pollen of L-flowers (Table 2). Pollen from L-flowers was 2·6–8·8 times more likely to be transferred to a non-reciprocal stigma than pollen from S-flowers. As expected for distylous species, anther–stigma distances were significantly smaller between reciprocal than between non-reciprocal organs (Fig. 2b). Furthermore, anther–stigma distances between both reciprocal flowers and non-reciprocal flowers were generally smaller for L-pollen donors than for S-pollen donors (reciprocal organs: effect size = −1·74 ± 0·37 [mean ± standard error]; $t = -4{\cdot}75$, $P < 0{\cdot}001$; non-reciprocal organs: effect size = −5·1 ± 0·37; $t = -13{\cdot}87$, $P < 0{\cdot}001$). However, the difference between the two pollen donors was smaller for reciprocal than for non-reciprocal organs.

(B) ARE POLLEN GRAINS FROM L- AND S-FLOWERS DIFFERENTIALLY PLACED ON POLLINATOR BODIES?

Bees picked up 67 013 pollen grains in total: 36 091 pollen grains from L-flowers and 30 922 from S-flowers, indicating that more pollen was transferred to the bees from low than from high anthers ($F_{1,18} = 2829{\cdot}03$, $P \leq 0{\cdot}001$; see Table S2 in Supporting Information for all GLMM results). This result was expected, for low anthers produce more, smaller pollen grains, while high anthers produce fewer, bigger pollen grains in distylous species (Dulberger 1992). The number of pollen grains collected by the bees varied among insects, depending on the morph of the flower visited last, and whether bees walked (Bee02, Bee03, BeeS and BeeL) or flew between flowers (Bee01; Fig. 3a). However, regardless of how bees moved between flowers, more pollen was deposited on the proximal and middle pollen-carrying parts of the bees than on the distal part (Fig. 3b). Pollen of L- and S-flowers was placed on all three body parts (i.e. mixed pollen loads on the bees), but in different proportions. Significantly more pollen was transferred from low anthers to the middle than to the proximal (effect size = −0·77 ± 0·01, $t = -63{\cdot}31$, $P \leq 0{\cdot}001$) and distal parts of the pollen-carrying structures (effect size = −1·57 ± 0·02, $t = -95{\cdot}7$, $P \leq 0{\cdot}001$). Significantly more pollen was transferred from high anthers to the proximal than to the middle (effect size = −0·53 ± 0·01, $t = -44{\cdot}22$, $P \leq 0{\cdot}001$) and distal parts of the pollen-carrying structures (effect size = −2·7 ± 0·03, $t = -92{\cdot}62$, $P \leq 0{\cdot}001$). Therefore, proportionally more pollen from low than from high anthers occurred on distal (effect size = 1026·25 ± 833·63, $t = 4{\cdot}6$, $P = 0{\cdot}010$) and middle pollen-carrying structures (effect size = 3847·75 ± 833·63, $t = 4{\cdot}62$, $P = 0{\cdot}010$), while more pollen from high than from low anthers occurred on proximal pollen-carrying structures (effect size = −1196·03 ± 262·89, $t = -4{\cdot}55$, $P = 0{\cdot}009$; Fig. 3b), as expected.

Table 2. Probability of an individual pollen grain to be exported by long (L)- and short-styled (S) flowers to a reciprocal and non-reciprocal stigma in *Primula elatior* (EL) and *P. vulgaris* (VU). Probabilities were calculated after Lloyd & Webb (1992); see text using our experimental data of pollen transfer and previously published data on number of pollen grains produced per flower (Reference). All values are multiplied by 10^{-3}

		Stigma type		
	Species	Reciprocal	Non-reciprocal	Reference
L-flowers	EL	4·86	3·60	Schou (1983)
	VU	1·39	2·73	Ornduff (1979)
		2·15	4·22	Piper & Charlesworth (1986)
S-flowers	EL	11·34	1·37	Schou (1983)
	VU	11·22	0·46	Ornduff (1979)
		11·64	0·48	Piper & Charlesworth (1986)

(C) IS MORE POLLEN TRANSFERRED WITHIN FLOWERS THAN BETWEEN FLOWERS OF THE SAME MORPH?

The final number of stigma pairs (intra-floral vs. intra-morph) analysed in 2012 for *P. elatior* was 19, with 10 pairs for L-pollen donors and nine pairs for S-pollen donors. Overall, anthers of L-flowers exported more pollen than anthers of S-flowers ($F_{1,17} = 38{\cdot}02$; $P \leq 0{\cdot}001$; Fig. 4a; see also Fig. 2a), with 35 561 pollen grains transported to stigmas of L-flowers and 5 819 pollen grains to stigmas of S-flowers. On average, significantly more pollen grains were transferred within a flower (28 371 vs. 4 829 for L- and S-flowers, respectively) than between flowers of the same morph (7 190 vs. 990 for L- and S-flowers, respectively; $F_{1,34} = 6\,285{\cdot}26$; $P \leq 0{\cdot}001$; Fig. 4a). A significant interaction ($F_{1,34} = 32{\cdot}3$; $P \leq 0{\cdot}001$) indicated that the difference between intra-floral and intra-morph pollen transfer was larger in L-flowers than in S-flowers. Consequently, self-pollination was overall significantly higher in L-flowers than in S-flowers (effect size = −3·40 ± 0·34, $t = -10{\cdot}06$, $P \leq 0{\cdot}001$; Fig. 4b; see also D below).

(D) ARE HERKOGAMY AND SELF-POLLINATION INVERSELY CORRELATED?

