**The impact of individual inaccuracy of reciprocal herkogamy on legitimate
pollen deposition and seed set in a distylous self-incompatible herb**

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Abstract

1. Heterostyly is a floral polymorphism that has been observed in at least 28 different plant families across the globe. Heterostyly is characterized by reciprocal arrangement of the anthers and stigmas and the presence of a heteromorphic self-incompatibility system, which together have been hypothesized to promote cross-pollination and to avoid interference of self-pollen and the deleterious effects of inbreeding.
2. Recent analyses have shown that the reciprocal positioning of the sexual organs in heterostylous plant species is not always perfect. Inaccuracies in the positioning of styles and anthers within populations are expected to affect both male and female fitness of plants by disturbing pollen uptake and stigmatic deposition by insects. However, at present little is known about how inaccuracies in the reciprocal positioning of the sexual organs affect stigmatic pollen deposition and subsequent seed set in heterostylous plant species.
3. In this study, we quantified the level of reciprocity in a grassland and forest population of the distylous herb *Primula veris* and tested the hypothesis that inaccuracies in the positioning of the sexual organs were related to stigmatic deposition of pollen by insects and ultimately seed set in the field.
4. Total inaccuracy in reciprocal positioning of the sexual organs was about four times larger in the forest population than in the grassland population (20.67 and 5.47, respectively). Individual inaccuracies in stigma positioning varied between 0.02 and 67.93 (L-morph) and 0.03 and 45.34 (S-morph) in the forest population, and between 0.00 and 13.19 (L-morph) and 0.00 and 26.15 (S-morph) in the grassland population.
5. Legitimate pollen deposition decreased significantly with increasing inaccuracy, both in the grassland and forest population, but effects were most pronounced in the forest population. In both populations, short-styled individuals suffered larger reductions in legitimate pollen deposition with increasing inaccuracies than long-styled individuals. Seed set was significantly and positively affected by the number of legitimate pollen and decreased significantly with

increasing inaccuracy, especially in the forest population where inaccuracies were most pronounced.

6. *Synthesis*. Overall, these results indicate that deviations from perfect reciprocity significantly affect the reproductive success of heterostylous plants by diminishing legitimate stigmatic pollen deposition and concurrently reducing seed set. As a result of their lower fitness, maladaptive genotypes should be selected against in natural populations, leading to maintenance of floral polymorphisms in general and the heterostylous syndrome in particular.

Key words: disassortative mating, floral polymorphism, heterostyly, pollination, *Primula veris*, reciprocity, reproductive ecology.

1 Introduction

Heterostyly is a floral polymorphism that has been observed in at least 28 different plant families (Barrett & Shore, 2008; Naiki, 2012). In heterostylous plant species, populations consist of two (distyly) or three (tristyly) floral morphs that are characterized by reciprocal arrangement of the sexual organs and a suite of ancillary polymorphisms, such as differences in pollen grain size and number, size of the stigmatic papillae and shape of the stigma, which is thought to promote attachment of legitimate pollen to the stigmatic surface (Ganders, 1979; Richards, 1997). In addition, most heterostylous plant species are characterized by a heteromorphic self-incompatibility system that reduces self- and intra-morph pollen germination and pollen-tube growth (Barrett & Cruzan, 1994). Pollen transferred between flowers of the different morphs will therefore lead to successful seed set, whereas pollen deposition within the same flower or between flowers of the same morph will yield little or no seeds.

In his classic book '*The different forms of flowers on plants of the same species*', Darwin (1877) suggested that the reciprocal arrangement of stigmas and anthers is sufficient to promote disassortative (i.e. between-morph) pollination and to optimize reproductive success and individual fitness, the so called "cross-promotion theory". Since pollen is deposited onto distinct positions of the pollinator's body that correspond to the heights of the receiving stigmas in the reciprocal morphs, it can be logically assumed that the efficiency of pollen transfer from one morph to the other is maximized. However, since most heterostylous plant species are characterized by a heteromorphic self-incompatibility system, which in most cases effectively blocks germination of self- and intra-morph pollen and impedes further pollen tube growth, the precise role of reciprocal herkogamy in promoting pollinator-mediated cross-pollination between floral morphs is hard to disentangle. Zhou et al. (2015) used parentage analyses based on microsatellite markers to test the cross-promotion hypothesis in the distylous, self-compatible herb *Luculia pinceana* (Rubiaceae). Their results showed that levels of disassortative mating (62.3%) were significantly larger than

assortative mating (37.7%), but still a substantial number of offspring resulted from intra-morph mating, providing only partial support for Darwin's cross-promotion hypothesis and underpinning the importance of a strong diallelic self-incompatibility system preventing self- and intra-morph fertilization and ensuring complete disassortative mating (Zhou et al., 2015). In *Narcissus assoanus*, a perennial bulbous plant species that shows dimorphic variation in style length, but non-reciprocity of anther positions, detailed assessments of seed set variation in experimental pollination arrays showed that inter-morph cross-pollination was more efficient than intra-morph cross pollination (Cesaro & Thompson, 2004), providing further support for Darwin's cross promotion hypothesis.

