

# Experimental insights on Darwin's cross-promotion hypothesis in tristylous purple loosestrife (*Lythrum salicaria*)<sup>1</sup>

Joana Costa<sup>2,5</sup>, Sílvia Castro<sup>2,3</sup>, João Loureiro<sup>2</sup>, and Spencer C.H. Barrett<sup>4</sup>

**PREMISE OF THE STUDY:** Darwin proposed that the reciprocal arrangement of anthers and stigmas in heterostylous plants promotes cross-pollination through pollen segregation on pollinators' bodies. The floral tube in most heterostylous species constrains the feeding posture of pollinators determining the site of contact with sex organs located at different heights within a flower. Here, we evaluate Darwin's hypothesis in tristylous *Lythrum salicaria*, a species with a partially tubular corolla, and examine the extent to which the location of sex organs within a flower influence compatible and incompatible pollination. We predicted that the proficiency of cross-pollination would increase for more inserted sex organs due to the restrictions imposed by the floral tube on pollinator positioning.

**METHODS:** We used experimental trimorphic and monomorphic arrays and emasculated flowers to quantify intermorph pollen transfer and capture among all sex-organ heights, and estimated the contribution of intraflower self-pollination, geitonogamous self-pollination, and intramorph outcross pollination to total intramorph pollination.

**KEY RESULTS:** As predicted, disassortative pollination varied significantly with sex-organ height and was highest for short-level organs and lowest for long-level organs. In monomorphic arrays, most intramorph pollination resulted from outcross pollination followed by intraflower and geitonogamous self-pollination.

**CONCLUSIONS:** Our results provide evidence for the Darwinian hypothesis. Reciprocal herkogamy promoted varying degrees of disassortative pollination with the magnitude strongly influenced by sex-organ height within a flower.

**KEY WORDS** disassortative pollination; floral design; heterostyly; pollen capture; pollen transfer proficiency; sex-organ height.

The remarkable diversity of floral traits among angiosperm species largely results from natural selection on mechanisms promoting the advantages of cross-pollination and limiting the costs of self-pollination. In many animal-pollinated species with tubular flowers, floral design determines the spectrum of suitable flower visitors by imposing restrictions on their posture while probing flowers for nectar (Darwin, 1862; Faegri and Van der Pijl, 1971; Harder and

Johnson, 2009). The floral polymorphism heterostyly provides a noteworthy example of the structural fit between flowers and pollinators.

Populations of heterostylous species are characterized by the occurrence of two (distyly) or three (tristyly) style morphs that differ reciprocally in the positioning of anthers and stigmas, a condition known as reciprocal herkogamy (Webb and Lloyd, 1986). In addition, they commonly possess a heteromorphic incompatibility system, in which seed production mainly occurs after pollinations between anthers and stigmas of equivalent height (Barrett and Cruzan, 1994), as well as a suite of ancillary polymorphisms of pollen and stigmas (Dulberger, 1992; Costa et al., 2017). Darwin (1877) proposed the first adaptive explanation for the function of heterostyly. He interpreted reciprocal herkogamy as a floral mechanism promoting cross-pollination between anthers and stigmas positioned at equivalent heights, thus reducing pollen wastage on

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<sup>2</sup> Centre for Functional Ecology, Department of Life Sciences, University of Coimbra, Calçada Martim de Freitas, 3000-456 Coimbra, Portugal;

<sup>3</sup> Botanic Garden of the University of Coimbra, Calçada Martim de Freitas, 3000-456 Coimbra, Portugal; and

<sup>4</sup> Department of Ecology and Evolutionary Biology, University of Toronto, 25 Willcocks Street, Toronto, Ontario, Canada M5S 3B2

<sup>5</sup> Author for correspondence (e-mail: joana.costa@uc.pt)

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incompatible stigmas. He further suggested that disassortative pollination was achieved owing to segregated pollen deposition on the bodies of pollinators during their visits to flowers.

This hypothesis is widely recognized as “Darwin’s cross-promotion hypothesis” (see Lloyd and Webb, 1992b; Barrett and Shore, 2008), and its empirical evaluation has been conducted in numerous studies by quantifying pollen capture in natural populations of distylous species (e.g., *Jepsonia heterandra* Eastw. [Saxifragaceae], Ganders, 1974; *Pulmonaria obscura* Dumort. [Boraginaceae], Olesen, 1979; *Primula elatior* Hill [Primulaceae], Schou, 1983; *Linum perenne* L. [Linaceae], Nicholls, 1986; *Palicourea padifolia* (Willd. ex Schult.) C.M. Taylor & Lorence [Rubiaceae], Ree, 1997; reviewed in Barrett and Shore, 2008; Weller, 2009). The distinctive pollen size dimorphism of most distylous species allows unambiguous identification of the source of pollen on stigmas, thus enabling estimates of the amounts of intermorph vs. intramorph pollen transfer and capture.

Studies of the composition of stigmatic pollen loads are more limited in tristylous species because of their restricted taxonomic distribution. Also, because discrete pollen size trimorphism is uncommon, this complicates identification of the three pollen types on stigmas of the floral morphs. Specifically, tristylous species often exhibit considerable overlap in the size of pollen produced by mid- and short-level anthers (e.g., *Eichhornia crassipes* (Mart.) Solms [Pontederiaceae], Barrett, 1977; *E. paniculata* (Spreng.) Solms, Barrett, 1985; *Decodon verticillatus* (L.) Elliott [Lythraceae], Eckert and Barrett, 1994; *Oxalis suksdorfii* Trel. [Oxalidaceae], Ornduff, 1964; *O. alpina* (Rose) Rose ex R. Knuth, Weller, 1979). Nevertheless, studies of naturally occurring pollen loads in *Lythrum salicaria* L. [Lythraceae] (Mulcahy and Caporello, 1970) and *L. junceum* Banks & Sol. (Ornduff, 1975a) were conducted in an effort to assess Darwin’s cross-promotion hypothesis. Because it is not possible to distinguish pollen from mid- and short-level stamens in these species, any conclusions reached could only concern pollen transfer between long-level organs. Discrete pollen-size trimorphism apparently only occurs in *Pontederia* [Pontederiaceae], and species in this genus provide an experimental system suitable for investigations on the effectiveness of reciprocal herkogamy in promoting disassortative pollination under field conditions. Indeed, several studies have examined pollen loads in natural populations of *P. cordata* L. (Price and Barrett, 1982; Barrett and Glover, 1985; Glover and Barrett, 1986) and *P. sagittata* C. Presl (Glover and Barrett, 1983), the former providing strong evidence for Darwin’s cross-promotion hypothesis (reviewed in Lloyd and Webb, 1992b).