A few flowers were excluded from the analyses because they had shrivelled styles. The final number of analysed flowers was 40 L- and 39 S-flowers in 2009 and 2012 for

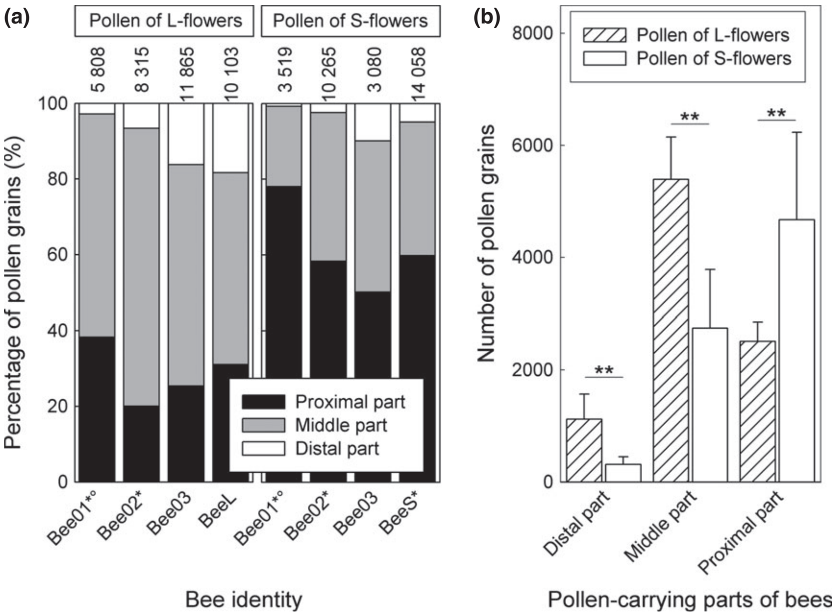

Fig. 3. Stratification of pollen from long-styled (L) and short-styled (S) flowers on three different pollen-carrying parts of *Anthophora plumipes* males visiting flowers of *Primula vulgaris* (see Fig. 1b). Occurrence of pollen grains on distal-, middle- and proximal pollen-carrying parts for individual bees (a) and mean values with standard errors (b). Numbers on top of a bar indicate total number of deposited pollen grains. Bee01, Bee02 and Bee03 visited a series of L- and S-flowers in alternating order; BeeS visited a series of S-flowers, and BeeL a series of L-flowers. Asterisks indicate bees that visited an S-flower as last flower; the open circle indicates a bee that flew, rather than walked, between flowers. Results from comparisons between number of pollen grains transferred from low and high anthers of L- and S-flowers, respectively, on the distal, middle and proximal body parts of the bees: $^{**}P \leq 0{\cdot}01$. Sequential Bonferroni correction was used to account for multiple tests.

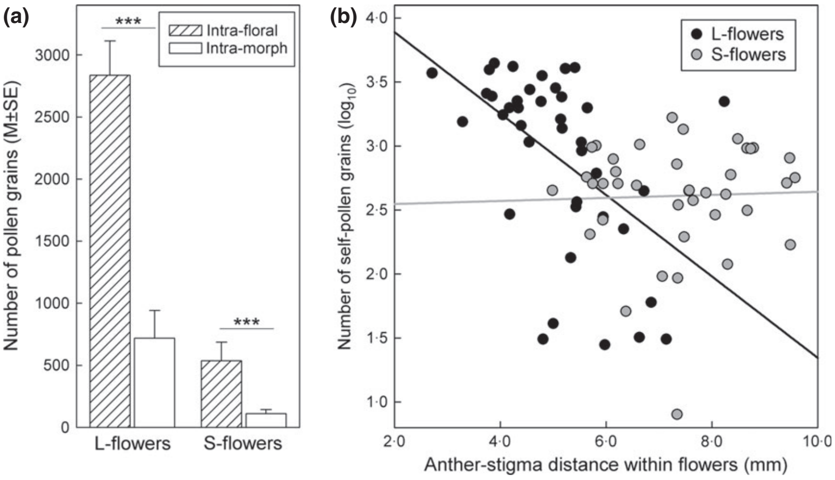

Fig. 4. Mean values (M) and standard errors (SE) for the number of pollen grains transported within flowers (intra-floral) and between flowers of the same morph (intra-morph) in long-styled (L) and short-styled (S) flowers of *Primula elatior* (a; results of the experiment performed in 2012; significance values: $^{***}P \leq 0{\cdot}001$) and correlations between number of pollen grains transported within flower and intra-floral anther–stigma distance (b; lines represent linear regressions; for results of the Poisson regressions from experiments performed in 2009 and 2012, see text). Sequential Bonferroni correction was used to account for multiple tests

P. elatior only. Overall, 67 334 and 21 979 self-pollen grains were transported to stigmas of L-flowers and S-flowers, respectively. S-flowers had generally larger anther–stigma distances than L-flowers (effect size = 2·25 ± 0·22, $t = 10{\cdot}14$, $P \leq 0{\cdot}001$; Fig. 4b). Self-pollination was inversely correlated with anther–stigma distance in L-flowers, as expected (coefficient = −0·37 ± 0·02 [mean ± standard error], $t = -24{\cdot}58$, $P \leq 0{\cdot}001$; Fig. 4b). In S-flowers, however, self-pollination was positively correlated with anther–stigma distance (coefficient = +0·52 ± 0·02, $t = 21{\cdot}7$, $P \leq 0{\cdot}001$), thus contradicting our expectations.