Whether disassortative pollen deposition occurs or not depends for a large part on the insects that are involved in pollen transfer. Most plant species are not pollinated by a single pollinator species, but rather by a whole range of species (Waser et al., 1996; Schleuning et al., 2012; Batstone et al., 2018) that often belong to different genera or even orders (Waser et al., 1996; Olesen et al., 2008). As a result, floral visitors of the same plant species are likely to differ in overall body size and morphology, resulting in variable patterns of pollen uptake and deposition (Adler & Irwin, 2006; Brys et al., 2008; Taki et al., 2009; Simón-Porcar et al., 2014). Deschepper et al. (2018), for example, recently showed that at least fifteen different insect species were found to visit flowers of the distylous *Primula veris*. More detailed analyses of the pollen assemblages on various parts of the insect's bodies revealed that the proportion of pollen of long-styled (L-morph) individuals deposited on the proboscis of insects significantly increased with proboscis length, whereas the proportion of pollen of short-styled (S-morph) individuals on the head of the insect decreased with increasing proboscis length, indicating that key features of the insect such as proboscis length can have a major impact on pollen transfer patterns and thus the effectiveness of reciprocal herkogamy in optimizing pollen transfer (Deschepper et al. 2018). In the distylous *Pulmonaria officinalis*, the only pollinator species for which the amount of pollen of long-styled flowers exceeded that of pollen of short-styled flowers on the proboscis was the long-tongued *Anthophora plumipes*. In all other insect species investigated, no such relationship was observed (Brys et al., 2008). Since stigmas of

short-styled flowers rely more on long-tongued pollinators, the long-tongued *A. plumipes* was considered as the most efficient pollinator to transfer pollen from anthers of long-styled flowers to styles of short-styled flowers (Brys et al., 2008).

Apart from insect-specific differences in pollen uptake, flowers of heterostylous plant species have also been found to show deviations from perfect reciprocity (Eckert & Barrett, 1994; Armbruster et al., 2017), potentially further affecting pollen deposition patterns. Recent observations in nine *Pulmonaria* species have shown that most species showed deviations from perfect reciprocity, with total inaccuracies varying between 2.9 and 20.3% relative to the squared mean of all anther and stigma heights in the population (Jacquemyn et al., 2018). In almost all cases, most of the inaccuracy was generated by the high-level organs. These results largely confirm earlier observations of Armbruster et al. (2017), who showed that in two *Primula* species (*Primula elatior* and *P. vulgaris*) most of the inaccuracy in reciprocity was due to variation in the high-level organs. However, in a third species (*P. veris*), a strikingly different pattern was observed with total inaccuracy and imprecision being affected by high and low organs to similar extents (Armbruster et al., 2017). Detailed assessment of pollen deposition patterns on stigmas of *Pulmonaria* further showed that the percentage of legitimate pollen deposition significantly decreased with increasing individual inaccuracy, suggesting that deviations from perfect reciprocal herkogamy may be much more common in heterostylous plant species than previously assumed and have a significant impact on pollen transfer patterns (Jacquemyn et al., 2018).

To get better insights in the ecological and morphological factors determining pollen deposition and seed set in distylous species, we investigated pollen deposition patterns and seed set in a grassland and forest population of the distylous herb *Primula veris*. Previous research has shown that populations in forest habitat showed distorted reciprocity, whereas nearby populations in grasslands displayed almost perfect reciprocal positioning of the anthers and stigmas (Brys & Jacquemyn, 2015) (Fig. 1). The wide range of individual inaccuracies found in forest populations

allowed us to assess in more detail how inaccuracies in reciprocal positioning of the sexual organs affect pollen deposition and subsequent seed set in heterostylous plant species and to compare it with a situation where deviations from perfect reciprocity were much smaller (Fig. 1) Moreover, the study of two distinct populations allowed assessing the extent to which differences in local environmental conditions or pollinator communities impact on pollen deposition and seed set. Specifically, we quantified inaccuracy of reciprocity in both a large grassland and forest population using a recently developed adaptive inaccuracy concept (Armbruster et al., 2017). We then related individual inaccuracies to patterns of legitimate and illegitimate pollen deposition and seed set (Fig. 1). We hypothesized that:

- 1) legitimate pollen deposition is significantly higher in the grassland population than in the forest population;
- 2) legitimate pollination decreases with increasing individual inaccuracy;
- 3) seed set is significantly negatively related to individual inaccuracy.