Two issues need to be considered when experimentally testing Darwin’s cross-promotion hypothesis on the function of heterostyly using pollen load data. First, as originally pointed out by Ganders (1974, 1979), heterostyly should not influence the rate of intraflower self-pollination any more or less than would a similar separation of stigmas and anthers in a monomorphic (nonheterostylous) species. This is evident, because the nature of the floral polymorphism does not enter into the process of self-pollination within a flower (see Ganders, 1979, p. 621, fig. 5). Second, geitonogamous pollination (pollen transfer between flowers of a plant) will almost certainly occur in populations of heterostylous species with large floral displays, because pollinators tend to frequently move between flowers on the same individual (Lloyd and Webb, 1992b). Because the distinction between the sources of outcross pollen on stigmas, i.e., intermorph vs. intramorph, is crucial for a rigorous evaluation

of Darwin’s cross-promotion hypothesis (see Ganders, 1979), intraflower and geitonogamous components of the stigmatic pollen load should ideally be excluded by the emasculation of flowers on a plant. This procedure eliminates the sources of self-pollination, allowing a more accurate measure of the effectiveness of reciprocal herkogamy in promoting disassortative pollination (Ganders, 1974, 1979; Barrett and Glover, 1985; Lloyd and Webb, 1992b; Weller, 2009).

Here, we examine Darwin’s cross-promotion hypothesis in tristylous purple loosestrife (*Lythrum salicaria*) and assess the influence of sex-organ heights on patterns of disassortative pollination. A primary motivation for investigating *L. salicaria* was to improve on previous investigations of the species, which only considered pollen transfer between long-level organs, because of the inability to distinguish pollination events involving mid- and short-level pollen (Mulcahy and Caporello, 1970). In addition, we were also interested in evaluating these authors’ conclusion that heterostyly functions primarily to reduce intraflower self- and geitonogamous pollination, with the promotion of disassortative pollination assuming a secondary role (Mulcahy and Caporello, 1970, p. 1030). By using experimental arrays and the emasculation of flowers, we were able to distinguish pollen transfer and capture among the three sex-organ levels and to estimate the components of intramorph pollination, thus more fully evaluating Darwin’s cross-promotion hypothesis.

Our study addressed the following specific questions: (1) How does variation in the degree of insertion of stamen and stigma levels affect intermorph pollen transfer and capture? We predicted that short-level organs borne deep within the floral tube would have the highest proficiency of compatible pollen transfer and capture, and that long-level organs would have the lowest. This prediction is based on Lloyd and Webb’s proposal that the floral tube in heterostylous species restricts insect feeding posture, and that the path followed by the pollinator’s probe results in more precise pollen transfer between inserted than exerted sex organs (Lloyd and Webb, 1992b, p. 200, fig. 1; Stone and Thomson, 1994; Santos-Gally et al., 2013). (2) What are the levels of intramorph self- and intramorph outcross pollination and do they vary among floral morphs? By using monomorphic arrays and emasculated flowers, we compared the relative amounts of the three potential sources of intramorph pollen that can occur resulting from within-flower self-pollination, geitonogamous self-pollination, and intramorph outcross pollination. This approach enabled us to evaluate the suggestion of Mulcahy and Caporello (1970, p. 1030) that tristylous probably functions to “reduce selfing within flowers and between flowers on the same plant.” Our pollen load data allowed us to test Darwin’s cross-promotion hypothesis in the only tristylous species that he investigated in detail (Darwin, 1864, 1868, 1877), and also to evaluate the role of sex-organ height in affecting the pollination process.

## MATERIALS AND METHODS

**Study species**—*Lythrum salicaria* is a Eurasian wetland perennial that produces hundreds of purple-pinkish flowers (Mal et al., 1992) with a floral tube that is 5.0–6.5 mm long and 2.0–2.5 mm wide (Velayos, 1997). The floral tube is only partially developed extending from the base of the ovary until approximately the height of mid-level organs. Consequently, short-level organs are completely concealed within the floral tube, mid-level organs are located at the

mouth of the floral tube and long-level organs are exerted well beyond the floral tube (Fig. 1). This species possesses trimorphic incompatibility and is largely self- and intramorph incompatible (Darwin, 1877; Colautti et al., 2010). We collected seeds from two natural populations in Portugal (geographical coordinates, Coimbra: 40.20050, -8.42867; Penacova: 40.26967, -8.27467) in September 2013 and these were germinated in March 2014. Subsequently, individual plants were transferred singly to 2 L pots filled with a standard horticultural soil mix and fertilized every two weeks (Substral®, Mainz, Germany) until used in the pollination experiments described below. We conducted the experiments in mid-July 2015 in an experimental garden located at the Botanic Garden of the University of Coimbra, Portugal. This corresponds to the natural flowering time of populations in this region of the native area. Plants from the two populations were nearly identical in phenotype, were of comparable stature and flowering phenology, and we therefore did not distinguish the source of plants in the experimental arrays. No flowering plants of *L. salicaria* occurred in a radius of several km from the experimental garden and thus there was little opportunity for outcross pollen contamination.

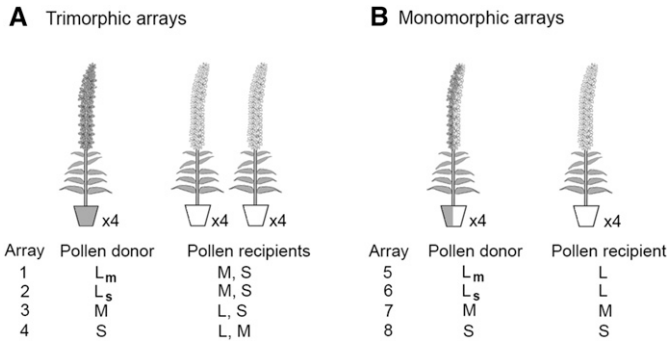
Determination of pollen-size distributions and pollen production among anther levels of *L. salicaria* is necessary for analyses of the composition of stigmatic pollen loads and calculation of pollen transfer proficiencies, respectively. We therefore characterized pollen size by measuring the polar axis and equatorial diameter of 20 hydrated pollen grains per anther level from five plants of each style morph (i.e., long-, mid-, and short-styled morph, hereafter referred to as L-, M-, and S-morph, respectively; Fig. 1) under a Leitz HM-LUX 3 light microscope (Leitz, Stuttgart, Germany) at 400× magnification by using a calibrated ocular micrometer. To quantify pollen production per anther level for each style morph, we randomly collected one flower from 10 plants of the three style morphs before anther dehiscence. One anther per level was later transferred to a microscope slide and dissected under a binocular microscope to release all pollen grains from pollen sacs; pollen grains were then counted under a Leitz light microscope at 100× magnification as the slide was shifted back and forth in a linear fashion across the entire coverslip. Previous investigations of pollen production in *L. salicaria* reported no significant differences in production among anthers within the same stamen level (Caswell and Davis, 2011). Thus, we multiplied the value obtained from the single anther sampled per stamen level by six to account for the number of anthers per stamen level within each flower. We used the data on pollen production to calculate pollen transfer proficiencies (see Statistical Analysis section).