(E) DOES INCOMPATIBLE POLLEN ON A STIGMA DECREASE TOTAL SEED SET?

Seed set varied significantly among the six pollination treatments ($F_{5,95} = 93{\cdot}1$; $P \leq 0{\cdot}001$), but not significantly between the two morphs ($F_{5,95} = 0{\cdot}59$; $P = 0{\cdot}445$). Seed set was low after intra-morph and self-pollinations in both morphs (Fig. 5). Seed set after mixed pollinations was high, with the following exception: when incompatible pollen was applied two hours before compatible pollen in L-flowers, then seed set was significantly lower than after inter-morph pollinations (Fig. 5a). This effect did not

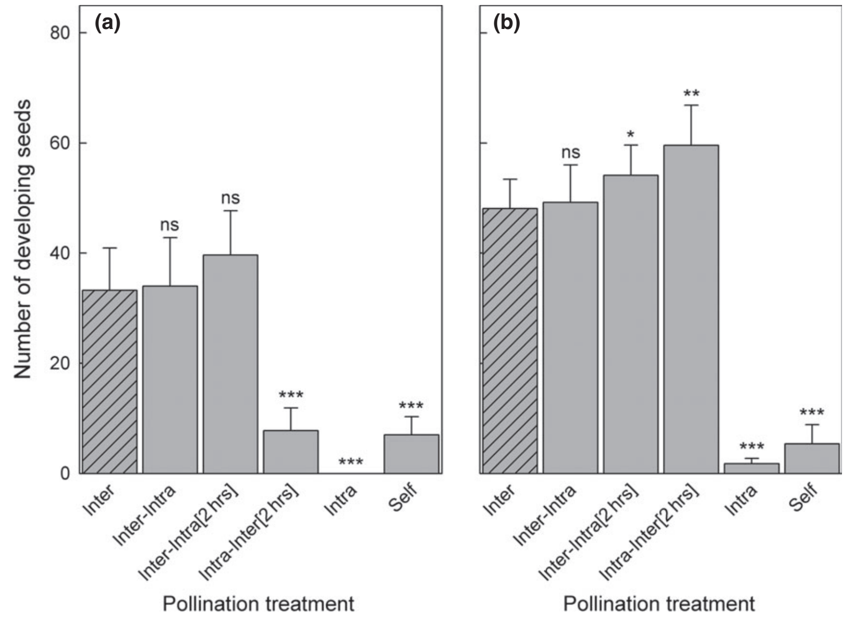

Fig. 5. Mean seed set and standard errors of (a) long-styled and (b) short-styled morphs of *Primula vulgaris* after inter-morph (Inter), intra-morph (Intra), intra-flower (Self) and mixed pollinations. In mixed pollinations, both inter-morph and intra-morph pollinations were performed on the same stigma. Mixed pollen loads were obtained by applying (i) both pollen types simultaneously (Inter-Intra), (ii) first compatible pollen and then two hours later incompatible pollen (Inter-Intra[2 hr]) and (iii) first incompatible pollen and then two hours later compatible pollen (Intra-Inter [2 hr]). We compared seed set after inter-morph pollination (Inter; most left hatched bar) with each of the other pollination treatments to test whether incompatible pollen had a negative effect on seed set: significance levels: $***P \leq 0{\cdot}001$, $**P \leq 0{\cdot}01$, $*P \leq 0{\cdot}05$, or not significantly different (ns). Sequential Bonferroni correction was used to account for multiple tests.

occur in S-flowers (Fig. 5b), which most likely caused the significant *treatment* × *morph* interaction ($F_{5,95} = 32{\cdot}63$; $P \leq 0{\cdot}001$). Finally, none of the unpollinated flowers set seeds (negative control), so unintended pollinations are unlikely.

Discussion

Here, we investigate the functional roles of disassortative pollination and sexual interference under controlled experimental conditions in two distylous species of primroses that are closely related to each other. We used between five and 18 bees of the hairy-footed flower bee (*Anthophora plumipes*) as pollen vectors. This common visitor of both *P. elatior* and *P. vulgaris* represents the long-tongued bee pollinator-type of both plant species. Although it would be ideal to use more *Anthophora* bees and consider additional primrose-visiting insects in the experiments, our results represent significant progress in understanding the ecological functioning of heterostyly. Our experiments demonstrate that the reciprocal spatial separation of anthers and stigmas and the self-/intra-morph incompatibility system of distyly are working in concert to optimize cross-pollination and minimize sexual interference. Importantly, the main patterns of pollen-transfer probability estimated from our experiments (Table 2) resemble those found in natural populations of *P. vulgaris* and other distylous species (reviewed by Stone & Thomson 1994), providing external corroboration for the overall soundness of our approach.

DISASSORTATIVE POLLINATION

Pollen transfer in distylous species is considered disassortative when more pollen is exported to reciprocal than to non-reciprocal stigmas (e.g. Ganders 1974; Fig. 1a). Therefore, the disassortativeness of pollen movement can be increased by increasing pollen export to reciprocal stigmas, restricting pollen transfer to non-reciprocal stigmas, or both. In the species of *Primula* tested in our study, high anthers exported pollen more disassortatively than low anthers, that is, they transported both more pollen to reciprocal stigmas and less pollen to non-reciprocal stigmas than low anthers (Fig. 2a). Such asymmetries between the two morphs have been documented in other distylous species (e.g. Stone & Thomson 1994; Matsumura & Washitani 2002; Ornelas *et al.* 2004), indicating that exposed male organs are more efficient at exporting pollen than organs hidden in the corolla tube and that exposed female organs are more efficient at capturing pollen than sunken organs.

