

Decrease of sexual organ reciprocity between heterostylous primrose species, with possible functional and evolutionary implications

Barbara Keller*, Jurriaan M. de Vos and Elena Conti

Institute of Systematic Botany, University of Zurich, 8008 Zurich, Switzerland

*For correspondence. E-mail barbara.keller@systbot.uzh.ch

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- **Background and Aims** Heterostyly is a floral polymorphism that has fascinated evolutionary biologists since Darwin's seminal studies on primroses. The main morphological characteristic of heterostyly is the reciprocal placement of anthers and stigmas in two distinct (distyly) floral morphs. Variation in the degree of intermorph sexual reciprocity is relatively common and known to affect patterns of pollen transfer within species. However, the partitioning of sexual organ reciprocity within and between closely related species remains unknown. This study aimed at testing whether intermorph sexual reciprocity differs within vs. between primrose species that hybridize in nature and whether the positions of sexual organs are correlated with other floral traits.
- **Methods** Six floral traits were measured in both floral morphs of 15 allopatric populations of *Primula elatior*, *P. veris* and *P. vulgaris*, and anther–stigma reciprocity was estimated within and between species. A combination of univariate and multivariate approaches was used to test whether positions of reproductive organs were less reciprocal between than within species, to assess correlations between sexual organ positions and other corolla traits, and to quantify differences between morphs and species.
- **Key Results** The three species were morphologically well differentiated in most floral traits, except that *P. veris* and *P. vulgaris* did not differ significantly in sexual organ positions. Overall, lower interspecific than intraspecific sexual organ reciprocity was detected. This decrease was marked between *P. elatior* and *P. vulgaris*, intermediate and variable between *P. elatior* and *P. veris*, but negligible between *P. veris* and *P. vulgaris*.
- **Conclusions** Differences in anther and stigma heights between the analysed primrose species were of the same magnitude or larger than intraspecific differences that altered pollen flow within other heterostylous systems. Therefore, it is possible to suggest that considerable reductions of sexual organ reciprocity between species may lower interspecific pollen flow, with potential effects on reproductive isolation.

Key words: reciprocal herkogamy, sexual organ reciprocity, *Primula*, primrose, distyly, heterostyly, floral morphology, hybridization, reproductive isolation, pollen flow, pre-mating barriers, speciation.

INTRODUCTION

The co-occurrence of different kinds of plants with male and female sexual organs placed reciprocally in distinct flowers of the same species (i.e. heterostyly) has fascinated evolutionary biologists since Darwin's seminal studies on the primroses *Primula elatior*, *P. veris* and *P. vulgaris* (Darwin, 1862, 1877). Over the past 150 years, numerous investigations were aimed at describing the morphological and physiological characteristics of heterostyly and elucidating its ecological, genetic and evolutionary significance, primarily within species (Barrett, 2002; Barrett and Shore, 2008; McCubbin, 2008; Weller, 2009; Cohen, 2010). While some studies have described heterostyly in closely related species (e.g. Kálmán *et al.*, 2007; Chen, 2009 in *Primula*; Massinga *et al.*, 2005 in *Pentstemon*) and their hybrids (Kálmán *et al.*, 2004; Zhu *et al.*, 2009 in *Primula*), to our knowledge the patterns of intra- and interspecific variation of the morphological traits unique to the floral syndrome have never been explicitly compared, despite the potential importance of such comparisons to explain the ecological and evolutionary role of heterostyly. In the present paper, we aim at filling this gap of knowledge in heterostyly, after providing a brief review of this specialized floral polymorphism.

Populations of heterostylous plant species are usually composed of two (distyly) or three (tristyly) genetically determined floral morphs, with distyly being more common than tristily (Ganders, 1979; Naiki, 2012). The two kinds of flowers of distylous species are referred to as the long-styled (LS) and short-styled (SS) morphs, also known as pins and thrums, respectively (Darwin, 1877). In pin and thrum flowers, male and female reproductive organs are positioned reciprocally, so that anthers are placed low in the corolla of pins and high in the corolla of thrums, while stigmas have the opposite configuration. This structural arrangement is called reciprocal herkogamy (Webb and Lloyd, 1986) and represents the main morphological component of distyly. Distylous flowers may also differ in pollen grain size and number, morphology of the stigmatic papillae, stigma shape and corolla mouth size (Dulberger, 1992; Richards, 1997; McCubbin, 2008).

Additionally, the heterostyly syndrome often entails a physiological response that inhibits self- and intramorph pollen tube germination and growth (Dulberger, 1992). The combination of reciprocal herkogamy and incompatibility ensures that successful fertilization occurs predominantly between anthers and stigmas placed at reciprocal positions in the two floral morphs (i.e. legitimate pollinations; Darwin,

1877; Barrett and Shore, 2008). Distyly is usually controlled by a simple Mendelian mechanism in which pin plants are homozygous (*ss*) and thrum plants are heterozygous (*Ss*), a genetic system that, with disassortative mating between the morphs, maintains equal morph ratios in sufficiently large populations (Dowrick, 1956; Lewis and Jones, 1992).

By focusing on the key functional role of reciprocal herkogamy, Darwin (1877) proposed that heterostyly evolved to promote outcrossing, a hypothesis later expanded by Lloyd and Webb (1992a, b) and Barrett (2002), and verified on artificial flowers by Stone and Thomson (1994). Specifically, it is thought that reciprocal herkogamy evolved to reduce gamete wastage due to self- or intramorph pollination, and increase the efficiency of pollen transfer between anthers and stigmas placed at the same level in the reciprocal floral morphs via disassortative pollination. Controlled pollination experiments in *Palicourea fendleri* (Rubiaceae; Lau and Bosque, 2003) demonstrated that pin pollen was transported more efficiently to thrum than pin flowers, while thrum pollen landed in greater quantities on thrum than pin stigmas. The authors interpreted this difference in pollen transfer as a product of the greater spatial matching between pin anthers and thrum stigmas than between thrum anthers and pin stigmas, confirming that the degree of reciprocity in the placement of sexual organs plays a crucial role in controlling pollen movement between distylous flowers.

Considering the importance of reciprocal herkogamy in the heterostyly syndrome, different methods (e.g. Lau and Bosque, 2003) and single-index metrics have been developed to quantify it (Richards and Koptur, 1993; Eckert and Barrett, 1994; Sánchez et al., 2008). By using these metrics, high levels of spatial matching between reciprocal sexual organs were detected in the distylous *Guettarda scabra*, *Oldenlandia umbellata*, *Palicourea fendleri* (Rubiaceae) and *Lithodora moroccana* (Boraginaceae), while *Anchusa hybrida* (Boraginaceae) displayed substantial deviation from reciprocity (Richards and Koptur, 1993; Sánchez et al., 2008). *Lithodora prostrata* and *L. fruticosa* (Boraginaceae), on the other hand, were characterized by stigma height dimorphism only (Sánchez et al., 2008), and *Glandora prostrata* (Boraginaceae) by relaxed stylar dimorphism (Ferrero et al., 2011). Furthermore, population-level variation in the degree of reciprocal herkogamy can be large (Ferrero et al., 2011), sometimes ranging from fully dimorphic to style dimorphic (Sánchez et al., 2010) or even trimorphic populations (Sosenski et al., 2010; Baena-Díaz et al., 2012) within the same species.