**Experimental trimorphic arrays**—To investigate intermorph compatible (legitimate) and incompatible (illegitimate) pollen transfer and capture, we set up four trimorphic arrays containing 12 plants, four of each style morph, placed approximately 20 cm apart in a six by two grid. Plants were trimmed to approximately the same number of flowers (between 15–20 open flowers per plant). In each array, one style morph was assigned as a “pollen donor”, and its flowers were left intact, while the remaining two morphs were “pollen recipients” and their flowers were all emasculated (Fig. 2A). We emasculated flowers to exclude possibilities for intramorph pollination in each array and to unambiguously identify the source of intermorph pollen on recipient stigmas. We removed anthers 3 h before anthesis by using fine forceps and both the anther filaments and corolla were left intact during this procedure.



FIGURE 1 Flowers of *Lythrum salicaria*. (A) long-styled morph, (B) mid-styled morph, (C) short-styled morph.



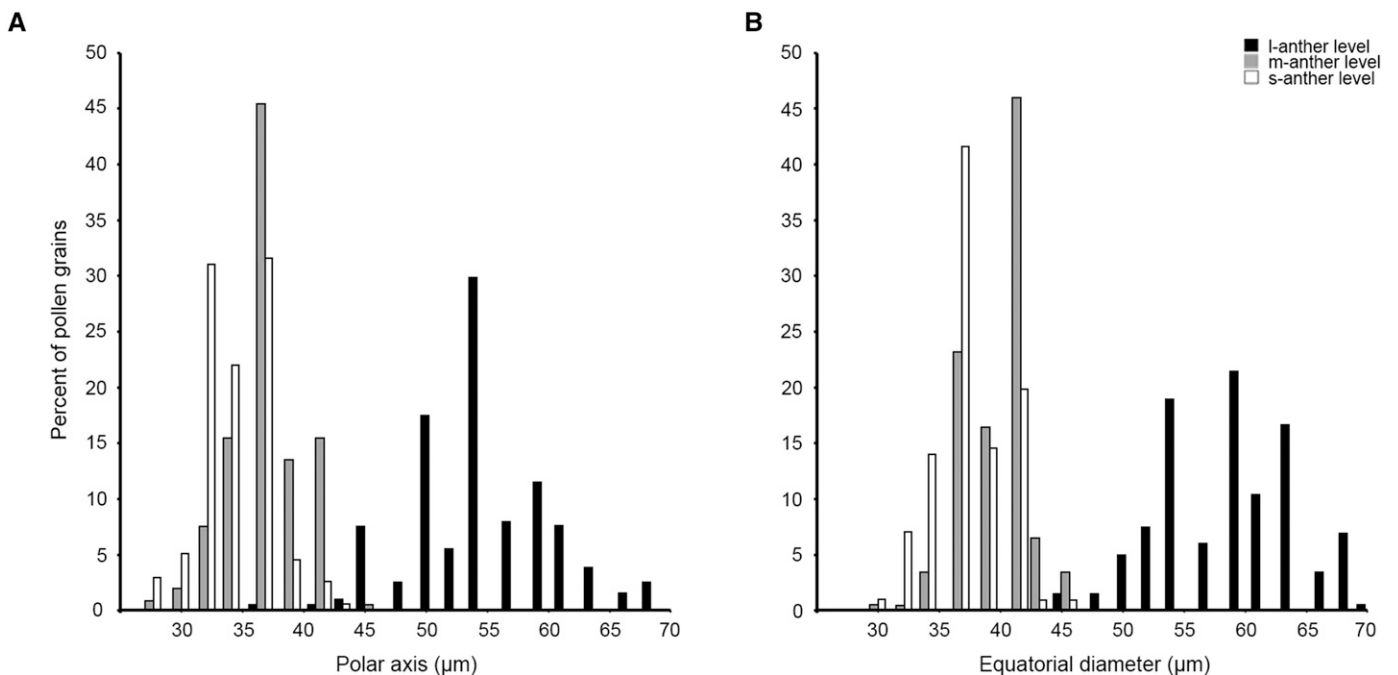


**FIGURE 2** Experimental arrays of *Lythrum salicaria* used in this study. (A) Trimorphic arrays. The shaded inflorescences represent pollen donor with intact flowers, whereas unshaded inflorescences represent pollen recipients with all flowers emasculated. (B) Monomorphic arrays. The half-shaded inflorescence represents the pollen donor with half of its flowers intact and half emasculated, whereas unshaded inflorescences indicate pollen recipients with all flowers emasculated. L, M, and S indicate the long-, mid-, and short-styled morphs, respectively. L<sub>m</sub> and L<sub>s</sub> indicate L-morph plants with only mid-level anthers and L-morph plants with only short-level anthers as pollen donors, respectively. The number of pollen donors and recipient plants in an array is given next to each plant.

Because of the overlap in size of pollen grains produced by mid- and short-level anthers of the L-morph (Fig. 3), two separate experimental arrays were required when using this morph as a pollen donor. In contrast, only a single array was necessary when using the M- and S-morphs as pollen donors. For the two arrays using the L-morph as a pollen donor, only a single anther level (either mid- or short-level) donated pollen to recipients in each array (Fig. 2A), with the alternate anther level being removed by emasculation.

Plants in the arrays were left to experience open pollination for 2 h, after which time we removed stigmas from emasculated recipient flowers with fine forceps, and these were then squashed underneath a coverslip on a microscope slide using glycerin as a mounting medium. Under a Leitz light microscope at 400× magnification, we counted the number of compatible and incompatible conspecific pollen grains on stigmas. Our arrays received abundant pollinator visits and therefore 2 h were sufficient to obtain pollinated stigmas from all plants. The sampled stigmas could potentially receive intermorph pollen from reciprocal and non-reciprocal anther levels. The four experimental arrays were randomly assigned to a given day and replicated twice, with one array during the morning (10 am to 12 pm; GMT) and the other during the afternoon (2 to 4 pm; GMT). The arrays involved different plants thus allowing us to account for within-day variation in pollen transfer and capture. Following the completion of the first set of arrays, we performed a second set so that the four trimorphic arrays were replicated twice in a randomized block design with a two-day interval between the blocks, thus each array was replicated four times.