The observed asymmetries of pollen transfer between high and low sexual organs of distylous flowers can be explained in terms of both flower–flower and flower–pollinator interactions. Promotion of inter- over intra-morph pollen transfer in distylous species partially depends on spatial proximity of reciprocal sexual organs vs. distance between non-reciprocal organs (Barrett 2002a; Barrett & Shore 2008). Empirically, this was demonstrated by Baena-Díaz *et al.* (2012), who found a significant relationship between pollen-export efficacy and anther–stigma separation in their study of the evolutionary transition from tristyly to distyly in *Oxalis alpina*. In *P. elatior* and *P. vulgaris*, anther–stigma distances were generally smaller between reciprocal than between non-reciprocal organs (Fig. 2b), thus meeting theoretical predictions for disassortative pollination. Yet, only high anthers exported the vast majority of pollen (*P. elatior*: 89%; *P. vulgaris*: 96%) to the spatially closer, reciprocal stigmas, while low anthers exported 43% (*P. elatior*) and 66% (*P. vulgaris*) of all pollen to the more distant, non-reciprocal stigmas (Fig. 2). This asymmetry between the two pollen donors could be explained either by smaller anther–stigma distances between high than between low reciprocal organs or by larger anther–stigma distances between high anthers and low stigmas than between low anthers and high stigmas, or both. In *Primula*, low organs are spatially closer than high organs (see also Fig. 2b), thus contradicting the first expectation above. Conversely, low anthers are spatially closer to high stigmas than high anthers to low stigmas, thus confirming the second expectation above. Consequently, the distance between non-reciprocal organs, rather than proximity between reciprocal organs, better explains the unequal performance of high and low anthers as pollen donors.

The morphological fit between flowers and pollinators interacts with the spatial correspondence between sexual organs and pollen production in the anthers in shaping the mechanics of pollen movement (e.g. Armbruster *et al.* 2009). The process of pollen transfer can be divided into two steps: pollen placement from anthers onto the pollinator's body and pollen delivery from the pollinator's body to the receiving stigma. In *P. vulgaris,* the significantly higher amount of pollen placed onto pollinator's bodies from low than from high anthers and the globally higher amount of pollen transported from low anthers to recipient stigmas (Fig. 2) may reflect the fact that low anthers produce 2·1–3·2 times more pollen than high anthers (Ornduff 1979; Piper & Charlesworth 1986). It has been suggested that the higher production of pollen in anthers placed deeply in the corolla tube may represent an adaptation to flower–pollinator interaction (Ganders 1979; Dulberger 1992). Indeed, the size and shape of both flowers and pollinators determine how deeply insects can probe flowers for nectar and on which body parts pollen is deposited (e.g. Armbruster *et al.* 2009). Many insect-pollinated – including distylous – species have rather narrow corolla tubes that constrain how the pollinator interacts with the flower (e.g. Massinga, Johnson & Harder 2005; Brys *et al.* 2008; Ferrero *et al.* 2011). The deeper a pollinator probes a tubular flower, the more pollen it extracts from the anthers (Campbell, Waser & Price 1996). Consequently, pollinators might be able to extract less pollen from low than from high anthers of distylous species. In our case study, the higher production of pollen in low anthers more than compensates for potentially reduced pollen extraction during floral visitation, for bees end up transferring significantly more pollen from low than from high anthers (Figs 2 and 3).

While narrow and long corolla tubes are thought to constrain the way pollinators approach and probe flowers, thus promoting more precise pollen transfer from anthers to pollinator to stigma, this same floral morphology may also cause the smearing and dislocation of pollen onto parts of the pollinator's body that slide past floral sexual organs during probing, especially for organs placed at the mouth of the corolla tube (Armbruster *et al.* 2006, 2009). Indeed, it has been proposed that this 'smearing effect' might be especially marked for high anthers during pollen pickup and for high stigmas during pollen delivery, thus causing asymmetries in pollen-transfer efficacy between morphs, with high organs being less disassortative (Armbruster *et al.* 2006, 2009). However, our results do not support this hypothesis, for we find that most pollen of both *P. vulgaris* morphs is placed onto parts of the pollinator's body that correspond to the position of the anthers in pollen-donor flowers (Fig. 3a). In fact, more pollen from high than from low anthers occurred on proximal pollen-carrying structures of the bees, and more pollen from low than from high anthers occurred on middle and distal pollen-carrying structures of the bees (Fig. 3b). Our results corroborate the findings of other studies of pollen stratification on heads and proboscises of long-tongued bees that visit distylous species with comparable floral morphologies (Olesen 1979; Washitani *et al.* 1995; Brys *et al.* 2008), although the degree of segregation of the two pollen types depended strongly on proboscis length (Brys *et al.* 2008). In summary, we find that pollen transfer is globally more disassortative between high than between low sexual organs of distylous flowers (Table 2; Fig. 2a), contrary to some theoretical predictions (Armbruster *et al.* 2006, 2009), but in accord with experimental results in other distylous species (Olesen 1979; Washitani *et al.* 1995; Brys *et al.* 2008).