2 Material and Methods

2.1 Study species

Primula veris is a distylous herb belonging to the Primulaceae. The genus *Primula* contains about 500 species and is widely distributed throughout Asia and Europe, but has comparatively few representatives in North America (Richards, 2003). Relatively few taxa are circumboreal or widespread in Eurasia. In Europe, *P. veris*, *P. elatior* and *P. vulgaris* are the most widespread species (Jacquemyn et al., 2009; Schmidt-Lebuhn et al., 2012). *Primula veris* is a typical shade intolerant grassland herb, finding its optimum in many mesotrophic and calcicolous lowland grassland communities across Europe (Brys & Jacquemyn, 2009). Flowering generally starts at the beginning of May and ends 3–4 weeks later. Plants produce a rosette of several leaves and mostly one or two flowering stalks that bear between four and fifteen pale yellow flowers displayed in an umbel (Brys

& Jacquemyn, 2009). Flowers are distylous and denoted as strictly self-incompatible (Wedderburn & Richards, 1990), implying that only cross-pollination between the two genetically determined long-styled (further denoted as L-morph) and short-styled (further denoted as S-morph) individuals produces successful seed set (Richards, 1989) and that fruit and seed set generally decline when populations become strongly reduced in size or skewed in morph ratio (Kéry et al., 2000; Brys et al., 2003).

Flowers of *P. veris* are fragrant, produce ample nectar and are visited by a variety of insects, including long-tongued bumblebees and bees, but also by a large number of smaller insects, such as thrips and small beetles (Woodell, 1960; Brys & Jacquemyn, 2009; Deschepper et al., 2018). However, it is most successfully pollinated by insects with long proboscises that are able to exploit the nectar (Proctor & Yeo, 1973), which is secreted at the bottom of the flower. Although the anthers of both morphs do not differ in size (Darwin, 1877), pollen of the L-morph is significantly smaller (on average 18.1 μm) than that of the S-morph (on average 29.5 μm) (Brys & Jacquemyn, 2009). The smaller L-morph pollen is thought to adhere more easily to the short S-morph papillae than to the longer papillae in the L-morph, and vice versa (Richards, 1989). L-morph individuals also produce more pollen grains per flower than S-morph individuals (average pollen production per flower: 211000 and 87000, respectively) (Ornduff, 1980), resulting in a biased availability of S- and L-morph pollen in isoplethic populations.

2.2 Study populations and floral measurements

The study was conducted in southern Belgium, where *P. veris* can be found in both grassland and forest habitats (Deschepper et al., 2017). Previous research (Brys & Jacquemyn, 2015) has shown indications of marked differences in flower morphology and associated reciprocity of sexual organs between forest and grassland populations. Flowers of plants growing in forest population generally have significantly longer and wider corolla tubes, larger corolla lobes and corolla diameter, and a

higher positioning of the anthers compared with flowers of plants growing grasslands (Brys & Jacquemyn, 2015). Some plants in forest populations also show a strong deviation from perfect reciprocity that is mainly driven by pronounced variation in stigma length of L-morph individuals, whereas this is not observed in grassland populations (Fig. 1) (Brys and Jacquemyn 2015). For the purpose of this study, we selected two large *P. veris* populations, one in grassland and one in forest. Both populations contained > 1000 flowering individuals. In Spring 2017, in both populations we haphazardly selected >200 individuals throughout the entire population. Each plant was marked with a numbered plastic stick and from each plant one flower was harvested, immediately stored in plastic containers filled with ethanol and brought to the laboratory for further analysis. For each flower, the height of the stigma and anther position, and the length and width of the corolla tube were measured using digital calipers. Anther position was measured as the distance from the base of the corolla to the middle of the anthers. Additionally, herkogamy was determined as the distance from the top of the stigma to the middle of the anthers (see Brys & Jacquemyn (2015) for more details on floral measurements).

2.3 Pollen deposition and seed set

From the same flowers, styles were harvested and stored on FAA (45% ethanol, 5% acetic acid, 5% commercial formalin in distilled water) until they were stained to quantify the number of S- and L-pollen grains that were naturally deposited on the stigmatic surface. To assess total stigmatic pollen loads and the distribution of S- and L-morph pollen that were deposited, styles were softened in 8 M NaOH for 24 h, after which they were rinsed three times with tap water and stained for 30 min in a 0.5 mg/ml aniline blue solution in phosphate buffer pH 8.5. Subsequently, styles were gently squashed in a mounting medium for fluorescence (Vectashield) under a cover slip and for each stigma, pollen loads were determined using an epifluorescence microscope at 200x by counting the number of L- and S-morph pollen at both sides of the glass slide.

In spring, we also counted for each marked plant the total number of flowers. When fruits were mature (end of June), we harvested three fruits per plant. For each of these fruits, the total number of ovules and seeds were counted and averaged per plant. Because some plants and flowers showed signs of florivory (mainly by snails), we only considered intact flowers in order to obtain reliable estimates of successful pollination and seed set.

2.4 Data analysis

2.4.1 Inaccuracy of reciprocity

To measure the level of reciprocity in each population, we used the adaptive accuracy concept outlined by Armbruster et al. (2017). Essentially, it proposes a measure of inaccuracy, which assesses the fitness cost of the deviation of phenotypes from the optimal phenotype. Because pollen from one morph can in theory only be transported to the stigma of the opposite morph if they are positioned at the same level (Fig. 1), this inaccuracy value is defined based on the positions of the anthers and stigmas in the whole population relative to this optimal position (Armbruster et al., 2017). If all anthers and stigmas are at the same (optimal) position, inaccuracy will be zero. It can therefore be expected that the larger the inaccuracy values, the larger the deviations from disassortative mating and the larger the fitness costs will be. For each population, we first calculated the average height of high anthers on long stamens (A), the average height of high stigmas on long styles (S), the average height of low anthers on short stamens (a) and the average height of low stigmas on short styles (s). Inaccuracy was then calculated for each population as:

$$\text{Inaccuracy}_{\text{high organs}} = (\bar{A} - \bar{S})^2 + V_A + V_S \quad (1)$$

$$\text{Inaccuracy}_{\text{low organs}} = (\bar{a} - \bar{s})^2 + V_a + V_s \quad (2)$$

where V_A , V_S , V_a and V_s are the corresponding variances (Armbruster et al., 2017). Subsequently, total inaccuracy was calculated as the sum of the inaccuracy of the high and low organs. To compare levels of inaccuracy between the habitats, we calculated for each population and morph individual

stigma and anther inaccuracies as the square of the difference between the individual height of the stigma (anther) and the population mean height of compatible anthers (stigmas) (Armbruster et al., 2017).

Analysis of variance (ANOVA) was used to test whether individual inaccuracies differed significantly between individuals of the grassland and forest population and between floral morphs. To meet the assumptions of ANOVA, individual inaccuracies were log-transformed prior to analysis. The same analysis was used to investigate whether the level of herkogamy, i.e. the spatial distance between anthers and stigmas (Opedal, 2018), differed between individuals in the grassland and forest population and between short- and long-styled plants. In both analyses, the interaction between morph type and habitat was also included in the model.

2.4.2 Relationship between inaccuracy, pollen deposition and seed set

We used simple ordinary least-squares regression to assess the relationship between female inaccuracies on the one hand and legitimate and illegitimate pollen deposition on the other hand. The relationships between female inaccuracy and seed set were quantified using the same analysis. However, it should be noted that patterns of pollen transfer and subsequent seed set may also depend on the morphology of the insects that pick up and deposit pollen (Deschepper et al. 2018). Differences in local pollinator communities can therefore be expected to affect patterns of pollen transfer in natural populations as well. To investigate whether pollen deposition and seed set differed between long- and short-styled plants and to examine whether the relationship between inaccuracies, pollen deposition and seed set differed between short- and long-styled plants and the population from which the plants were sampled, an analysis of covariance (ANCOVA) was performed with floral morph (L- vs. S-morph) and population (grassland vs. forest populations) as fixed factors and inaccuracy as a continuous independent variable. All two- and three-way interactions were included in the model as well.

3 Results

3.1 Inaccuracy of reciprocity

The number of flowers per plant did not significantly differ between the forest and grassland population ($F_{1,475} = 0.81$; $P > 0.05$) (number of flowers (average $\pm$ SD): 7.80 ± 3.68 and 7.42 ± 4.38 , respectively). Flowers of plants growing in forest habitat had significantly larger corolla tubes ($F_{1,475} = 157.9$; $P < 0.0001$) and petal widths ($F_{1,475} = 21.4$; $P < 0.0001$) than flowers from grassland plants, whereas petal length and corolla size ($F_{1,475} = 5.13$; $P = 0.02$) were slightly smaller in forest habitat (Table S1). As a result, the positioning of the anthers was for both style morphs higher in flowers of forest plants than in flowers of grassland plants (Table 1) (L-morph: 7.81 ± 0.78 vs. 6.55 ± 0.49 mm; S-morph: 13.95 ± 1.40 vs. 12.60 ± 1.48 mm). Style length, on the other hand, was significantly smaller for long-styled plants in forest habitat than in grassland habitat (11.18 ± 2.62 and 13.04 ± 1.00 mm, respectively). The difference in style length of short-styled individuals between plants from forest and grassland was much smaller (5.51 ± 1.31 and 5.68 ± 0.99 mm, respectively). As a result, the level of herkogamy was significantly different between plants growing in the forest and grassland population ($F_{1,467} = 42.4$, $P < 0.0001$) and between L- and S-morph individuals ($F_{1,467} = 667.6$, $P < 0.0001$). Moreover, the difference between morphs was significantly dependent on the habitat where the plants were growing ($F_{1,467} = 338.7$, $P < 0.0001$). L-styled plants growing in forest had the lowest level of herkogamy (3.39 ± 2.68 mm), whereas the average level of herkogamy of S-morph individuals in the forest population was 8.54 ± 2.27 mm. In the grassland population, no such difference in the average level of herkogamy was found between L- and S-morph plants (6.41 ± 1.25 and 6.95 ± 1.18 mm, respectively) (Fig. S1).