**Experimental monomorphic arrays**—To investigate intramorph pollination, including self-pollen transfer and capture, we set up four monomorphic arrays containing eight plants of one style morph, placed approximately 20 cm apart in a four by two grid. Plants were trimmed to have approximately the same number of flowers (between 15–20 open flowers per plant). In each array, four plants were assigned as pollen donors and half of their flowers were left intact and half were emasculated. This procedure was undertaken so that we could investigate intraflower and geitonogamous pollination separately. The remaining four plants were assigned as pollen recipients and their flowers were all emasculated (Fig. 2B). We emasculated pollen recipient flowers so that intramorph pollination between plants was not confounded with intramorph



**FIGURE 3** The frequency distribution of pollen size produced by each anther level ( $n = 200$  pollen grains) for experimental arrays of *Lythrum salicaria*. (A) Polar axis of pollen. (B) Equatorial diameter of pollen.

pollination within plants. As for trimorphic arrays, two experimental arrays were required for the L-morph in which emasculation of one of the two anther levels was performed (Fig. 2B), and this enabled us to distinguish the source of pollen in stigmatic pollen loads. We used the same emasculation procedure as for trimorphic arrays.

Plants were left for open pollination for 2 h and then stigmas were removed with fine forceps from pollen donors (intact and emasculated flowers) and recipient plants (all flowers emasculated) and treated in the same manner as described previously in Experimental Trimorphic Arrays section. We used the same randomized block design previously described for trimorphic arrays, and each monomorphic array was replicated four times. On pollen donor plants, intact flowers received intramorph outcross pollen, geitonogamous, and intraflower self-pollen (Total pollen load = Outcross + Geitonogamous + Intraflower Self), whereas emasculated flowers of pollen donor plants captured intramorph outcross and geitonogamous pollen (Total pollen load = Outcross + Geitonogamous). Emasculated flowers of pollen recipient plants (all flowers emasculated) captured only intramorph outcross pollen (Total pollen load = Outcross).

By subtracting the total average pollen load of emasculated flowers on recipient plants (i.e., Outcross) from the total average pollen load of emasculated flowers from donor plants (i.e., Outcross + Geitonogamous), we estimated the amount of geitonogamous pollination for each style morph. Similarly, by subtracting the total average pollen load of emasculated flowers of donor plants (i.e., Outcross + Geitonogamous) from the total average pollen load of intact flowers from donor plants (i.e., Outcross + Geitonogamous + Intraflower Self), we estimated the amount of intraflower self-pollination for each style morph.

**Pollinator visitation**—To determine if insects visiting *L. salicaria* flowers in experimental arrays showed a preference for intact compared to emasculated flowers, we conducted pollinator observations in trimorphic arrays. We recorded the identity of insect visitors to flowers and the number of intact vs. emasculated flowers that were visited during 10 min intervals for a total of 180 min of observation across the two experimental blocks.

**Statistical analysis**—We assessed differences in pollen production between anther levels of each style morph and in total pollen production among style morphs either by a one-way analysis of variance (ANOVA) or the nonparametric Kruskal-Wallis test depending on data distribution. We investigated differences in: (1) pollen size (i.e., polar axis and equatorial diameter) among anther levels, and (2) pollinator visitation between intact and emasculated flowers by using a generalized linear model with a Gamma distribution and a log link function.

In our analysis of pollination patterns, we used pollen capture to refer to the stigmatic pollen loads (female function), while pollen transfer refers to the probability of a single pollen grain of a given anther level being deposited on the stigma of each style morph, i.e., pollen transfer proficiency (male function). We calculated the pollen transfer proficiency ( $T_{ij}$ ) for each experimental array following Lloyd and Webb (1992b):

$$T_{ij} = \frac{(\text{average stigma load})_{ij} \times (\text{number of flowers})_j}{(\text{pollen/flower})_i \times (\text{number of flowers})_i}$$

where  $i$  and  $j$  are the style morphs in a given array, and average stigma load<sub>ij</sub> is the average number of type  $i$  pollen grains on each type  $j$  stigma. This formula accounts for any variability in pollen and flower production among style morphs (Lloyd and Webb, 1992b).

We investigated differences in pollen transfer proficiencies and capture given as stigmatic pollen loads per flower among style morphs for: (1) compatible and incompatible intermorph pollinations, and (2) intramorph pollinations, by generalized linear mixed models (hereafter GLMM) with a Gamma distribution and a log link function. Here, and in the following GLMM analyses, pollen transfer proficiency and stigmatic pollen loads are the dependent variables. In the intermorph comparisons, anther level and style morph were specified as fixed factors in a model either across anther levels or style morphs, for pollen transfer and capture, respectively. In the analyses conducted for assessing pollen transfer of each anther level and pollen capture by style morph, either style morph or anther level was treated as a fixed factor, respectively. Intramorph pollen type (i.e., intramorph outcross, geitonogamous and intraflower self-pollen) and style morph were fixed factors in a model either across intramorph pollen types or style morphs for pollen transfer and capture, respectively. Style morph and intramorph pollen type were used as fixed factors in separate analyses of pollen transfer of each intramorph pollen type and capture by style morph, respectively. Here, and in the following GLMM analyses, time of day (i.e., morning or afternoon) nested within day was treated as a random factor. Prior to statistical analysis, stigmatic pollen load data were  $\log_{10}(x+2)$  transformed (Zar, 2010).

To evaluate Darwin's cross-promotion hypothesis from both male and female perspectives, we compared the proficiency of transfer of each component of the stigmatic load (i.e., intermorph compatible, intermorph incompatible, intramorph outcross, geitonogamous, and intraflower self-pollen) to the three stigmas (i.e., long-, mid-, and short-level) and their capture by individual style morphs. The component of the stigmatic pollen load was defined as a fixed factor in the GLMMs.

We performed all statistical analyses with R software version 3.0.1 (R Development Core Team, 2013) using the following packages: "car" for Type-III analysis of variance as an integrated part of the generalized linear models and GLMM models (Fox and Weisberg, 2015), "lme4" for generalized mixed models (Bates et al., 2014), "multcomp" for multiple comparisons after Type-III analysis of variance (Hothorn et al., 2015), "nlme" for linear mixed models (Pinheiro et al., 2015), and "stats" for Shapiro-Wilk normality test, Kruskal-Wallis test, and generalized linear models (R Development Core Team, 2013).

## RESULTS

**Pollen size and production**—There were significant differences in the polar axis (mean  $\pm$  SE, long-level:  $54.05 \pm 0.39$ ; mid-level:  $36.20 \pm 0.20$ ; short-level:  $33.75 \pm 0.20$ ;  $\chi^2_{2,597} = 3286.60$ ,  $P < 0.001$ ) and equatorial diameter (mean  $\pm$  SE, long-level:  $58.08 \pm 0.37$ ; mid-level:  $39.06 \pm 0.19$ ; short-level:  $36.67 \pm 0.20$ ;  $\chi^2_{2,597} = 3908.70$ ,  $P < 0.001$ ) of pollen grains produced by the three anther levels. However, there was considerable overlap in the overall size of pollen produced by mid- and short-level anthers, whereas pollen from long-level anthers was significantly larger and there was virtually no size overlap with pollen from mid- and short-level anthers (Fig. 3).