As seen above, our results conform to the predictions of the disassortative-pollination hypothesis in both floral morphs of *P. elatior* and in S-flowers of *P. vulgaris*. However, the L-flowers of *P. vulgaris* do not disperse pollen disassortatively, for low anthers of *P. vulgaris* export more pollen to non-reciprocal than to reciprocal stigmas (Fig. 2a). The locations of reciprocal and non-reciprocal sexual organs help to explain this exception. The difference between the spatial proximity of non-reciprocal vs. reciprocal sexual organs is smaller when low anthers of *P. vulgaris* are compared with high and low stigmas, respectively, of the same species than in any other comparisons (Fig. 2b). This small difference in the degree of spatial matching between reciprocal and non-reciprocal sexual organs may be insufficient to favour pollen movement towards reciprocal, compatible stigmas, overall decreasing male function in *P. vulgaris*, with potentially critical ecological consequences. Given the lack of disassortativeness of low sexual organs in *P. vulgaris*, reproductive output is expected to be lower in this species than in *P. elatior,* if compatible pollen is limited. Pollen can become a limiting factor for reproduction in heterostylous primroses when pollinator service (e.g. Piper & Charlesworth 1986) and/or compatible mating partners are scarce, as in small populations (Ågren 1996; Waites & Ågren 2004). Additionally, small populations of heterostylous species often have skewed morph ratios, further reducing the number of compatible mates (e.g. Kéry, Matthies & Schmid 2003; Brys, Jacquemyn & Hermy 2007; Brys, Jacquemyn & Beeckman 2008). Indeed, insufficient pollinator services have been documented in *P. vulgaris* (Piper & Charlesworth 1986), and small populations of both species are known to suffer from low reproductive output (*P. elatior*: Jacquemyn, Brys

& Hermy 2001, 2002; *P. vulgaris*: Brys *et al.* 2004, 2007). Considering the lower male component of fitness of long-styled flowers of *P. vulgaris* documented in our study (Fig. 2a), small populations of this species may be more vulnerable to reproductive failure and stochastic population fluctuations than those of *P. elatior*, if pollinator service becomes unreliable and/or compatible mates are rare.

SEXUAL INTERFERENCE

Interference between male (pollen production and dispersal) and female (pollen receipt and seed set) functions represents a central problem in reproduction for hermaphroditic plants, especially if species are self-incompatible, and various forms of herkogamy have evolved to resolve it (e.g. Webb & Lloyd 1986; Barrett, Jesson & Baker 2000). In herkogamous, monomorphic flowers, a conflict ensues between avoidance of sexual interference (requiring increased separation of anthers and stigmas within flowers) and optimization of cross-pollination (requiring enhanced spatial fit of anthers and stigmas between flowers). This problem is thought to be largely solved in heterostylous species *via* the optimization of both morphological (i.e. reciprocal positions of sexual organs) and physiological (i.e. self- and intra-morph pollen incompatibility) interactions (e.g. Barrett 2002a). Indeed, our results show that distyly reduces sexual interference (Figs 2, 4–5). Female function is optimized *via* disassortative pollination transferring most pollen to reciprocal stigmas, as discussed above (Fig. 2a), and by the effectiveness of the self- and intra-morph incompatibility system in limiting seed production after intra-floral or intra-morph pollinations (Fig. 5). Our results also show that pollen incompatibility hardly limits seed production after pollinations with mixtures of compatible and incompatible pollen (Fig. 5), confirming the findings of studies on other heterostylous species (Shore & Barrett 1984; Barrett & Glover 1985; Nicholls 1987; Nishihiro & Washitani 1998). Male function is primarily optimized through the spatial separation of anthers and stigmas between non-reciprocal flowers (Fig. 2b), as indicated by significantly lower pollen transfer between flowers of the same morph than within the same flower (Fig. 4a), also confirming earlier studies (Ganders 1979; Piper & Charlesworth 1986).

Anther–stigma separation is thought to decrease both autonomous and facilitated self-pollen deposition in all forms of herkogamy (Webb & Lloyd 1986). For example, in the homostylous, self-compatible *Primula halleri,* De Vos *et al.* (2012) demonstrated a negative correlation between herkogamy and selfed seed set. In the non-distylous, herkogamous flowers of *Ipomoea wolcottina* (Parra-Tabla & Bullock 2005) and *Erythronium grandiflorum* (Thomson & Stratton 1985), which are structurally similar to L-flowers of distylous species, anther–stigma distance was negatively correlated with self-pollen deposition. However, to our knowledge, no study prior to ours had empirically investigated the correlation between herkogamy and self-pollen deposition in distylous species (Fig. 4b). Our results confirm the expected inverse relationship between herkogamy and self-pollination for L-, but not for S-flowers (Fig. 4b; Webb & Lloyd 1986). At the same time, anther–stigma distance is significantly larger in S- than in L-flowers (Fig. 4b), likely explaining the significantly lower self-pollen transfer in S-flowers (Fig. 4b), despite the lack of a negative correlation between herkogamy and self-pollination in the latter (Fig. 4b). The difference in correlation between herkogamy and self-pollination detected for L- vs. S-flowers might be explained in terms of a 'threshold' effect inherent to floral structure. Indeed, the distance between sexual organs in S-flowers might exceed a certain threshold, beyond which more separation does not further decrease deposition of self-pollen on the stigma. Furthermore, the different dynamics of pollen transfer from flower to pollinator and insect probing-behaviour in S- vs. L-flowers might also contribute to lower levels of self-pollination in the former vs. the latter. In summary, our results demonstrate that reciprocal herkogamy reduces self-interference by optimizing pollen transfer to compatible stigmas (Fig. 2) and minimizing self- and intra-morph pollination, especially in S-flowers (Fig. 4), while pollen incompatibility mechanisms reduce seed set from self- and intra-morph pollinations (Fig. 5).