The position of sexual organs is influenced by various floral traits, as multiple, distinct floral traits or entire flowers can be functionally integrated (Ordano et al., 2008; Armbruster et al., 2009; Rosas-Guerrero et al., 2011). For example, correlations between sexual organ placement and corolla tube length, corolla mouth diameter or corolla size were found in *Primula* (e.g. Nishihira et al., 2000; Webster and Gilmartin, 2006; Kálmán et al., 2007) and correlations between the spatial fit of reciprocal sexual organs and floral integration were reported in *Nivenia* (Iridaceae; Sánchez et al., 2010), *Lithodora* and *Glandora* (Boraginaceae; Ferrero et al., 2011). Because reciprocity between sexual organs is both a compound and a complex trait, defined by the positions of sexual organs in complementary floral morphs and possibly affected by a

number of floral traits, it should be studied in relation to other relevant characteristics of heterostylous flowers.

While reciprocal herkogamy and related floral traits have been intensively examined at the intraspecific level, no studies have addressed how variation in these traits is partitioned within and between closely related species. Variation in floral traits probably plays a crucial role in hybridization and reproductive isolation, because the precise morphology of flowers can influence the mechanics of pollen uptake and deposition by pollinators, possibly affecting gene flow between species (i.e. mechanical isolation; Campbell and Aldridge, 2006). Campbell and Aldridge (2006) also underscored the lack of studies aimed at clarifying how mechanical isolation may affect species boundaries.

As a first step towards filling this knowledge gap, we investigated the morphological prerequisites for potential mechanical isolation between heterostylous species, by focusing on the spatial positioning of sexual organs and related floral traits. We examined the phylogenetically close *P. elatior*, *P. veris* and *P. vulgaris*, which are included in the small, well-defined *Primula* sect. *Primula* (six species in total; Mast et al., 2006; Schmidt-Lebuhn et al., 2012) of the genus *Primula* (approx. 420 species, of which approx. 90 % are distylous and often self- and intramorph incompatible; Wedderburn and Richards, 1992; Richards, 1997, 2003). Floral morphology and heterostyly have been studied in depth within each of the three selected species (Darwin, 1862, 1877; Fey, 1929; Gurney et al., 2007; Kálmán et al., 2007), providing a detailed context for our comparative study. We measured six floral traits from several populations of *P. elatior*, *P. veris* and *P. vulgaris*, adapted an existing method for the estimation of reciprocity between sexual organs (Lau and Bosque, 2003) to the application of statistical tests, and tested whether: (1) the degree of reciprocity between sexual organs differed within vs. between species; (2) the positions of sexual organs were correlated with other floral traits; and (3) the studied floral traits differed between morphs and species. Considering the functional significance of reciprocal herkogamy, the discovery of potential interspecific discontinuities in this trait may have important implications for evolutionary processes in heterostylous species, including interspecific pollen flow, hybridization and local adaptation.

MATERIALS AND METHODS

Study species, field sampling and floral measurements

Study species. *Primula elatior* (L.) Hill, *P. veris* L. and *P. vulgaris* Huds. are perennial, rosette-forming diploid species ($2n = 22$) with distylous flowers that bloom in spring. *Primula elatior* and *P. vulgaris* have pale yellow corollas with broad, overlapping, widely v-notched corolla lobes; additionally, the corolla shape of *P. vulgaris* is more flat-faced than in *P. elatior* (Richards, 2003). *Primula veris* is mainly characterized by an inflated calyx, golden-yellow corolla and a distinctive floral scent (Richards, 2003). The three species can be pollinated by the same pollinators (Woodell, 1960) and their distributional ranges overlap in Europe (Richards, 2003). In Switzerland, where our study was conducted, *P. elatior* and *P. vulgaris* occur predominantly north and south of the Alps, respectively, while *P. veris* is widespread (Lauber and Wagner,

2007), and all three species can form hybrids when they come into contact (Fey, 1929; B. Keller, pers. obs.), as they do in the rest of Europe (Woodell, 1965, 1969; Kálmán *et al.*, 2004; Gurney *et al.*, 2007; Taylor and Woodell, 2008; Brys and Jacquemyn, 2009; Jacquemyn *et al.*, 2009).

Field sampling. We sampled five allopatric populations each of *P. elatior*, *P. veris* and *P. vulgaris* in Switzerland during the flowering period of spring 2008 (Table 1). A population was considered as allopatric if it was located at least 150 m apart from any other population, as pollen flow in *Primula* is leptokurtic (Richards, 1997) and the kinship coefficient decreases sharply with geographic distance (Van Rossum and Triest, 2006). No phenotypic hybrids or introgressants were observed within the sampled populations. We included only allopatric populations in our study to reduce the possibility that morphological measurements from introgressed individuals might prevent us from characterizing the floral traits typical of each species and performing interspecific comparisons. We sampled approximately equal numbers of pin and thrum plants dispersed throughout each population. Final sample sizes per population ranged between 12 and 22 individuals for *P. elatior*, 20 and 21 individuals for *P. veris* and eight and 20 individuals for *P. vulgaris* (Table 1). From each plant, we randomly picked three open flowers and preserved them in 70 % ethanol for morphological analyses.

Floral measurements. We dissected flowers by longitudinally cutting the calyx and corolla tube. A few flowers were excluded from the analyses, because they had shrivelled, brown, dwarf styles and ovaries, obvious developmental disorders or were severely damaged by herbivores. The final number of analysed flowers was thus 251 (133 pins, 118 thrums) in *P. elatior*, 288 (148 pins, 140 thrums) in *P. veris* and 245 (123 pins, 122 thrums) in *P. vulgaris* (Table 1). We

took digital images of dissected flowers and measured the following traits (see Fig. 1A): (1) distance from the base of the gynoeceum to (a) the middle of anthers (anther position: AP); (b) the tip of the stigma (stigma position: SP); and (c) the top of the corolla tube (corolla tube length: TL); (2) the diameter of the corolla mouth (corolla mouth diameter: MD); (3) the length of the longest corolla limb (corolla limb length: LL); and (4) the maximal distance between opposite corolla limbs (flower width: FW). The total length of the corolla was calculated as the sum of the tube and limb length (total corolla length: CL). Since no intrafloral variation among the five anther positions was detected, we measured the position of only one anther per flower. All measurements were taken to an accuracy of 0.01 mm using ImageJ 1.40 (<http://rsbweb.nih.gov/ij/>).

Intra- and interspecific sexual organ reciprocity

GLMM approach. To test whether sexual organ reciprocity (SOR) differed significantly within vs. between species, we used the approach described in Lau and Bosque (2003) and modified it for the application of statistical tests as follows. We first constructed frequency distributions for all sexual organ measurements in each species (frequency histograms, intervals of 0.2 mm). We then statistically defined SOR as the probability of each individual sexual organ measurement falling inside or outside the frequency distributions of the reciprocal sexual organs, either from the same (intraspecific SOR) or from a different species (interspecific SOR). We applied three generalized linear mixed effects models (GLMMs) with binomial error distribution and a logit link function. Each GLMM used the frequency distributions of sexual organs for one of the three species as reference distributions. This allowed us subsequently to test with planned

TABLE 1. *Sampled populations, individuals and flowers, with corresponding reciprocity index R (Sánchez et al., 2008), for Primula elatior, P. vulgaris and P. veris in Switzerland*