There was no significant difference in pollen production per flower between anther levels of the L-morph (mean  $\pm$  SE, mid-level: 7905.00  $\pm$  529.34; short-level: 7917.60  $\pm$  711.88;  $F_{1,18} = 0.002$ ,  $P = 0.99$ ). In contrast, pollen production per flower differed significantly between anther levels of the M-morph (mean  $\pm$  SE, long-level: 5566.80  $\pm$  474.16; short-level: 8614.30  $\pm$  748.68;  $F_{1,18} = 14.82$ ,  $P = 0.001$ ) and the S-morph (mean  $\pm$  SE, long-level: 4884.40  $\pm$  402.48; mid-level: 7671.30  $\pm$  938.17;  $H_{1,18} = 7.00$ ,  $P = 0.008$ ). In general, long-level anthers produced significantly less pollen than mid- and short-level anthers ( $P < 0.05$ ), the latter two not differing in pollen production. We found no significant differences in total pollen production per flower among style morphs (mean  $\pm$  SE, L-morph: 15,822.60  $\pm$  1066.00; M-morph: 14,181.10  $\pm$  1014.47; S-morph: 12,555.70  $\pm$  1251.63;  $F_{2,27} = 2.14$ ,  $P = 0.14$ ).

**Pollinator visitation**—The primary visitor to flowers of *L. salicaria* during the experiment was the long-tongued bee *Anthidium manicatum* (L.) [Megachilidae], with individuals of this species accounting for 81.5% of the total insect visits. Less frequently, we observed *Apis mellifera* L. [Apidae], syrphid flies [Syrphidae] and *Xylocopa violacea* L. [Apidae] visiting flowers in the arrays. There was no significant difference in the level of visitation to intact and emasculated flowers (mean number of flowers visited during 10 min intervals  $\pm$  SE, 12.02  $\pm$  1.77 and 9.50  $\pm$  1.20 for intact and emasculated flowers, respectively;  $\chi^2_{1,106} = 3.75$ ,  $P = 0.05$ ).

**Compatible and incompatible intermorph pollen transfer and capture**—We detected a significant interaction between anther level and recipient style morph in pollen transfer proficiencies ( $P < 0.001$ ; Table 1). Pollen produced by the exerted long-level anthers had a significantly higher probability of being transferred to stigmas of the L-morph than to stigmas of the M- or S-morphs ( $\chi^2_2 = 34.49$ ,  $P < 0.001$ ; Fig. 4A; Appendix S1, see Supplementary Data with this article). In contrast, pollen from mid-level anthers was transferred to stigmas of all three morphs equivalently ( $\chi^2_2 = 1.51$ ,  $P = 0.47$ ; Fig. 4A; Appendix S1). The most proficient transfer of pollen

was evident from the inserted short-level anthers, which was preferentially transferred to stigmas of the S-morph ( $\chi^2_2 = 34.83$ ,  $P < 0.001$ ; Fig. 4A; Appendix S1).

Intermorph pollen capture was significantly different among the style morphs ( $P < 0.001$ ; Table 1), and there was a significant interaction between recipient style morph and anther level ( $P < 0.001$ ; Table 1). Stigmas of the L-morph captured significantly more incompatible than compatible intermorph pollen ( $\chi^2_2 = 31.31$ ,  $P < 0.001$ ; Fig. 4B; Appendix S1), whereas stigmas of the S-morph captured significantly more compatible than incompatible intermorph pollen ( $\chi^2_2 = 897.44$ ,  $P < 0.001$ ; Fig. 4B; Appendix S1). Stigmas of the M-morph captured similarly large amounts of pollen from the mid- and short-level anthers, but less pollen from long-level anthers ( $\chi^2_2 = 345.39$ ,  $P < 0.001$ ; Fig. 4B; Appendix S1).

**Intramorph pollen transfer and capture**—The components of intramorph pollen differed significantly in their transfer proficiency to each style morph ( $P = 0.003$ ; Table 1). The probability of intramorph outcross pollen transfer was significantly higher for the L-morph than for the M- and S-morphs ( $\chi^2_2 = 23.42$ ,  $P < 0.001$ ; Fig. 5A; Appendix S2). In contrast, we detected no significant differences in geitonogamous pollen transfer ( $\chi^2_2 = 2.11$ ,  $P = 0.35$ ; Fig. 5A; Appendix S2) or intraflower self-pollen transfer ( $\chi^2_2 = 0.73$ ,  $P = 0.70$ ; Fig. 5A; Appendix S2) among style morphs.

There were significant differences in pollen capture among style morphs ( $P = 0.001$ ; Table 1) and among the three components of the intramorph pollen load ( $P = 0.002$ ; Table 1). Geitonogamous pollen capture was significantly lower than intramorph outcross and intraflower pollen capture in each of the three style morphs (L-morph,  $\chi^2_2 = 8.64$ ,  $P = 0.01$ ; M-morph,  $\chi^2_2 = 11.33$ ,  $P = 0.003$ ; S-morph,  $\chi^2_2 = 16.91$ ,  $P < 0.001$ ; Fig. 5B; Appendix S2). There were small differences in capture of intramorph outcross pollen and intraflower self-pollen, but these were not statistically significant in each of the three style morphs ( $P > 0.05$ ; Fig. 5B).