These differences in style length and anther positioning resulted in significantly larger values of inaccuracy of reciprocity in forest plants than in grassland plants (total inaccuracy: 19.105 and 4.90 mm², respectively; mean² standardized inaccuracy: 20.67 and 5.47%) ($F_{1,467} = 309.8$, $P < 0.0001$) (Table 1). Individual stigma position inaccuracies varied between 0.02 and 67.93 (L-morph) and 0.03

and 45.34 (S-morph) in forest populations and between 0.00 and 13.19 (L-morph) and 0.00 and 17.66 (S-morph) in grassland populations (Fig. 2a-d). Individual anther position inaccuracies were smaller and varied between 0.00 and 25.27 (L-morph) and 0.32 and 29.54 (S-morph) in the forest population and between 0.00 and 7.63 (L-morph) and 0.00 and 7.25 (S-morph) (Fig. 2e-h). Individual stigma position inaccuracies were for both morph types strongly correlated to herkogamy in the forest population (L-morph: $r = -0.84$, $P < 0.0001$; S-morph: $r = 0.59$, $P < 0.0001$), whereas in grassland populations correlations between individual inaccuracies and herkogamy were less pronounced, especially in the S-morph (L-morph: $r = 0.48$, $P < 0.0001$; S-morph: $r = 0.23$, $P = 0.04$) (Fig. S2).

3.2 Pollen deposition and seed set

Total pollen deposition depended significantly on floral morph and on the population from which flowers were sampled (Table 2a). In the forest population, the total number of pollen grains deposited on L-morph stigmas was significantly higher than that deposited on S-morph stigmas (L: 409.4 ± 208.3 ; S: 160.3 ± 87.0). Similar patterns were observed in the grassland population. L-morph stigmas captured significantly more pollen grains than S-morph stigmas (L-morph: 366.8 ± 179.6 ; S-morph: 138.0 ± 66.1). In contrast, the number of legitimate pollen grains deposited depended significantly on floral morph, population, inaccuracy and the interactions between morph and population and between inaccuracy and population (Table 2b). In the forest population, the number of legitimate pollen deposited on S-morph stigmas was significantly higher than that on L-morph stigmas (S: 105.1 ± 73.0 ; L: 58.0 ± 40.0). Similarly, S-morph stigmas captured more legitimate pollen than L-morph stigmas (S-morph: 80.9 ± 45.1 ; L-morph: 67.2 ± 34.8) in the grassland population, although the difference in absolute numbers was not as large as in the forest population. In both populations, the number of legitimate pollen deposited on stigmas significantly decreased with increasing inaccuracy, but the effects were less pronounced in the grassland population than in the forest population, where inaccuracies were larger. S-morph individuals showed stronger declines in

legitimate pollen deposition with increasing inaccuracies than L-morph individuals (Fig. 3a,b), but effects were again stronger in the forest than in the grassland population. In L-morph individuals in the grassland population, no significant relationship between legitimate pollen deposition and inaccuracy was found (Fig. 3b). Legitimate and illegitimate pollen deposition were not significantly related to each other in the forest population (L-morph: $F_{1,99} = 1.43$, $p = 0.23$, S-morph: $F_{1,70} = 1.28$, $p = 0.26$), but the relationship was significant in the grassland population (L-morph: $F_{1,106} = 10.7$, $p = 0.001$, S-morph: $F_{1,88} = 4.43$, $p = 0.038$). In the latter, illegitimate pollen deposition increased with increasing levels of legitimate pollen deposition, especially in the L-morph (Fig. S3).

Patterns of seed set largely mirrored those of legitimate pollen deposition. Seed set was significantly affected by floral morph, population, inaccuracy and the interaction between floral morph and population (Table 2c). In both populations, seed set significantly declined with increasing inaccuracy, but effects were strongest in the forest population (Fig. 4a,b). In the forest population, the decline in seed set with increasing inaccuracy was most pronounced in individuals of the S-morph. Nonetheless, S-morph individuals produced significantly more seeds than L-morph individuals (Fig. 4a). In the grassland population, the overall decline in seed set with increasing inaccuracy was less pronounced, and there was no significant difference in average seed set between long- and short-styled individuals (Fig. 4b). Regression of seed set against the number of legitimate and illegitimate pollen deposited on stigmas showed that seed set significantly increased with increasing number of legitimate pollen deposited, but was independent of the number of illegitimate pollen deposited or even increased (Fig. 5).

4 Discussion

4.1. Inaccuracies in reciprocal positioning of the sexual organs

Because it is generally known that flowers of different individual plants within natural populations of heterostylous species differ in size and overall morphology (Eckert & Barrett, 1994), it can be

expected that floral traits related to the heterostylous syndrome (e.g. stigma height and anther positioning) also show substantial levels of among-plant variation. However, quantifying these deviations has proven to be challenging and several measures (Richards & Koptur, 1993; Eckert & Barrett, 1994, Faivre & McDade, 2001; Lau & Bosque, 2003; Sánchez et al., 2008, 2013), each with its own advantages and disadvantages, have been proposed to characterize the degree of spatial correspondence of compatible sexual organs between flowers of the opposite morphs in heterostylous plant populations (reviewed by Armbruster et al., 2017). In this study, we used a recently developed adaptive accuracy concept to assess the level of reciprocity and to relate individual inaccuracies in positioning of sexual organs to legitimate pollen deposition and ultimately seed set in natural populations of the distylous self-incompatible *P. veris*. Our results showed that inaccuracies in the grassland population were small and similar for the high and low-level sexual organs, confirming previous results that have been reported for *P. veris* (Armbruster et al., 2017). In the forest population, on the other hand, individual inaccuracies were much larger, leading to a fourfold difference in total inaccuracy compared to the grassland population.