Species name	Locality (abbreviation of Canton)	Longitude (E)	Latitude (N)	No. of sampled plants			No. of sampled flowers			R*
				Pins	Thrums	Total	Pins	Thrums	Total	
<i>P. elatior</i>	Küsnacht (ZH)	8°35'36.97"	47°19'41.52"	6	6	12	18	17	35	0.006
	Kollbrunn (ZH)	8°47'42.36"	47°27'46.04"	10	9	20	30	26	56	0.005
	Zurich (ZH)	8°33'25.20"	47°23'57.48"	10	10	20	29	28	57	0.026
	Zurich (ZH)	8°29'02.76"	47°22'07.78"	9	7	16	22	19	41	0.030
	Thörigen (BE)	7°44'04.56"	47°09'57.60"	12	10	22	34	28	62	0.013
	Total/mean ± s.d.			47	42	89	133	118	251	0.016 ± 0.011
<i>P. veris</i>	Seewis (GR)	9°38'7.44"	46°58'45.48"	10	10 [†]	20	30	26	56	0.005
	Montreux (VD)	6°54'52.20"	46°26'34.80"	11	9	20	31	25	56	0.003
	Kollbrunn (ZH)	8°47'36.60"	47°27'47.88"	10	11	21	28	31	59	0.012
	Pfungen (ZH)	8°38'15.83"	47°31'22.46"	10	10	20	30	30	60	0.007
	Glarus (GL)	9°03'11.16"	47°02'23.28"	10	10	20	29	28	57	0.009
	Total/mean ± s.d.			51	50	101	148	140	288	0.007 ± 0.003
<i>P. vulgaris</i>	Pompagles (VD)	6°28'49.80"	46°39'55.44"	5	3	8	15	9	24	0.007
	Arogno (TI)	8°59'51.36"	45°57'37.08"	9	9	18	26	27	53	0.011
	Vaglio (TI)	8°57'26.64"	46°03'46.44"	10	10	20	27	29	56	0.014
	Collonges (VL)	7°02'22.56"	46°10'32.88"	10	10	20	27	29	56	0.010
	Lausanne (VD)	6°34'57.00"	46°31'21.36"	10	10	20	28	28	56	0.024
	Total/mean ± s.d.			44	42	86	123	122	245	0.013 ± 0.007

* A value of 0 would represent perfect reciprocity; values <0.05 are considered to indicate distyly.

† One plant was excluded from all analyses involving stigma position, since stigmas were missing in all three flowers analysed.

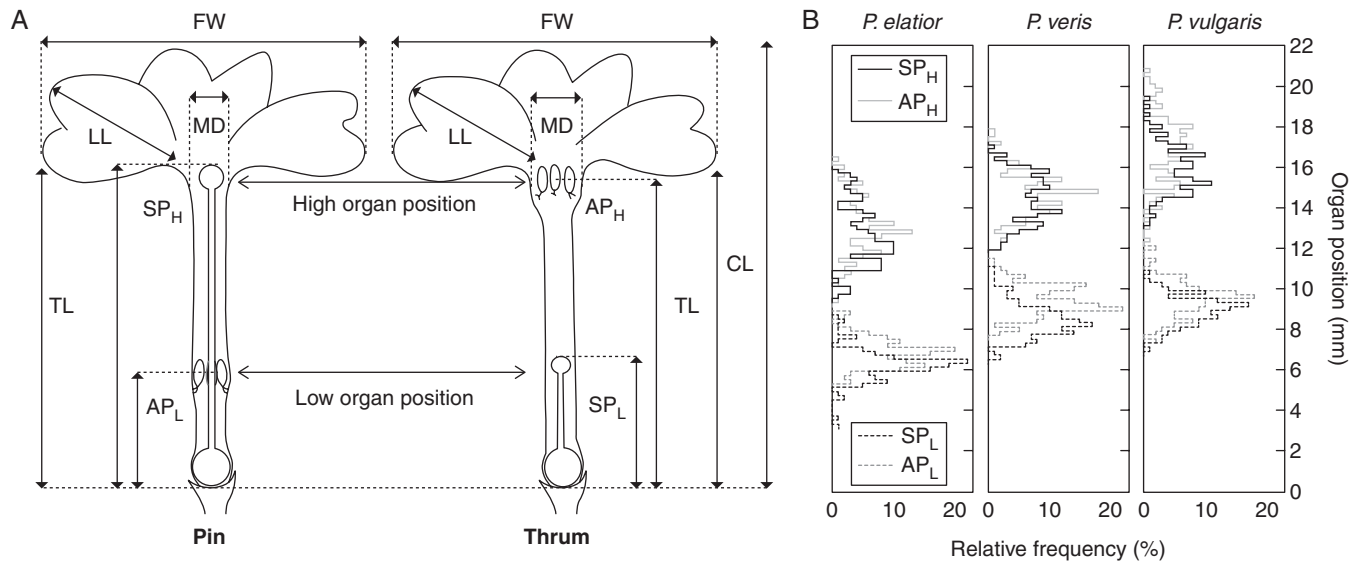


FIG. 1. Floral traits measured in the three studied *Primula* species. (A) Diagram of a pin (long-styled) and thrum (short-styled) morph of *P. vulgaris*. (B) Frequency distributions of anther and stigma positions of all sampled individuals in *P. elatior*, *P. veris* and *P. vulgaris*. Sexual organ traits: AP_H, anther position high (thrum); SP_H, stigma position high (pin); AP_L, anther position low (pin); SP_L, stigma position low (thrum). Corolla traits: TL, corolla tube length; LL, corolla limb length; MD, corolla mouth diameter; FW, flower width; CL, total corolla length (CL = TL + LL).

contrasts whether SOR differed significantly within vs. between species for sexual organs placed either high (i.e. AP_H and SP_H) or low (AP_L and SP_L) within the corolla tube (see the legend of Fig. 1A for abbreviations). For example, we tested whether thrum anthers of *P. elatior* had a higher probability of falling under the frequency distribution of pin stigmas from the same species (i.e. reference distribution) than the corresponding anthers of *P. veris*. In all GLMMs we used comparison type (i.e. one intraspecific and two interspecific organ comparisons), organ level (i.e. high or low), organ type (i.e. anthers or stigmas) and all two- and three-way interactions as fixed effects. To account for hierarchical data structure, we included as random effects: (1) population identity nested in species identity; (2) plant identity nested in population identity and species identity; and (3) flower identity nested in plant identity, population identity and species identity. Due to unbalanced data sets, we used Satterthwaite's method to determine the approximate denominator degree of freedom and applied sequential Bonferroni correction to account for multiple tests. All statistical analyses were performed using SPSS version 19.0.0 (SPSS, Inc.), unless otherwise stated.

Indices of reciprocity. To enable direct comparisons with other published studies, we additionally calculated the degree of spatial matching between reciprocal sexual organs using two different indices. The index of Sánchez *et al.* (2008) compares each organ position with that of every other organ position in the sampled flowers and provides a single combined metric of reciprocity (R) for high and low organs. The index can be interpreted as a measure of the average population-level deviation from perfect reciprocity, with zero indicating perfect reciprocity, and values <0.05 indicating distyly. The index of Richards and Koptur (1993) calculates the reciprocity between high (R_H) and low (R_L) organ positions, separately.

It is calculated from species-wide means of floral measurements using equations $R_H = (AP_H - SP_H)/(AP_H + SP_H)$ and $R_L = (AP_L - SP_L)/(AP_L + SP_L)$. R_H and R_L values can vary between 1 and -1 , with zero indicating perfect reciprocity. Positive values indicate that the mean anther position is higher than the mean stigma position, while negative values indicate the opposite.