Conclusion

The results of our study allow us to confirm the general predictions of both disassortative-pollination and sexual-interference hypotheses, while explaining exceptions and specific details of asymmetric patterns in the mechanics of heterostyly. By experimentally demonstrating that heterostyly functions as expected, our findings also suggest that the conditions for the maintenance of this floral polymorphism, as outlined by Barrett (2002a), are met. Further studies are underway to investigate whether the dynamics of pollen movement in heterostylous species might also contribute to reproductive isolation (Haller *et al.* unpublished data; Keller *et al.* unpublished data).

Acknowledgements

We thank the Georges and Antoine Claraz-Schenkung for financial support; Barbara Weigelt and Manuel Rey for describing the precise functioning of the mouth parts of *Anthophora plumipes* bees; the Institute of Plant Biology for the use of their slide scanning microscope; and the reviewers and associate editor for their valuable comments.

Data accessibility

The raw data of all experiments are deposited in the Dryad repository: http://doi.org/10·5061/dryad.mn6np (Keller *et al.* 2014).

References

Ågren, J. (1996) Population size, pollinator limitation, and seed set in the self-incompatible herb *Lythrum salicaria*. *Ecology*, **77**, 1779–1790.

Armbruster, W.S., Perez-Barrales, R., Arroyo, J., Edwards, M.E. & Vargas, P. (2006) Three-dimensional reciprocity of floral morphs in wild flax

(*Linum suffruticosum*): a new twist on heterostyly. *New Phytologist*, **171**, 581–590.

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

Baena-Díaz, F., Fornoni, J., Sosenski, P., Molina-Freaner, F.E., Weller, S.G., Pérez-Ishiwara, R. *et al.* (2012) Changes in reciprocal herkogamy during the tristyly–distyly transition in *Oxalis alpina* increase efficiency in pollen transfer. *Journal of Evolutionary Biology*, **25**, 574–583.

Barrett, S.C.H. (1992) *Evolution and Function of Heterostyly*. Springer-Verlag, Berlin.

Barrett, S.C.H. (2002a) The evolution of plant sexual diversity. *Nature Reviews Genetics*, **3**, 274–284.

Barrett, S.C.H. (2002b) Sexual interference of the floral kind. *Heredity*, **88**, 154–159.

Barrett, S.C.H. (2003) Mating strategies in flowering plants: the outcrossing-selfing paradigm and beyond. *Philosophical Transactions of the Royal Society B: Biological Sciences*, **358**, 991–1004.

Barrett, S.C.H. & Glover, D.E. (1985) On the Darwinian hypothesis of the adaptive significance of tristyly. *Evolution*, **39**, 766–774.

Barrett, S.C.H., Jesson, L.K. & Baker, A.M. (2000) The evolution and function of stylar polymorphisms in flowering plants. *Annals of Botany*, **85**, 253–265.

Barrett, S.C.H. & Shore, J.S. (2008) New insights on heterostyly: comparative biology, ecology and genetics. *Self-incompatibility in Flowering Plants – Evolution, Diversity, and Mechanisms* (ed. V.E. Franklin-Tong), pp. 3–32. Springer-Verlag, Berlin.

Björkman, T. (1995) The effectiveness of heterostyly in preventing illegitimate pollination in dish-shaped flowers. *Sexual Plant Reproduction*, **V8**, 143–146.

Brys, R. & Jacquemyn, H. (2010) Floral display size and spatial distribution of potential mates affect pollen deposition and female reproductive success in distylous *Pulmonaria officinalis* (*Boraginaceae*). *Plant Biology*, **12**, 597–603.

Brys, R., Jacquemyn, H. & Beeckman, T. (2008) Morph-ratio variation, population size and female reproductive success in distylous *Pulmonaria officinalis* (*Boraginaceae*). *Journal of Evolutionary Biology*, **21**, 1281–1289.

Brys, R., Jacquemyn, H. & Hermy, M. (2007) Impact of mate availability, population size, and spatial aggregation of morphs on sexual reproduction in a distylous, aquatic plant. *American Journal of Botany*, **94**, 119–127.

Brys, R., Jacquemyn, H., Endels, P., van Rossum, F., Hermy, M., Triest, L. *et al.* (2004) Reduced reproductive success in small populations of the self-incompatible *Primula vulgaris*. *Journal of Ecology*, **92**, 5–14.

Brys, R., Jacquemyn, H., De Bruyn, L. & Hermy, M. (2007) Pollination success and reproductive output in experimental populations of the self-incompatible *Primula vulgaris*. *International Journal of Plant Sciences*, **168**, 571–578.

Brys, R., Jacquemyn, H., Hermy, M. & Beeckman, T. (2008) Pollen deposition rates and the functioning of distyly in the perennial *Pulmonaria officinalis* (*Boraginaceae*). *Plant Systematics and Evolution*, **273**, 1–12.

Campbell, D.R., Waser, N.M. & Price, M.V. (1996) Mechanisms of hummingbird-mediated selection for flower width in *Ipomopsis aggregata*. *Ecology*, **77**, 1463–1472.

Charlesworth, D. & Charlesworth, B. (1987) Inbreeding depression and its evolutionary consequences. *Annual Review of Ecology and Systematics*, **18**, 237–268.

Crane, P.R., Friis, E.M. & Pedersen, K.R. (1995) The origin and early diversification of angiosperms. *Nature*, **374**, 27–33.