In the distylous genus *Pulmonaria*, Jacquemyn et al. (2018) recently calculated inaccuracy values for a large number of populations over nine species across Europe, and documented mean²-standardized total inaccuracy values that usually varied between 4 and 11. Only in the case of an Estonian population of *Pulmonaria obscura*, an inaccuracy value >20 was observed. Interestingly, this species showed the largest variation in accuracy values among populations, ranging from 4.2 to 20.3, indicating that in some heterostylous species populations can show strong deviations from perfect reciprocal positioning of the sexual organs. Consistent with previous research, most of the inaccuracy occurred through variation in the high-level organs, whereas low level organs seemed to contribute less (Armbruster et al., 2017; Jacquemyn et al., 2018). In case of the *P. veris* populations studied here, variation in style length of L-morph individuals contributed most to the inaccuracy, suggesting that style length is a more labile trait than anther positioning. It remains, however, unclear which factor(s) drive the observed differences in style length variation between L- and S-

morph individuals in the forest population. In the distylous *Jasminum fruticans*, Thompson & Dommée (2000) suggested that modifier genes are most likely responsible for a comparable variations in stigma positioning in S-morph individuals. Similarly, in the tristylous *Eichornia paniculata*, modifier genes have been suggested to be responsible for the observed deviation in stamen elongation, resulting in the occurrence of homostylous variants in populations of this species (Fenster & Barrett, 1994).

4.2 Patterns of pollen transfer

When inaccuracies in reciprocal positioning of stigmas and anthers arise within populations of heterostylous plant species, it can be expected that pollen flow patterns become distorted and that less legitimate pollen will be deposited on the respective stigmas, especially in plants that show the largest deviations from perfect reciprocity. Our results indeed showed a clear and strong relationship between individual levels of inaccuracy and legitimate pollen deposition, particularly in the forest population, where the inaccuracy values were largest. Although individual inaccuracies were significantly smaller in the studied grassland population, there was still a detectable and significant effect of individual inaccuracy on legitimate pollen deposition. Similar results have been reported for *Pulmonaria*, where also a significant negative relationship between inaccuracy and legitimate pollen deposition was observed (Jacquemyn et al., 2018), indicating that increasing deviations from perfect match in spatial positioning between anthers and stigmas decrease the probability of legitimate pollen transfer. However, in that study the observed relationship was rather weak, most likely because the observations of many populations of different species growing in different habitats across Europe were pooled, the number of plants investigated within populations was low and inaccuracies were in most populations small or comparable to those observed in the grassland population studied here. Our data further showed that population itself explained a substantial amount of the variation in pollen deposition and seed set, suggesting that differences in local habitat conditions (e.g. light conditions) may affect patterns of pollen deposition and seed set.

However, a more likely explanation for the observed population effect may be found in local differences in the pollinator communities. Deschepper et al. (2018), for example, showed that forest and grassland populations harboured different insect species visiting flowers of *P. veris*. Interestingly, the interaction term between population and inaccuracy was less pronounced, suggesting that the effect of inaccuracy in the reciprocal positioning of the sexual organs is similar in both habitats.

4.3 Differences between L- and S-morph

S-morph individuals were more affected by deviations from perfect reciprocity than long-styled individuals, resulting in stronger declines in legitimate pollen deposition with increasing inaccuracy. This can be explained by the fact that stigmas of S-morph individuals are hidden deep within the tube-like flower and consequently are more difficult to reach for insects. L-morph stigmas, on the other hand, sometimes protrude the flower and are therefore easier to reach by foraging insects. This was also reflected in the total number of pollen deposited on stigmas, with L-morph stigmas capturing significantly more pollen in total than S-morph stigmas. S-morph stigmas, on the other hand, received significantly more legitimate pollen than L-morph stigmas, which is most likely due to the fact that in the studied populations L-morph individuals produce significantly more pollen than S-morph individuals do (114 656 vs. 24 375 pollen grains in the forest population, and 51 000 vs. 11 156 pollen grains in the grassland population, respectively) (Brys and Jacquemyn, 2015).

Keller et al. (2014) performed detailed pollination experiments with *Primula elatior* and *P. vulgaris* and their primary pollinator in Central Europe (*Anthophora plumipes*). Similar to the results presented here, stigmas of L-morph flowers captured significantly more pollen per visit than stigmas of S-morph individuals. However, in contrast to our results, they found that more pollen of S-morph anthers was deposited to reciprocal stigmas of L-morph flowers. One reason why more legitimate pollen of the S-morph was deposited on stigmas of the L-morph may be that Keller et al. (2014) used emasculated flowers, whereas in our case flowers were left unmanipulated. When insects forage for

nectar in L-morph flowers, they will pick up L-morph pollen on the distal part of the proboscis. When leaving the flower, it is likely that a substantial part of L-morph pollen comes into contact with and adheres to the stigma. Another reason for this difference may be that experiments were conducted with only one single pollinator that is characterized by a long proboscis and thus possesses ideal characteristics that facilitate inter-morph pollination in plant species with relatively long corolla tubes. Under natural field conditions, however, plants are most likely visited by a whole range of insects with varying proboscis lengths, which may alter patterns of legitimate pollen transfer (Brys et al., 2008a; Ferrero et al., 2011; Simón-Porcar et al., 2014). In the case of the *P. veris* populations studied here, Deschepper et al. (2018) recently showed that at least 15 different insect species visited *P. veris* flowers, ranging from small beetles, hoverflies, bumble bees to bees. This study further showed that the length of the proboscis had a major impact on the number of pollen that was picked up and the location where it was deposited on their bodies. The longer the proboscis was, the more pollen grains of each morph type were deposited at spatially separated parts of their bodies.