Using the single-value index of Sánchez *et al.* (2008), we calculated reciprocity in each of the five populations sampled in *P. elatior*, *P. veris* and *P. vulgaris*, checked for outliers within each species with Grubbs' test (as implemented in QuickCalcs; GraphPad Software, Inc.) and estimated whether R was significantly below the threshold value of 0.05 for distyly with one-sample t -tests. R -values were transformed logarithmically to stabilize variances, and a one-way analysis of variance (ANOVA) was performed to establish whether the R -values of the three species differed significantly from each other. We assessed interspecific reciprocity by pooling the measurements of anther and stigma positions from all five sampled populations of each species and calculating the respective interspecific values for R , R_H and R_L (for instance using mean values of low anther positions of *P. elatior* and low stigma positions of *P. veris* to calculate R_L between the two species).

Correlations among floral traits and differences between morphs and species

To estimate whether anther and stigma positions were correlated with each other and with other corolla traits, we calculated Pearson correlation coefficients between anther and stigma positions and between the positions of each sexual organ type with corolla tube length, corolla mouth diameter, corolla limb length and flower width. To investigate morph-specific correlations among traits, we analysed pin and thrum

flowers separately. For these and all subsequent analyses, we used mean values of floral traits per plant if more than one flower per plant was measured. Trait measurements were transformed logarithmically to stabilize variances. To summarize quantitative variation and co-variation of all floral traits among the three species, we performed principal component analyses (PCAs). Differences between the principal component (PC) scores of the three species along the first and second PCs in thrums were tested with one-way ANOVAs followed by Tukey post-hoc tests. Due to heteroscedasticity of variances in PC scores in pins, we used Welch's ANOVAs followed by Games and Howell's post-hoc tests, because they do not assume homogeneous variances.

To test whether individual floral traits differed significantly between morphs and species, we used a model with normal distribution and identity link function in the GLMM environment of SPSS. As fixed effects we used species membership, morph identity and the interaction between species membership and morph identity. Since some traits associated with flower size (i.e. TL, LL and FW) differed between species, we further analysed sexual organ traits (AP and SP) using total corolla length (CL; see Fig. 1A) as a proxy for flower size and incorporated it in the models as a continuous predictor. This simple allometric scaling method allowed us to test whether anther and stigma positions differed between morphs and species proportionately to overall flower size (Packard and Boardman, 1988). Differences in floral traits between species pairs were assessed with pairwise contrasts. When a significant interaction between morph and species was found, differences in floral traits between individual species pairs were estimated separately for pins and thrums. We used population identity nested in species identity as a random effect to account for hierarchical data structure in all models, and applied sequential Bonferroni correction to account for multiple tests.

RESULTS

Intra- and interspecific sexual organ reciprocity

GLMM approach. The three GLMMs used to estimate whether SOR differed significantly within vs. between species provided a good fit for the data. In fact, the models correctly assigned $\geq 80\%$ of the individual sexual organ measurements either inside or outside (i.e. binary trait) each reference distribution (correct assignments: 83.3, 80.0 and 81.3 % to the reference distributions of *P. elatior*, *P. veris* and *P. vulgaris*, respectively). The three-way interaction between comparison type, organ level and organ type was either highly (Supplementary Data Table S1B, C) or marginally significant (Supplementary Data Table S1A), justifying execution of subsequent contrast tests.

Within species, the distributions of male and female sexual organs overlapped considerably at both the high and low level (Fig. 1B). Accordingly, SOR was high, ranging from 0.926 ± 0.025 (mean \pm s.e.) in *P. vulgaris* (Fig. 2C) to 0.555 ± 0.075 in *P. veris* (Fig. 2B). High-placed sexual organs (i.e. thrum anthers and pin stigmas) were generally more reciprocal than low-placed organs (i.e. pin anthers and thrum stigmas), with the following exception: in *P. vulgaris*, thrum stigmas were more reciprocal to pin anthers (0.870 ± 0.041) than thrum

anthers to pin stigmas (0.716 ± 0.072 ; Fig. 2C). Within high-placed sexual organs, thrum anthers had a higher probability of falling under the reference distribution of pin stigmas than vice versa (i.e. pin stigmas vs. thrum anthers) in *P. elatior* (0.876 ± 0.033 vs. 0.779 ± 0.049 ; Fig. 2A) and *P. veris* (0.917 ± 0.024 vs. 0.795 ± 0.050 ; Fig. 2B), while the situation was reversed in *P. vulgaris* (0.716 ± 0.072 vs. 0.926 ± 0.025 ; Fig. 2C). Within low-placed sexual organs, thrum stigmas had a higher probability of falling under the reference distribution of pin anthers than vice versa in *P. elatior* (0.761 ± 0.054 vs. 0.679 ± 0.062 ; Fig. 2A) and *P. vulgaris* (0.870 ± 0.041 vs. 0.702 ± 0.073 ; Fig. 2C), while the situation was reversed in *P. veris* (0.555 ± 0.075 vs. 0.725 ± 0.060 ; Fig. 2B).

Sexual organ reciprocity was lower between than within species in 21 out of the 24 pairwise comparisons, and the difference was significant in 17 out of the 21 cases (Fig. 2), ranging from 0.792 ± 0.053 to almost zero in four cases, all detected between low-placed organs. In general, the reduction of reciprocity was most pronounced between the sexual organs of *P. vulgaris* and *P. elatior*, with values ranging between 0.251 ± 0.055 and 0.009 ± 0.004 (Fig. 2A, C). The decrease of reciprocity was also marked between *P. veris* and *P. elatior*, although pin stigmas of *P. veris* and thrum anthers of *P. elatior* were not significantly less reciprocal than the corresponding sexual organs of *P. elatior* (Fig. 2A). The reduction of SOR was modest between *P. veris* and *P. vulgaris*, where the difference between interspecific and intraspecific SOR was non-significant in five out of eight comparisons (Fig. 2B, C). Likewise, the only statistically significant exception to the trend of lower inter- than intraspecific SOR was detected between thrum stigmas of *P. vulgaris* and pin anthers of *P. veris*, which were more reciprocal (0.792 ± 0.053) than the corresponding sexual organs of *P. veris* (0.555 ± 0.075 ; Fig. 2B, right panel).

The partitioning of SOR between high-placed and low-placed sexual organs was more complex than the situation described above for intraspecific SOR. High sexual organs were more reciprocal than low sexual organs in all interspecific comparisons between *P. vulgaris* and *P. elatior* and between *P. elatior* and *P. veris*. In contrast, high organs were less reciprocal than low organs in two of the eight comparisons involving *P. vulgaris* and *P. veris*: thrum anthers of *P. vulgaris* and pin stigmas of *P. veris* (0.334 ± 0.071 ; Fig. 2B); pin stigmas of *P. veris* and thrum anthers of *P. vulgaris* (0.675 ± 0.074 ; Fig. 2C).