Dafni, A., Kevan, P.G. & Husband, B.C. (2005) *Practical Pollination Biology*. Enviroquest, Cambridge, Ontario.

Darwin, C. (1862) On the two forms, or dimorphic condition, in the species of *Primula*, and on their remarkable sexual relations. *Journal of the Linnean Society (Botany)*, **6**, 77–96.

Darwin, C. (1877) *The Different Forms of Flowers on Plants of the Same Species*. Murray, London.

De Vos, J.M., Keller, B., Isham, S.T., Kelso, S. & Conti, E. (2012) Reproductive implications of herkogamy in homostylous primroses: variation during anthesis and reproductive assurance in alpine environments. *Functional Ecology*, **26**, 854–865.

Dulberger, R. (1992) Floral polymorphisms and their functional significances in the heterostylous syndrome. *Evolution and Function of Heterostyly* (ed. S.C.H. Barrett), pp. 41–84. Springer-Verlag, Berlin.

Ferrero, V., Castro, S., Sanchez, J.M. & Navarro, L. (2011) Stigma-anther reciprocity, pollinators, and pollen transfer efficiency in populations of heterostylous species of *Lithodora* and *Glandora* (*Boraginaceae*). *Plant Systematics and Evolution*, **291**, 267–276.

Ganders, F.R. (1974) Disassortative pollination in distylous plant *Jepsonia heterandra*. *Canadian Journal of Botany-Revue Canadienne De Botanique*, **52**, 2401–2406.

Ganders, F.R. (1976) Pollen flow in distylous populations of *Amsinckia* (*Boraginaceae*). *Canadian Journal of Botany-Revue Canadienne De Botanique*, **54**, 2530–2535.

Ganders, F.R. (1979) The biology of heterostyly. *New Zealand Journal of Botany*, **17**, 607–635.

Hernández, A. & Ornelas, J.F. (2007) Disassortative pollen transfer in distylous *Palicourea padifolia* (*Rubiaceae*), a hummingbird-pollinated shrub. *Ecoscience*, **14**, 8–16.

Jacquemyn, H., Brys, R. & Hermy, M. (2001) Within and between plant variation in seed number, seed mass and germinability of *Primula elatior*: effect of population size. *Plant Biology*, **56**, 1–568.

Jacquemyn, H., Brys, R. & Hermy, M. (2002) Patch occupancy, population size and reproductive success of a forest herb (*Primula elatior*) in a fragmented landscape. *Oecologia*, **130**, 617–625.

Keller, B., de Vos, J.M. & Conti, E. (2012) Decrease of sexual organ reciprocity between heterostylous primrose species, with possible functional and evolutionary implications. *Annals of Botany*, **110**, 1233–1244.

Keller, B., Thomson, J.D. & Conti, E. (2014) Data from: Heterostyly promotes disassortative pollination and reduces sexual interference in Darwin's primroses: evidence from experimental studies. *Dryad Digital Repository*. http://doi.org/10.5061/dryad.mn6np.

Kéry, M., Matthies, D. & Schmid, B. (2003) Demographic stochasticity in population fragments of the declining distylous perennial *Primula veris* (*Primulaceae*). *Basic and Applied Ecology*, **4**, 197–206.

Lau, P. & Bosque, C. (2003) Pollen flow in the distylous *Palicourea fendleri* (*Rubiaceae*): an experimental test of the disassortative pollen flow hypothesis. *Oecologia*, **135**, 593–600.

Levin, D.A. (1968) The breeding system of *Lithospermum caroliniense*: adaptation and counteradaptation. *The American Naturalist*, **102**, 427–441.

Lloyd, D.G. & Webb, C.J. (1986) The avoidance of interference between the presentation of pollen and stigmas in angiosperms. 1. Dichogamy. *New Zealand Journal of Botany*, **24**, 135–162.

Lloyd, D.G. & Webb, C.J. (1992) The selection of heterostyly. *Evolution and Function of Heterostyly* (ed.S.C.H. Barrett), pp. 179–207. Springer-Verlag, Berlin.

Massinga, P.H., Johnson, S.D. & Harder, L.D. (2005) Heteromorphic incompatibility and efficiency of pollination in two distylous *Pentanisia* species (*Rubiaceae*). *Annals of Botany*, **95**, 389–399.

Mast, A.R., Kelso, S. & Conti, E. (2006) Are any primroses (*Primula*) primitively monomorphic? *New Phytologist*, **171**, 605–616.

Matsumura, C. & Washitani, I. (2002) Heterostylous morph differences in pollen transfer and deposition patterns in *Primula sieboldii* on a visitation by a queen bumblebee, measured with a semi-natural experimental system. *Plant Species Biology*, **17**, 1–12.

Meyer, B. (2007) Pollinator communities and plant-pollinator interactions in fragmented calcareous grasslands. PhD thesis, Georg-August-Universität, Göttingen.

Nicholls, M.S. (1986) Population composition, gender specialization, and the adaptive significance of distyly in *Linum perenne* (*Linaceae*). *New Phytologist*, **102**, 209–217.

Nicholls, M.S. (1987) Pollen flow, self-pollination and gender specialization: factors affecting seed-set in the tristylous species *Lythrum salicaria* (*Lythraceae*). *Plant Systematics and Evolution*, **156**, 151–157.

Nishihiro, J. & Washitani, I. (1998) Patterns and consequences of self-pollen deposition on stigmas in heterostylous *Persicaria japonica* (*Polygonaceae*). *American Journal of Botany*, **85**, 352–359.