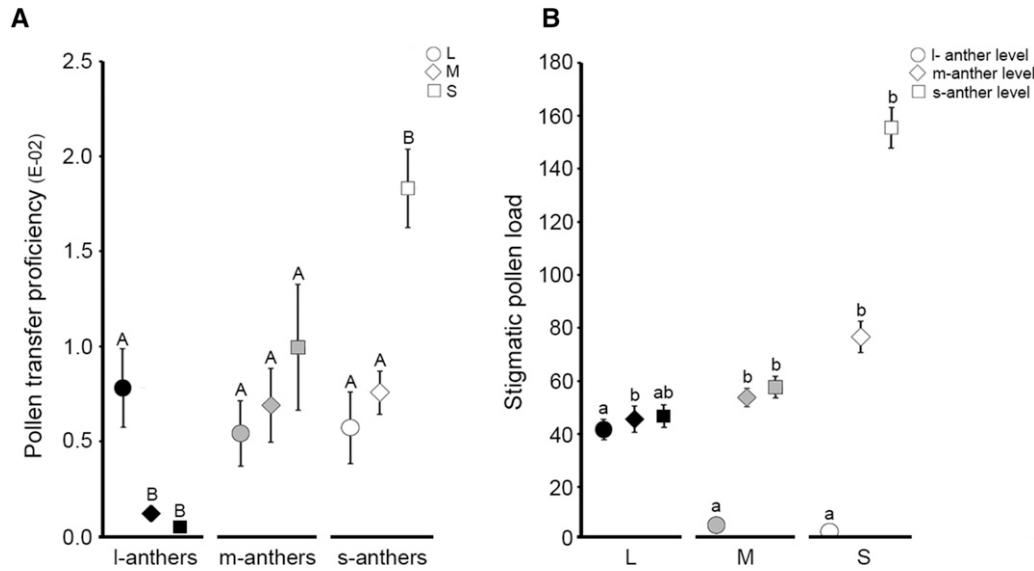
**Evidence for Darwin's cross-promotion hypothesis**—Darwin's cross-promotion hypothesis would be supported if values for compatible intermorph pollen transfer and capture were significantly higher than equivalent values from incompatible intermorph pollen transfer and capture. This was indeed the pattern for the M- and S-morphs, with higher pollen transfer and capture between anthers and stigmas that were reciprocally positioned (Fig. 6; Appendix S3). We found no differences between compatible and incompatible intermorph pollen transfer and capture for the L-morph (Fig. 6; Appendix S3).

## DISCUSSION

Our investigation of pollen transfer and capture in tristylous *Lythrum salicaria* revealed several main findings: (1) As predicted, disassortative pollen transfer and capture was greatest for anthers and stigmas located deep within the floral tube and diminished with increased levels of sex-organ exertion (Fig. 4); (2) Intramorph (incompatible) pollination occurred mainly between plants and was particularly high in the L-morph (Fig. 5); (3) Most self-pollination resulted from intraflower pollen transfer and capture, with levels of geitonogamous pollination being relatively low in each of the three style morphs (Fig. 5). Our results provide some support for Darwin's cross-promotion hypothesis (Fig. 6),

**TABLE 1.** Results of the Generalized Linear Mixed Models (GLMM) examining pollen transfer and capture in (a-b) trimorphic and (c-d) monomorphic arrays of *Lythrum salicaria*. Values in bold represent statistically significant differences, df = degrees of freedom.

	df	Wald $\chi^2$	P
(a) Trimorphic arrays: pollen transfer			
Style morph	2	<b>69.02</b>	<b>&lt; 0.001</b>
Anther level	2	0.20	0.905
Style morph $\times$ anther level	4	<b>68.95</b>	<b>&lt; 0.001</b>
(b) Trimorphic arrays: pollen capture			
Style morph	2	<b>296.97</b>	<b>&lt; 0.001</b>
Anther level	2	<b>52.42</b>	<b>&lt; 0.001</b>
Style morph $\times$ anther level	4	<b>278.12</b>	<b>&lt; 0.001</b>
(c) Monomorphic arrays: pollen transfer			
Style morph	2	3.84	0.146
Component of the pollen load	2	<b>11.74</b>	<b>0.003</b>
Style morph $\times$ component	4	2.78	0.595
(d) Monomorphic arrays: pollen capture			
Style morph	2	<b>13.26</b>	<b>0.001</b>
Component of the pollen load	2	<b>12.25</b>	<b>0.002</b>
Style morph $\times$ component	4	7.71	0.103



**FIGURE 4** Pollen transfer and capture in trimorphic arrays of *Lythrum salicaria*. Values are the mean and standard error of pollen transfer proficiencies and number of pollen grains per stigma for pollen transfer and capture, respectively (Appendix S1, see Supplementary Data with this article). (A) Pollen transfer proficiency of each anther level to the stigma. See Materials and Methods for the calculation of pollen transfer proficiencies. Different uppercase letters indicate statistically significant differences in pollen transfer proficiency. (B) Pollen capture based on stigmatic pollen load for each style morph. Different lowercase letters represent statistically significant differences in pollen capture of style morphs. L, M, and S indicate the long-, mid-, and short-styled morphs, respectively.

with disassortative pollination varying in a predictable way with sex-organ height. Below we consider the floral mechanisms governing the pollination process in *L. salicaria* and compare our findings with other studies of pollen capture in heterostylous species.

**Evidence for disassortative pollination**—The majority of the studies investigating Darwin’s cross-promotion hypothesis have examined the composition of stigmatic pollen loads in natural populations of heterostylous species (reviewed in Lloyd and Webb, 1992b; Barrett and Shore, 2008; Weller, 2009). These studies have most commonly compared the relative frequency of compatible (legitimate) and incompatible (illegitimate) pollen captured by stigmas with random expectations based on the frequency of pollen types in populations (e.g., Ganders, 1974; Olesen, 1979; Glover and Barrett, 1983). In our study, we compared compatible and incompatible intermorph pollen capture in experimental arrays using emasculated flowers, so that we could distinguish pollen transfer among the three sex-organ levels. This was necessary because of the strong overlap in size of pollen produced by mid- and short-level anthers (Fig. 3). Following the identification of pollen types in stigmatic pollen loads, we used analysis of variance to compare the amounts of compatible and incompatible pollen capture, and quantified the proficiency of pollen transfer as the likelihood that a single pollen grain is involved in compatible or incompatible pollination. This latter method, first introduced by Lloyd and Webb (1992b), provides a means of evaluating the effectiveness of heterostyly in promoting pollination success through male function. Since its introduction, this approach has been used to analyze patterns of pollen transfer in several distylous species (*Palicourea padifolia* (Willd. ex Schult.) C.M.Taylor & Lorence [Rubiaceae], Ree, 1997; *Persicaria japonica* (Meiss.) H. Gross ex Nakai

[Polygonaceae], Nishihiro and Washitani, 1998; *Gaertnera vaginata* Poir. [Rubiaceae], Paillet et al., 2002; *Pulmonaria officinalis* L. [Boraginaceae], Brys et al., 2008; *Palicourea demissa* Standl., Valois-Cuesta et al., 2012). The method complements earlier studies of stigmatic pollen loads, which provided a maternal perspective on the pollination process.