Indices of reciprocity. Within species, the global indices of reciprocity (R) calculated according to Sánchez *et al.* (2008) were < 0.05 in each of the 15 sampled populations (five per species; Table 1), indicating good spatial fit between the positions of pin and thrum sexual organs in all populations. Grubbs tests for outliers indicated no significant deviations of R -values among populations within each species ($P > 0.05$), allowing us to pool the data for subsequent comparisons. Mean R -values per species were significantly lower than the threshold level for distyly of 0.05 (*P. elatior*: $t = -6.634$, d.f. = 4, $P = 0.002$; *P. veris*: $t = -28.736$, d.f. = 4, $P < 0.001$; *P. vulgaris*: $t = -12.814$, d.f. = 4, $P < 0.001$) and they did not differ significantly from each other (one-way ANOVA: $F_{2,12} = 1.590$; $P = 0.244$). The indices of reciprocity

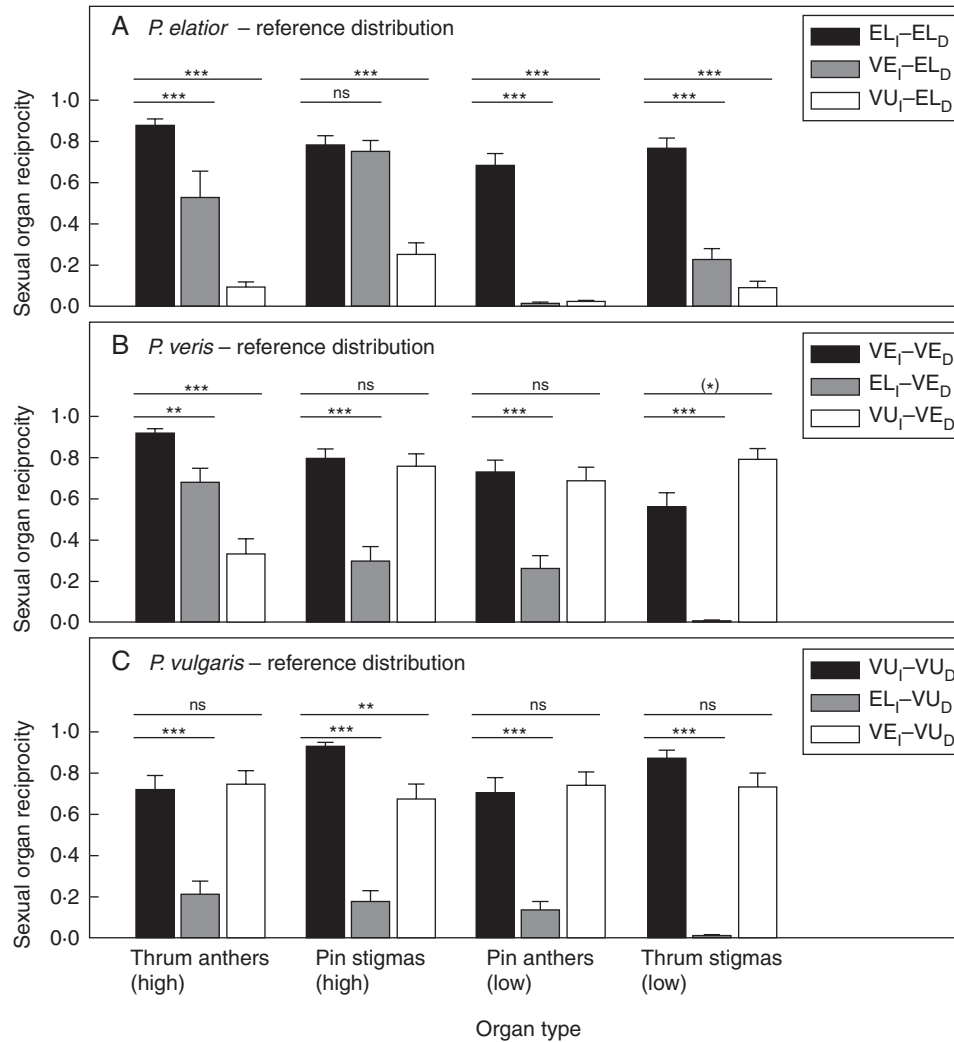


FIG. 2. Sexual organ reciprocity (SOR) with standard errors estimated from generalized linear mixed effect models (GLMMs). Values on the y-axis represent the probability that the individual sexual organs of a species (indicated on the x-axis and denoted by the subscript I in the keys) fall within the frequency distribution of the reciprocal sexual organs (i.e. reference distribution, denoted by the subscript D) either of the same species (intraspecific SOR) or of another species (interspecific SOR). Reference distributions of (A) *Primula elatior* (EL), (B) *P. veris* (VE) and (C) *P. vulgaris* (VU). For example, the black bar furthest left in (A) indicates that each thrum anther of *P. elatior* has a 0.876 mean probability of falling within the frequency distribution of *P. elatior* pin stigmas, with a standard error of 0.033. Significance levels: interspecific organ comparisons with a significantly lower (** $P < 0.01$, *** $P < 0.001$), higher [$^{(*)}P < 0.05$] or not significantly different (ns) probability than intraspecific organ comparisons. Sequential Bonferroni correction was used to account for multiple tests.

calculated according to Richards and Koptur (1993) for high (R_H) and low organs (R_L) indicated greater spatial matching for the former than the latter in *P. elatior* ($R_H = 0.022$ vs. $R_L = 0.049$) and *P. veris* ($R_H = 0.007$ vs. $R_L = 0.057$), while the difference was very small in *P. vulgaris* ($R_H = 0.030$ vs. $R_L = 0.032$; Table 2A), confirming the results inferred from intraspecific estimates of SOR (Fig. 2).

R -values calculated according to Sánchez *et al.* (2008) were higher between than within species (Table 2B), indicating an overall reduction of spatial fit between pin and thrum sexual organs in interspecific comparisons and confirming SOR results (Fig. 2). The largest decrease of reciprocity was detected between *P. elatior* and *P. vulgaris* (Table 2), as in SOR analyses (Fig. 2). R_H values were lower than R_L values between *P. elatior* and *P. veris*, and between *P. elatior* and *P. vulgaris*, reflecting a higher degree of reciprocity in high-

placed than in low-placed organs for these interspecific comparisons (Table 2). However, R_H values were higher than R_L values in comparisons between *P. veris* and *P. vulgaris*, as also observed in SOR estimates (Fig. 2).

Correlations among floral traits and differences between morphs and species

Anther and stigma positions were significantly correlated with each other in both pin and thrum flowers, and the position of sexual organs was most strongly correlated with corolla tube length in both floral morphs (Table 3). Anther positions were more weakly correlated with corolla limb length, corolla mouth diameter and flower width in thrums. Stigma positions were more weakly correlated with corolla limb length, corolla

TABLE 2. Intra- and inter-specific reciprocity between sexual organs in *Primula elatior* (EL), *P. veris* (VE), and *P. vulgaris* (VU) calculated by pooling measurements from all populations with A) index of Richards and Koptur (1993)* for the high (R_H : thrum anthers and pin stigmas) and low organs (R_L : pin anthers and thrum stigmas) separately, and B) the index of Sánchez et al. (2008) † for both organ levels combined (R).

	<i>P. elatior</i>	<i>P. veris</i>	<i>P. vulgaris</i>	EL–VE	VE–EL	EL–VU	VU–EL	VE–VU	VU–VE
(A) Richards and Koptur*									
R_H	0.022	0.007	0.030	–0.044	0.073	–0.093	0.145	–0.043	0.079
R_L	0.049	0.057	0.032	–0.105	0.208	–0.131	0.210	0.030	0.059
(B) Sánchez et al. †									
R	0.016	0.010	0.015	0.022	0.034	0.046	0.041	0.020	0.012

* Index varies between –1 and 1; perfect reciprocity = 0; positive values indicate that the mean anther position is higher than the mean stigma position, while negative values indicate the opposite. In interspecific comparisons (e.g. EL–VE), anthers of the species mentioned first are compared to stigmas of the second species.

† Index values of 0 would represent perfect reciprocity; values <0.05 are considered to indicate distyly. In interspecific comparisons (e.g. EL–VE), pin flowers of the species mentioned first are compared to thrum flowers of the second species.