Nishihiro, J., Washitani, I., Thomson, J.D. & Thomson, B.A. (2000) Patterns and consequences of stigma height variation in a natural population of a distylous plant, *Primula sieboldii*. *Functional Ecology*, **14**, 502–512.

Olesen, J.M. (1979) Floral morphology and pollen flow in the heterostylous species *Pulmonaria obscura* Dumort (*Boraginaceae*). *New Phytologist*, **82**, 757–767.

Ornduff, R. (1970) Incompatibility and the pollen economy of *Jepsonia parryi*. *American Journal of Botany*, **57**, 1036–1041.

Ornduff, R. (1979) Pollen flow in a population of *Primula vulgaris* Huds. *Botanical Journal of the Linnean Society*, **78**, 1–10.

Ornduff, R. (1980) Pollen flow in *Primula veris* (*Primulaceae*). *Plant Systematics and Evolution*, **135**, 89–93.

Ornelas, J.F., Jimenez, L., Gonzalez, C. & Hernandez, A. (2004) Reproductive ecology of distylous *Palicourea padifolia* (*Rubiaceae*) in a tropical montane cloud forest. I. Hummingbirds' effectiveness as pollen vectors. *American Journal of Botany*, **91**, 1052–1060.

Parra-Tabla, V. & Bullock, S.H. (2005) Ecological and selective effects of stigma-anther separation in the self-incompatible tropical tree *Ipomoea wolcottiana* (*Convolvulaceae*). *Plant Systematics and Evolution*, **252**, 85–95.

Piper, J. & Charlesworth, B. (1986) The evolution of distyly in *Primula vulgaris*. *Biological Journal of the Linnean Society*, **29**, 123–137.

Richards, A.J. (1997) *Plant Breeding Systems*, 2nd edn. Chapmann and Hall, London.

Richards, A.J. (2003) *Primula*, 2nd edn. Timber Press, Portland, Oregon.

Schmidt-Lebuhn, A.N., Keller, B., De Vos, J.M. & Conti, E. (2012) Phylogenetic analysis of *Primula* section *Primula* reveals rampant non-monophyly among morphologically distinct species. *Molecular Phylogenetics and Evolution*, **65**, 23–34.

Schou, O. (1983) The distyly in *Primula elatior* (L) Hill (*Primulaceae*), with a study of flowering phenology and pollen flow. *Botanical Journal of the Linnean Society*, **86**, 261–274.

Shore, J.S. & Barrett, S.C.H. (1984) The effect of pollination intensity and incompatible pollen on seed set in *Turnera ulmifolia* (*Turneraceae*). *Canadian Journal of Botany-Revue Canadienne De Botanique*, **62**, 1298–1303.

Stone, J.L. (1995) Pollen donation patterns in a tropical distylous shrub (*Psychotria suerrensis*; *Rubiaceae*). *American Journal of Botany*, **82**, 1390–1398.

Stone, J.L. (1996) Components of pollination effectiveness in *Psychotria suerrensis*, a tropical distylous shrub. *Oecologia*, **107**, 504–512.

Stone, J.L. & Thomson, J.D. (1994) The evolution of distyly: pollen transfer in artificial flowers. *Evolution*, **48**, 1595–1606.

Thomson, J.D. & Stratton, D.A. (1985) Floral morphology and cross-pollination in *Erythronium grandiflorum* (*Liliaceae*). *American Journal of Botany*, **72**, 433–437.

Valois-Cuesta, H., Soriano, P.J. & Ornelas, J.F. (2011) Asymmetrical legitimate pollination in distylous *Palicourea demissa* (*Rubiaceae*): the role of nectar production and pollinator visitation. *Journal of Tropical Ecology*, **27**, 393–404.

Waites, A.R. & Ågren, J. (2004) Pollinator visitation, stigmatic pollen loads and among-population variation in seed set in *Lythrum salicaria*. *Journal of Ecology*, **92**, 512–526.

Washitani, I., Kato, M., Nishihiro, J. & Suzuki, K. (1995) Importance of queen bumble bees as pollinators facilitating inter-morph crossing in *Primula sieboldii*. *Plant Species Biology*, **9**, 169–176.

Webb, C.J. & Lloyd, D.G. (1986) The avoidance of interference between the presentation of pollen and stigmas in angiosperms. 2. Herkogamy. *New Zealand Journal of Botany*, **24**, 163–178.

Wedderburn, F.M. & Richards, A.J. (1992) Secondary homostyly in *Primula* L.; evidence for the model of the 'S' supergene. *New Phytologist*, **121**, 649–655.

Weller, S.G. (1980) Pollen flow and fecundity in populations of *Lithospermum caroliniense*. *American Journal of Botany*, **67**, 1334–1341.

Wolfe, L.M., Massinga, P.H. & Johnson, S.D. (2009) A quantitative evaluation of the distylous syndrome in *Sebaea grandis* (*Gentianaceae*). *South African Journal of Botany*, **75**, 785–790.

Woodell, S.R.J. (1960) What pollinates primulas? *New Scientist*, **8**, 568–571.

Received 9 October 2013; accepted 4 March 2014
Handling Editor: Gaku Kudo

Supporting Information

Additional Supporting information may be found in the online version of this article:

Table S1. Results of generalized linear mixed effects models of (A) transferred pollen grains and (B) inter-floral anther-stigma distances between species, pollen donors, and stigma types.

Table S2. Result of generalized linear mixed effects models of pollen grains transferred to bees between pollen types and pollen-carrying parts of the bees.