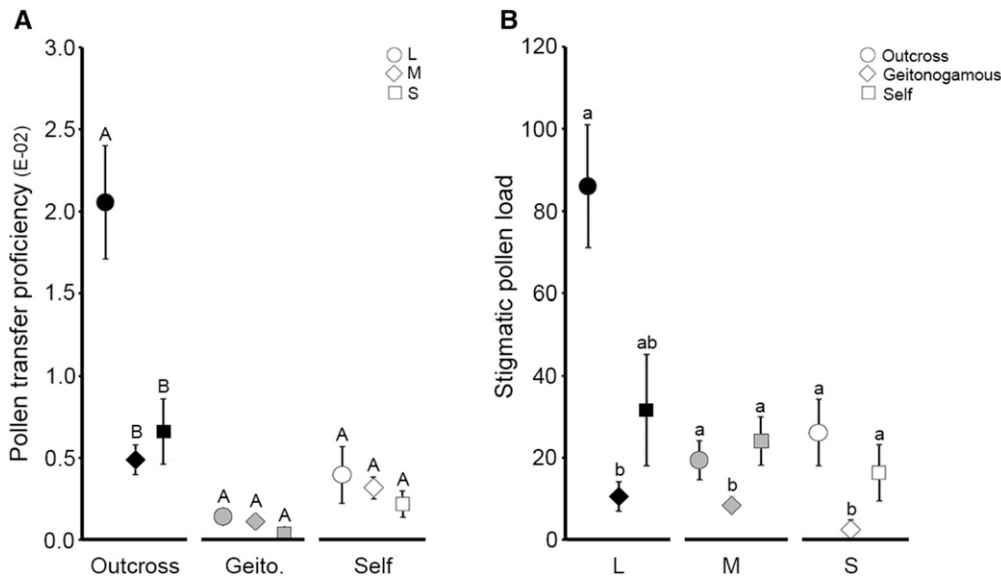
Previous investigations of tristylous *L. salicaria* (Mulcahy and Caporello, 1970) and *L. junceum* (Ornduff, 1975a) reported disassortative pollination in the L-morph. Despite high compatible pollen transfer proficiency by long-level anthers (Fig. 4A), we found that incompatible pollen accounted for approximately 50% of the intermorph pollen captured by stigmas of the L-morph. Earlier studies of pollen capture in distylous (e.g., Ornduff, 1979; Weller, 1980) and tristylous (Mulcahy and Caporello, 1970; Ornduff, 1975a) species also reported high levels of in-

compatible pollination in the L-morph. In contrast to the L-morph, our data indicated that disassortative pollen transfer was high for mid- and particularly short-level anthers, and stigmas of the M- and S-morphs captured mainly compatible intermorph pollen, particularly the S-morph. These results therefore provide support for Darwin’s cross-promotion hypothesis by demonstrating significant levels of disassortative pollination in the M- and S-morphs, although the amount of compatible pollen transferred and captured varied between them. In the L-morph, we found no strong evidence for disassortative pollination, although intermorph compatible pollen had a slightly larger transfer proficiency than intermorph incompatible pollen.

The morph-specific patterns of compatible intermorph pollination that we detected in *L. salicaria* are opposite to those previously reported in natural populations of tristylous *Pontederia cordata* (Barrett and Glover, 1985; Glover and Barrett, 1986) and *P. sagittata* (Glover and Barrett, 1983). In these species, compatible pollen capture occurred primarily in the L-morph, whereas the S-morph exhibited the lowest level of compatible pollen capture. Unfortunately, it is not possible to fully compare our results with those previously reported for *L. salicaria* (Mulcahy and Caporello, 1970). Despite the claim made by these authors that pollination was “predominantly legitimate” (Mulcahy and Caporello, 1970, p. 1027), this conclusion is not warranted based on the data presented in their article because of the inability to distinguish mid- and short-level pollen on stigmas.

It is unclear what factors are responsible for the contrasting patterns of pollen capture between the L- and S-morphs of *L. salicaria* and *Pontederia* species. Differences in floral design, the spatial distribution of floral morphs and the pollinator fauna visiting flowers may contribute to the observed differences (see Glover and Barrett,





**FIGURE 5** Pollen transfer and capture in monomorphic arrays of *Lythrum salicaria*. Values are mean and standard error of pollen transfer proficiencies and number of pollen grains per stigma for pollen transfer and capture, respectively (Appendix S2, see Supplementary Data with this article). (A) Pollen transfer proficiency of each intramorph pollen type (i.e., intramorph outcross, geitonogamous, and intraflower self-pollen) to stigmas. See Materials and Methods for the calculation of pollen transfer proficiencies. Different uppercase letters represent statistically significant differences in the proficiency of intramorph pollen transfer to the three style morphs. (B) Pollen capture based on stigmatic pollen load for each style morph. Different lowercase letters represent statistically significant differences in pollen capture within style morph. L, M, and S indicate the long-, mid-, and short-styled morphs, respectively. “Geito.” = geitonogamous pollen.

1983, 1986). It is also possible that our experimental treatments, including the removal of alternate anther levels in the L-morph, may have influenced pollen pickup and delivery in ways different from those that would occur in natural unmanipulated populations. However, we have no evidence from our data or observations of pollinators that this procedure significantly affected pollination events involving the L-morph as a pollen donor. Our experiments go beyond the previous dichotomy involving “long pollen” vs. “non-long pollen” in *Lythrum* pollen flow studies (Mulcahy and Caporello, 1970; Ornduff, 1975a), and provide for the first time, unambiguous measures of intermorph pollination among the three sex-organ levels of *L. salicaria*.

Stereomorphic depth-probed flowers (i.e., tubular flowers with concealed nectar at their base; Lloyd and Webb, 1992a) are thought to foster more precise pollen transfer and capture than open bowl-shaped flowers, thus promoting disassortative pollination in populations of heterostylous species (Lloyd and Webb, 1992a; Santos-Gally et al., 2013). The height at which the centrally placed stigma is located in flowers of heterostylous species determines the initial space available for the pollinator during entry to the flower (Lloyd and Webb, 1992b; Stone and Thomson, 1994). One of the main questions we addressed in our study was whether the degree of sex-organ insertion in the floral tube of *L. salicaria* influenced patterns of disassortative pollination. Because our experimental arrays were visited primarily by the solitary bee *Anthidium manicatum* (Pechuman, 1967), with 81.5% of flowers visited by this species, we can probably exclude pollinator identity as a significant source of variability in pollen transfer and capture, allowing us to isolate the influence of sex-organ height on the pollination process. Our experimental results revealed a consistent pattern in accord