TABLE 3. Pearson correlation coefficients between anther positions, stigma positions and four other corolla traits of *Primula elatior*, *P. veris* and *P. vulgaris* for all pin plants and all thrum plants

Floral traits	Pin plants		Thrum plants	
	Anther position	Stigma position	Anther position	Stigma position
Stigma position	0.723***	–	0.714***	–
Corolla tube length	0.863***	0.840***	0.985***	0.700***
Corolla limb length	0.122	0.436***	0.550***	0.255**
Corolla mouth diameter	–0.007	0.291***	0.153*	–0.041
Flower width	0.003	0.333***	0.469***	0.135

One-tailed *P*-values: **P* < 0.05, ***P* < 0.01, and ****P* < 0.001.

mouth diameter and flower width in pins, and with corolla limb length in thrums (Table 3).

The two PCAs produced two PCs (Supplementary Data Table S2). In both morphs, one of the PCs was mainly explained (i.e. high factor loadings) by the longitudinal floral traits anther position, stigma position and corolla tube length (pins, PC2; thrums, PC1), while the other PC was mainly explained by the lateral floral traits corolla mouth diameter, corolla limb length and flower width (pins, PC1; thrums, PC2; Supplementary Data Table S2; Fig. S1). Hence, the results of the two PCAs confirmed that the longitudinal floral traits were most strongly correlated with each other. The three species were morphologically well differentiated from each other for pin and thrum morphs along the two PCs (pins PC1: $F_{2,90.776} = 429.911$, $P < 0.001$; PC2: $F_{2,89.687} = 183.044$, $P < 0.001$; thrums PC1: $F_{2,131} = 162.794$, $P < 0.001$; PC2: $F_{2,131} = 240.751$, $P < 0.001$; all comparisons between individual species pairs $P < 0.002$). However, scatterplot diagrams showed that PCA scores slightly overlapped between *P. vulgaris* and *P. elatior* (Supplementary Data Fig. S1).

In general, each of the six examined floral traits differed significantly among the three species and between floral morphs (Supplementary Data Table S3A). Exceptions to this general pattern occurred in the following pairwise comparisons: (1) anther position did not significantly differ between the pin flowers of *P. veris* and *P. vulgaris* (Fig. 3A; as seen also in

Fig. 1B); (2) stigma position did not significantly differ between the thrum flowers of *P. veris* and *P. vulgaris* (Fig. 3B; as seen also in Fig. 1B); and (3) corolla mouth diameter did not significantly differ in thrum flowers of the three species (Fig. 3D). The first two results are consistent with the high levels of SOR estimated between the low sexual organs of *P. veris* and *P. vulgaris* (Fig. 2B, C). *Primula vulgaris* had the longest and largest flowers, *P. elatior* the shortest flowers, and *P. veris* the narrowest flowers (Fig. 3C, E, F). Corolla mouth diameter significantly differed among pin flowers of the three species, being widest in *P. vulgaris*, narrowest in *P. veris* and intermediate in *P. elatior* (Fig. 3D).

Anther and stigma positions were significantly affected by overall flower size (i.e. significant effect of total corolla length; Supplementary Data Table S3B). In analyses without correcting for flower size, anther positions of pin flowers and stigma positions of thrum flowers did not significantly differ between *P. veris* and *P. vulgaris*, whereas anther positions of thrum flowers and stigma positions of pin flowers were significantly lower in *P. veris* than in *P. vulgaris* (Fig. 3A, B). Conversely, after adjusting for the effect of overall flower size, relative anther and stigma positions were placed significantly higher in the corolla of *P. veris* than in the corolla of *P. vulgaris*, at both the high and low organ levels (all $P < 0.001$). Furthermore, in analyses without correcting for flower size, anther and stigma positions were significantly lower in *P. elatior* than in

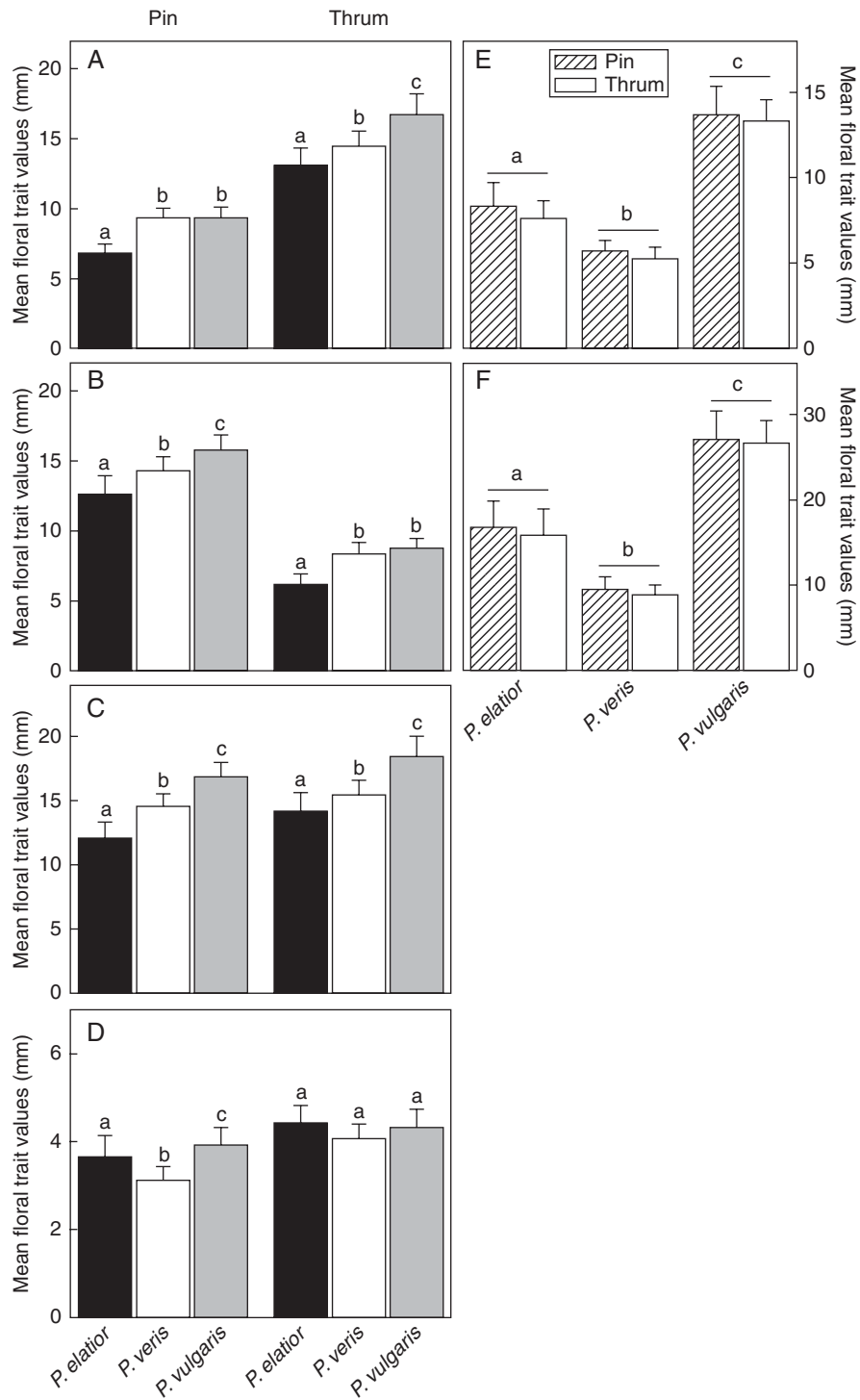


FIG. 3. Mean values of floral traits with standard deviations in pin and thrum flowers of *Primula elatior*, *P. veris* and *P. vulgaris*. (A) Anther position, (B) stigma position, (C) corolla tube length, (D) corolla mouth diameter, (E) corolla limb length and (F) flower width. Traits (A–D), showing significant species \times morph interaction (see Supplementary Data Table S3A), are each presented in two panels; traits (E), (F), without significant species \times morph interaction, in one panel each. Bars with different case letters indicate significantly different means of floral traits in pairwise comparisons (significance level: $P < 0.05$). Sequential Bonferroni correction was used to account for multiple tests.