Variation in local pollinator communities and associated proboscis length may therefore have disturbed the idealized pattern of inter-morph pollination. Similar results have been observed for the distylous *Pulmonaria officinalis* (Brys et al., 2008), where *A. plumipes* efficiently captured S- and L-morph pollen on different parts of the insect's body, whereas no such patterns were observed for the short-tongued bumblebees *Bombus terrestris* and *B. pascuorum*. In the style-dimorphic *Narcissus papyraceus*, pollen transfer patterns resulting from the action of long-tongued pollinators resembled that of heterostylous species, whereas short-tongued pollinators acted more as pollen thieves, leading to reductions in both male and female reproductive success (Simón-Porcar et al., 2014). This may also explain why population had a strong effect on patterns of pollen deposition and seed set. Nonetheless, despite the presumed action of multiple insects with different proboscis lengths, inaccuracies in reciprocity contributed to variation in pollen deposition in both populations, underpinning the importance of reciprocal herkogamy in determining legitimate pollen transfer.

4.4 *Inaccuracy of reciprocity and seed set*

Consistent with pollen deposition patterns, seed set declined with increasing inaccuracy, indicating a strong link between pollen deposition and seed set. Moreover, our results indicated the lower seed set in flowers with higher inaccuracy was the result of receipt of fewer compatible pollen, and not of more incompatible pollen that caused stigma clogging. Patterns were again strongest in the forest population, where inaccuracies were largest, and there was a clear difference between L- and S-morph individuals. Although S-morph individuals produced on average more seeds than L-morph plants, seed production declined stronger with increasing inaccuracy in S-morph individuals. In L-morph individuals, the decline of seed set with increasing inaccuracy was less pronounced. In the grassland population, this relationship was less stringent and no major difference in seed set was found between L- and S-morph individuals. In the forest population, plants of the L-morph needed on average 2.2 pollen to successfully produce a seed, whereas S-morph plants needed on average 3.5 pollen (a 40% difference). This may explain the stronger relationship between individual inaccuracy and seed set in S-morph individuals. In the grassland population, differences were less pronounced and L- and S-morph individuals needed similar pollen numbers to produce a seed (2.6 and 3.0 respectively).

The pronounced differences in seed set patterns between the grassland and forest population further suggest that, as long as inaccuracies are small and mismatches in the spatial positioning of anthers and stigmas are limited, no major effects on plant fitness can be observed. However, as soon as inaccuracies cross a certain threshold level, pronounced effects on seed set occur and seed set decreases substantially with increasing inaccuracy, providing empirical support for the adaptive inaccuracy concept proposed by Armbruster et al. (2017). These observations, however, raise the question why these individuals are still present within the forest population since theory suggests that plants displaying large mismatches in reciprocal positioning of the sexual organs should be rapidly removed from the population as a result of stabilizing selection around the fitness optimum (Armbruster et al., 2017). This was, however, not the case, and several cases of

homostylous individuals have been observed in this and other forest populations nearby (Brys & Jacquemyn, 2015). The long life span of the species (Lehtilä et al., 2016) and the overall low seed germination (Brys et al., 2005) may for some part explain why individuals with deviating style length are not immediately discarded from the population.

4.5 Conclusion

In this study, we investigated patterns of pollen deposition and seed set in two populations of the perennial forest herb *Primula veris*, one displaying a wide range of individual inaccuracies and one where deviations from perfect reciprocal positioning of the anthers and stigmas were much smaller. Our results further showed that in both populations deviations from perfect reciprocity had a significant effect on pollen deposition and seed set, indicating that even small mismatches in the spatial positioning of stigmas and anthers can have a strong impact on pollen transfer patterns and ultimately seed set of heterostylous plant species. Plants showing the highest inaccuracies in reciprocal positioning of the sexual organs suffered from severe legitimate pollen limitation and as a result showed the strongest reductions in seed set. As a result of their lower fitness, maladaptive genotypes should be selected against in natural populations, leading to maintenance of floral polymorphisms in general and the heterostylous syndrome in particular.

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490 **Authors' contributions**

491 R.B. and H.J. conceived the ideas and designed the methodology. R.B. and H.J. collected the data in
492 the field, R.B. performed all laboratory analyses. R.B. and H.J. performed the statistical analyses. H.J.
493 wrote the first draft of the manuscript.