P. vulgaris at both high and low organ levels (Fig. 3A, B), whereas, relative to flower size, the positions of pin and thrum anthers and pin stigmas did not significantly differ between *P. elatior* and *P. vulgaris* (all $P > 0.08$), and

thrum stigmas were placed significantly lower in the corolla of *P. elatior* than in the corolla of *P. vulgaris* ($P < 0.001$). Finally, anther and stigma positions were significantly lower in *P. elatior* than in *P. veris* at both the high and low

organ levels (all $P < 0.001$), when analysed either with or without correcting for flower size (Fig. 3A, B).

DISCUSSION

Reciprocal herkogamy is the main morphological trait characterizing heterostylous species. It is thought to promote outcrossing by increasing the efficiency of pollen transfer between anthers and stigmas placed at the same level in different floral morphs via disassortative pollination (Darwin, 1877; Barrett, 2002). Here, we investigated for the first time whether degrees of reciprocity between sexual organs differed within vs. between pairs of three closely related primroses that produce fertile hybrids in nature (e.g. Richards, 2003). While intermorph spatial matching of sexual organs was generally high within species, we found a pattern of lower reciprocity between species, although the reduction was more marked in some pairwise comparisons than in others (Fig. 2). Below, we discuss the complex partitioning of intra- vs. interspecific variation of reciprocity between sexual organs, the relationship between the placement of reproductive organs and other floral traits, and the potential functional and evolutionary consequences of the observed patterns.

Intraspecific patterns: high sexual organ reciprocity and correlations among floral traits

Our analyses determined that the positions of reproductive organs placed at the same level in pin and thrum flowers matched considerably well within each of the three studied primrose species (Figs 1 and 2), with no significant interpopulational or interspecific differences in the extent of reciprocal herkogamy (Tables 1 and 2B). The amounts of reciprocity found in Swiss populations of *P. vulgaris*, *P. veris* and *P. elatior* were generally similar to those calculated for Hungarian populations of the first two primrose species (Kálmán *et al.*, 2004, 2007), Asian primroses (Zhu *et al.*, 2009; see also Chen, 2009, on mean sexual organ position) and other distylous taxa (e.g. Richards and Koptur, 1993; Brys *et al.*, 2008; Sánchez *et al.*, 2008, 2010; Ferrero *et al.*, 2011). Furthermore, we detected almost no overlap between the distributions of high and low organs within each species (Fig. 1B), indicating strong intraspecific differentiation of anther and stigma positions between long-styled and short-styled individuals in allopatric Swiss populations. The values of global reciprocity for *P. vulgaris*, *P. veris* and *P. elatior* (Table 2B) were below the threshold of $R = 0.05$ for distyly (Sánchez *et al.*, 2008), confirming general expectations of both high reciprocal herkogamy (Barrett, 2002) and lack of perfect spatial matching (i.e. $R = 0$) between sexual organs in distylous flowers (Lloyd and Webb, 1992a; Faivre and McDade, 2001).

Despite the globally high levels of intraspecific reciprocal herkogamy, we detected some marked differences in the degree of reciprocity between reproductive organs placed at the two levels in pin and thrum flowers: high organs (i.e. thrum anthers and pin stigmas) were generally more reciprocal than low organs (i.e. pin anthers and thrum stigmas; Table 2A). These differences were most pronounced in *P. veris*, intermediate in *P. elatior* and smallest in *P. vulgaris*

(Table 2A; Fig. 2). Such variation in the degree of reciprocity between the two organ levels is relatively common in distylous species (e.g. Richards and Koptur, 1993; Thompson and Dommée, 2000; Hernández and Ornelas, 2007; Kálmán *et al.*, 2007; Zhu *et al.*, 2009).

Stigma and anther measurements were significantly correlated with each other in both pin and thrum flowers (Table 3), as already reported for the same (Kálmán *et al.*, 2007) and other distylous primroses (Nishihira *et al.*, 2000), as well as additional distylous taxa (e.g. Paillet and Thompson, 1997; Rossi *et al.*, 2004). The placement of sexual organs was also strongly linked with the length of the corolla tube in each of the three *Primula* species, especially for the anthers of thrum flowers (Table 3), a pattern similar to that found in *P. sieboldii* (Nishihira *et al.*, 2000) and *Psychotria poeppigiana* (Faivre and McDade, 2001). Additionally, the corolla tube was longer in thrum than pin morphs for all three primroses (Fig. 3C), suggesting that differential corolla growth may play an important role in improving reciprocity between high-placed organs, as proposed for *Jasminum fruticans* (Thompson and Dommée, 2000).

Morph-related differences in corolla tube length are relatively common in heterostylous species, especially in those with filaments fused to the corolla tube (Thompson and Dommée, 2000, and references therein), including primulas. Filaments are short and invariable in *Primula*, thus anther position is essentially determined by the length of the corolla tube below the point of anther insertion (Richards, 1997, 2003; Webster and Gilmartin, 2006). A few studies investigated how processes of corolla tube and style elongation affect the final positions of anthers and stigmas in mature pin and thrum flowers (Stirling, 1932, 1933, 1936; Faivre, 2000; Li and Johnston, 2001, 2010; Webster and Gilmartin, 2003, 2006). The growth rates of styles and anthers diverged between pin and thrum morphs in four distylous primroses (Stirling, 1932). The difference in the final height of sexual organs between floral morphs seems to be explained mainly by differential cell expansion for the styles and differential cell division for the portion of the corolla tube below the point of anther insertion (Heslop-Harrison *et al.*, 1981; Dulberger, 1992; Webster and Gilmartin, 2006). Detailed developmental studies would be required to clarify exactly how growth processes in the corolla and style determine the stronger correlation of high organs (i.e. pin stigmas and thrum anthers) with corolla tube length (Table 3) and the greater spatial matching between high- than low-placed organs in *P. elatior* and *P. veris* (Fig. 2A, B).

As expected, the position of sexual organs was less strongly correlated with lateral floral traits (i.e. corolla mouth diameter, corolla limb length and flower width) than with corolla tube length (Table 3). Additionally, the opening of the corolla tube was wider in thrums than in pins for each species (Fig. 3D). Both results are consistent with patterns described in other primroses (e.g. Fey, 1929; Nishihira *et al.*, 2000; Kálmán *et al.*, 2004, 2007) and heterostylous species (Ferrero *et al.*, 2011). Conversely, the length of the corolla limb and flower width did not differ significantly between floral morphs (Fig. 3E, F). The two latter traits are associated with pollinator attraction (see Rosas-Guerrero *et al.*, 2011), thus their lack of differentiation between pin and thrum

morphs is instrumental to the optimal functioning of disassortative pollination, which requires that pollinators do not discriminate between floral morphs when visiting distylous flowers (Charlesworth and Charlesworth, 1979; Ganders, 1979; Lloyd and Webb, 1992b; Barrett, 2002). The intermorph differences between sexual organ placement, corolla tube length and corolla mouth diameter most probably influence how pollinators interact with the flowers when they probe for nectar (placed at the bottom of the style in both morphs of primroses), remove pollen from the anthers and deliver pollen to the stigmas (Harder and Barrett, 1993; Richards, 1997, 2003; Armbruster et al., 2009), presumably playing a key role in optimizing pollen transfer between morphs.

Interspecific patterns: decrease of sexual organ reciprocity and correlations among floral traits

We observed a pattern of decreased sexual organ reciprocity between vs. within the three studied primroses (Table 2; Fig. 2), although exceptions existed in different pairwise comparisons. The use of GLMMs allowed us to test whether the inter- vs. intraspecific differences of reciprocity were statistically significant. Reproductive organs were significantly less reciprocal in all eight *P. vulgaris*–*P. elatior* comparisons (Fig. 2A, C), seven out of eight *P. elatior*–*P. veris* comparisons (Fig. 2A, B) and only two out of eight *P. veris*–*P. vulgaris* comparisons (Fig. 2B, C). Congruently with intraspecific results, we also observed greater reciprocity between high than low sexual organs in all *P. vulgaris*–*P. elatior* and *P. elatior*–*P. veris* comparisons, while pin stigmas of *P. veris* vs. thrum anthers of *P. vulgaris* (Fig. 2C) and, especially, thrum anthers of *P. vulgaris* vs. pin stigmas of *P. veris* (Fig. 2B) were less reciprocal than low-placed organs.

The relationship between reciprocal herkogamy and disassortative pollination has been investigated exclusively within species, including in *P. veris* (Ornduff, 1980), *P. vulgaris* (Ornduff, 1979; Piper and Charlesworth, 1986), *P. elatior* (Schou, 1983) and other heterostylous species (Ganders, 1975, 1979; Stone and Thomson, 1994; Stone, 1995; Ree, 1997; Paillet et al., 2002; Lau and Bosque, 2003; Massinga et al., 2005; Hernández and Ornelas, 2007; Brys et al., 2008; Baena-Díaz et al., 2012). Despite the lack of studies that specifically examined the functional effects of reduced reciprocal herkogamy between species, some addressed how different levels of reciprocity affected pollen transfer within species. These studies are illuminating on possible interspecific patterns and their evolutionary implications. For example, a pollination experiment on distylous *Palicourea fendleri* revealed that more legitimate pollen was transferred between sexual organs with a better spatial fit (Lau and Bosque, 2003). Similarly, an increase of sexual organ reciprocity between pin and thrum morphs in distylous populations of *Oxalis alpina* produced a 30% increment of compatible pollen transfer as compared with tristylous populations of the same species (Baena-Díaz et al., 2012). In the style-dimorphic and self-compatible *Narcissus assoanus*, the seed per flower ratio was significantly higher when anthers of the lower whorl and low-positioned stigmas were highly reciprocal (i.e. long-styled pollen donors vs. short-styled pollen recipients), compared with crosses in which sexual organs did not match

by an average distance of 2.73 mm (i.e. short-styled pollen donors vs. short-styled pollen receivers). In a population of distylous *Lithospermum californicum* with two height classes of pin stigmas, five times more thrum pollen was deposited on pin stigmas of the same height than on pin stigmas that were about 1.3 mm shorter (Ganders, 1975), highlighting the influence of even minor distances between sexual organs on pollen flow (De Vos et al., 2012).

All significant differences in mean anther and stigma positions between *P. elatior*, *P. veris* and *P. vulgaris* documented in our study (Fig. 3) were of the same magnitude or larger than differences that altered pollen flow in the studies mentioned above. Therefore, it is reasonable to suggest that, given the positive relationship between greater sexual organ reciprocity and efficiency of pollen transfer documented within species, considerable reductions of reciprocity between species may reduce interspecific pollen flow, with potential effects on reproductive isolation (Campbell and Aldridge, 2006), although experimental confirmation is needed. In the case of the investigated primroses, we would expect a stronger reduction of pollen movement between *P. elatior* and *P. vulgaris* than between *P. elatior* and *P. veris* and especially between *P. veris* and *P. vulgaris*, because sexual organ reciprocity was lowest between *P. elatior* and *P. vulgaris* (Fig. 2).

The high levels of interspecific SOR between *P. veris* and *P. vulgaris* in seven out of eight comparisons (Fig. 2B, C) are congruent with the small degree of spatial separation between reciprocal sexual organs of the two species (Figs 3A, B; Supplementary Data Table S1). The similarity in the mean heights of *P. veris* and *P. vulgaris* sexual organs, especially for those placed low in the corolla tube (Fig. 3A, B), occurred despite the fact that the corolla tubes of *P. vulgaris* were longer than those of *P. veris*, particularly in thrum flowers (Fig. 3C). After adjusting for the difference in floral size, the sexual organs of *P. veris* were positioned proportionately higher in the corolla than those of *P. vulgaris*, providing an allometric explanation for the high levels of interspecific SOR observed between these two species, especially for low-placed organs (Fig. 2B, C). Furthermore, the similar position of sexual organs in *P. veris* and *P. vulgaris* suggests that pollen movement might occur relatively easily between these two species, potentially leading to frequent hybridization. However, the two species displayed the largest pairwise differences in corolla limb length and flower width (Fig. 3E, F), traits involved in pollinator attraction (see Rosas-Guerrero et al., 2011). These interspecific differences, coupled with the distinct floral odour (Darwin, 1877; Fey, 1929) and brighter yellow colour of *P. veris* (Fey, 1929; Richards, 2003), reduced overlap in phenology of the two species (Clifford, 1958; Richards, 2003; Brys and Jacquemyn, 2009; Jacquemyn et al., 2009) and anomalies in seed development of the hybrids (Valentine, 1955), may contribute to their reproductive isolation.

While our study is the first to calculate interspecific SOR explicitly, species-specific differences in anther and stigma positions can be compared with other studies. In allopatric populations of *P. veris* and *P. vulgaris*, we detected a high degree of similarity for the mean heights of pin anthers and thrum stigmas, respectively, between the two species (Fig. 3A, B). Our results contrasted with the intramorph

divergence of anther positions between the two species reported for a hybrid zone in Hungary (Kálmán *et al.*, 2004). The differences in sexual organ placement between allopatric and sympatric populations of the two countries may reflect local adaptation to different pollinator communities (reviewed by Laine, 2009; see also Pérez-Barrales and Arroyo, 2010) and/or reinforcement and character displacement in contact zones (reviewed by Widmer *et al.*, 2008), especially since post-zygotic barriers to gene exchange were found to be relatively high in interspecific crosses (Valentine, 1955). Hence, heterospecific pollen transfer could cause fitness disadvantages for both species, potentially providing a basis for selection on anther and/or stigma position. However, specific studies are needed to specify if reinforcement indeed affects anther and stigma height in the two species.

To conclude, we discovered a marked decrease of intermorph reciprocity between *P. elatior* and *P. vulgaris*, a negligible reduction between *P. veris* and *P. vulgaris*, and a mosaic pattern between *P. veris* and *P. elatior*. The association between levels of sexual organ reciprocity and pollen transfer demonstrated in several intraspecific studies suggests that these interspecific discontinuities of reciprocal herkogamy may contribute to modulating pollen flow between species. Detailed pollination studies aimed at comparing intra- and interspecific levels of pollen transfer between these closely related primroses are necessary to test the predictions for reproductive isolation derived from the present analyses of how reciprocal herkogamy is partitioned within and between species.

SUPPLEMENTARY DATA

Supplementary data are available online at www.aob.oxfordjournals.org and consist of the following. Table S1: results of generalized linear mixed effects models of sexual organ reciprocity among species, organ levels and organ types. Table S2: results of principal component analyses. Table S3: results of generalized linear mixed effects models of floral traits between species and morphs. Figure S1: scatterplot diagrams of principal component analyses on six floral traits in pins and thrums.

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