494

495 **Data Accessibility**

496 Floral inaccuracies and pollen deposition data have been deposited in the Dryad Digital Repository:
497 <https://doi:10.5061/dryad.b9m3402>.

498

499 **Supporting Information**

500 Additional supporting information may be found online in the Supporting Information section at the
501 end of the article.

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List of figures

Fig. 1 Schematic overview illustrating variation in flower morphology and predicted patterns of pollen pick up and deposition by insects and ultimately seed set in a) grassland populations of *Primula veris* displaying near perfect reciprocity of the sexual organs and b) forest populations showing strong deviations in reciprocity of anthers and stigmas. Long-styled plants in the forest population show a continuous variation in stigma height that ranges from clear and typical L-morph individuals to plants that show a complete overlap in anther-stigma positioning. Although less pronounced, short-styled plants of the forest population also display substantial variation in stigma length and a resulting inaccuracy with the legitimate anthers of L-morph plants. Based on these observations, we hypothesize that plants growing in forest populations will show stronger deviations from perfect reciprocity and that plants displaying the largest inaccuracies in the positioning of the anthers and stigmas will capture less legitimate pollen compared to plants displaying low inaccuracies, ultimately leading to decreased seed set. In particular, we predict that strong variation in stigma length of L-morph individuals in forest populations will lead to increased levels of inaccuracy in the positioning of the sexual organs in the forest population and therefore to altered patterns of pollen capture and deposition by insects and ultimately to reduced seed set.

Fig. 2 Histograms of individual female and male inaccuracies of reciprocity for long- (gray bars) and short-styled (white bars) plants in the studied forest (a, b, e,f) and grassland population (c, d, g, h).

Fig. 3 Relationship between individual inaccuracy and legitimate pollen deposition in long-styled (L-morph) and short-styled (S-morph) individuals of the distylous herb *Primula veris* in a) a forest populations and b) a grassland population. Bars on the right hand side of the figure denote the average pollen deposition on long- (black) and short-styled (white) individuals.

Fig. 4 Relationship between individual inaccuracy and seed set in long-styled (L-morph) and short-styled (S-morph) individuals of the distylous herb *Primula veris* in a) a forest population and b) a grassland population. Bars on the right hand side of the figure denote the average seed set in long- (black) and short-styled (white) individuals.

Fig. 5 Relationships between pollen deposition and seed set in long-styled (L-morph) and short-styled (S-morph) individuals of the distylous herb *Primula veris* in a-b) a forest population and c-d) a grassland population.

Fig. 1

Fig. 2

Fig. 3

a)

b)

Fig. 4

a)

b)

Fig. 5

Table 1 Summary statistics summarizing the position of the sexual organs and estimates of inaccuracy of reciprocity in a forest and grassland population of the distylous *Primula veris* in Belgium. a) Means and variances of style length (s) and anther positioning (a) for high (capital letter) and low-level organs (lower case letters). b) Corresponding inaccuracy values calculated using values reported in a).

a)

	Mean S	Mean A	Mean s	Mean a	Average	Var(S)	Var(A)	Var(s)	Var(a)
Forest	11.182	13.952	5.505	7.807	9.612	2.628	1.402	1.310	0.784
Grassland	13.035	12.606	5.680	6.550	9.468	0.997	1.480	0.993	0.489

b)

	Inaccuracy High	Inaccuracy Low	Total Inaccuracy	Mean ² -standardized total inaccuracy
Forest	11.702 (61.3)	7.393 (38.7)	19.095	20.669
Grassland	2.661 (54.3)	2.239 (45.7)	4.900	5.466

Table 2 Results of analysis of covariance (ANCOVA) displaying the effects of floral morph, female inaccuracy and population (grassland vs. forest population) and their interactions on a) total pollen deposition, b) legitimate pollen deposition and c) seed set in the distylous *Primula veris*.

a)

	df	Mean sum of squares	F value	P
Morph	1	4957966	205.74	< 0.0001
Inaccuracy	1	395	0.016	0.89
Population	1	219611	9.113	0.003
Morph*Inaccuracy	1	69205	2.872	0.09
Morph*Population	1	9249	0.384	0.54
Inaccuracy*Population	1	9178	0.381	0.54
Morph*Inaccuracy*Population	1	554	0.023	0.88

b)

	df	Mean sum of squares	F value	P
Morph	1	78750	48.89	< 0.0001
Inaccuracy	1	47782	29.66	< 0.0001
Population	1	60225	37.39	< 0.0001
Morph*Inaccuracy	1	989	0.61	0.43
Morph*Population	1	42918	26.64	< 0.0001
Inaccuracy*Population	1	10242	6.36	0.01
Morph*Inaccuracy*Population	1	226	0.14	0.71

c)

	df	Mean sum of squares	<i>F</i> value	<i>P</i>
Morph	1	971	6.67	0.01
Inaccuracy	1	3884	26.66	< 0.0001
Population	1	3101	21.29	< 0.0001
Morph*Inaccuracy	1	17	0.11	0.73
Morph*Population	1	1372	9.42	0.002
Inaccuracy*Population	1	397	2.72	0.09
Morph*Inaccuracy*Population	1	580	3.98	0.05