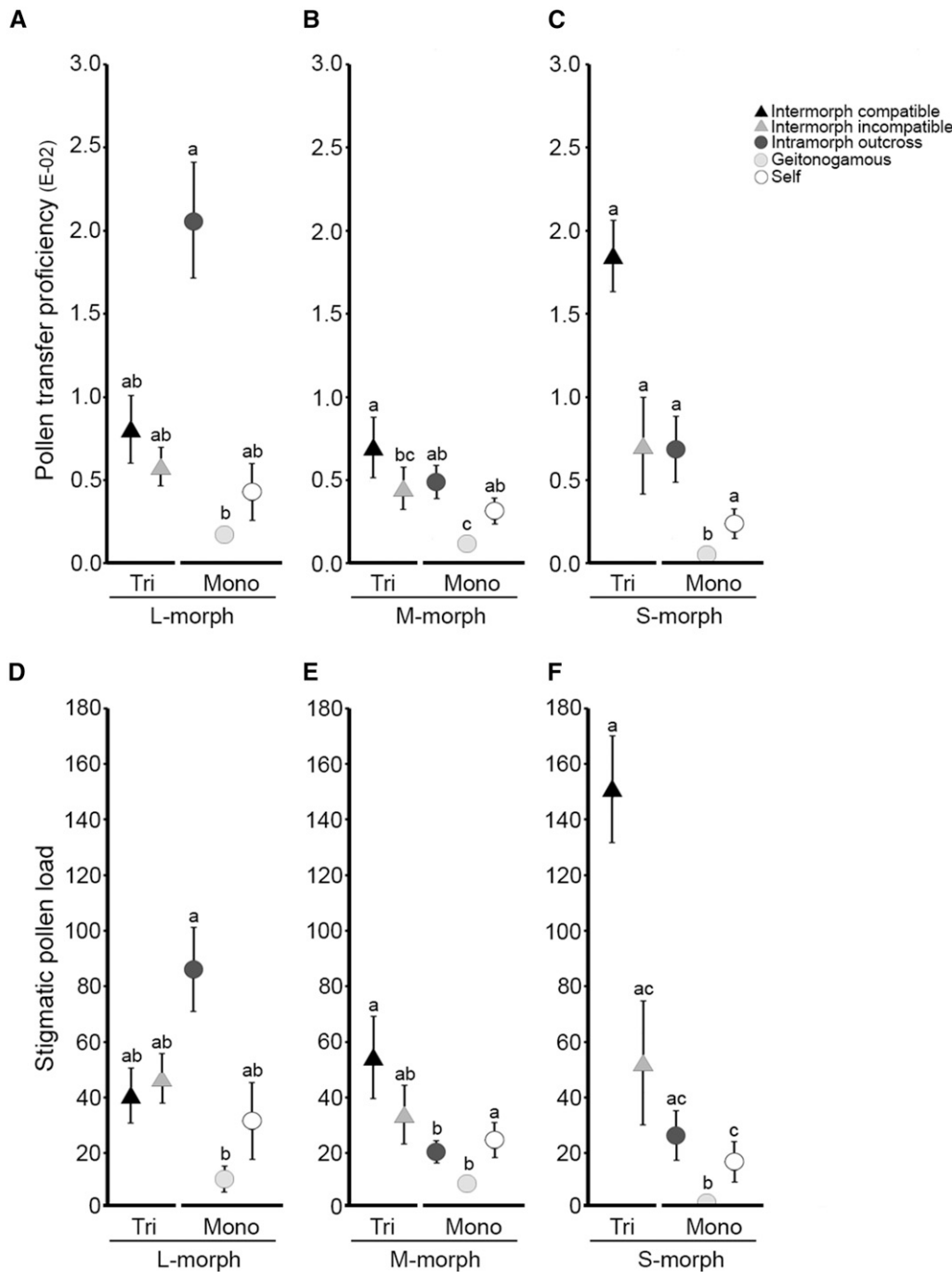
with our predictions. There was a decrease in the precision of pollen transfer between anthers and stigmas from short- to mid- to long-level sex organs. The floral tube of *L. salicaria* likely restricts pollinator movements during contact with short- and to a lesser extent mid-level organs. In contrast, the unspecialized dish-shaped terminal region of the flower (Fig. 1), where the highly exerted long-level organs are located, allows a wide range of contact points between the sex organs and the pollinator’s body, thus limiting the precision of intermorph pollen transfer (see also Ornduff, 1975b; Björkman, 1995).

#### **Decomposing the sources of intramorph pollination**

We used experimental monomorphic arrays to provide insight into the sources of intramorph pollination in *L. salicaria*. Three potential types of intramorph pollen can be transferred to stigmas by pollinators—intraflower self-pollen, geitonogamous self-pollen, and intramorph outcross pollen. We found that the main source of intramorph pollen on stigmas of each of the style morphs was pollen transferred from other plants in the arrays and not self-pollination, which was overall much reduced. Values of intraflower self-pollen transfer were higher than geitonogamous pollen transfer, but there were no significant differences in these two forms of self-pollination among the three style morphs. Of particular interest was the finding that stigmas of the L-morph captured significantly more intramorph outcross pollen than stigmas of the M- and S-morphs, thus raising the question of what factors might account for this pattern.

Monomorphic arrays of L-morph plants resemble a nonheterostylous species with approach herkogamous flowers. This form of herkogamy is the most common type of intraflower spatial separation of anthers and stigmas in angiosperms (Webb and Lloyd, 1986). When pollinators visit approach herkogamous flowers, they usually contact the exerted stigma before touching the anthers, thus promoting outcrossing (Webb and Lloyd, 1986; Lloyd and Webb, 1992b; Barrett, 2003, p. 995, fig. 3). Because L-morph flowers of *L. salicaria* exhibit approach herkogamy, the high level of intramorph outcross pollination we detected was probably caused by the initial contact between the pollinator’s body and stigma when bees entered the floral tube. In the only other experimental study of a tristylous species that has used monomorphic arrays of each of the style morphs, Kohn and Barrett (1992) reported the highest outcrossing rates in arrays of the L-morph of *Eichhornia paniculata*. Collectively, these results support the hypothesis that the morphology of the L-morph is superior to those of the other style morphs in favoring pollen transfer between plants, including intramorph outcross pollination.





**FIGURE 6** Pollen transfer and capture of each component of the stigmatic load in experimental arrays of *Lythrum salicaria*. Values are mean and standard error (Appendix S3, see Supplementary Data with this article). (A, B, C) Pollen transfer proficiencies to stigmas of the L-, M-, and S-morphs, respectively, of each pollen type (i.e., compatible intermorph, incompatible intermorph, intramorph outcross, geitonogamous, and intraflower self-pollen). See Materials and Methods for the calculation of pollen transfer proficiencies. (D, E, F), Pollen capture based on stigmatic pollen load for the L-, M-, and S-morphs, respectively. Different lowercase letters represent statistically significant differences in pollen transfer and capture within style morphs. L-, M-, and S-morph indicate the long-, mid-, and short-styled morphs, respectively. "Tri" and "Mono" for trimorphic and monomorphic arrays, respectively.

A criticism of the vast majority of studies investigating Darwin's cross-promotion hypothesis in natural populations of heterostylous species has been the failure to distinguish between self- and

intramorph outcross pollen on stigmas. This is necessary because reciprocal herkogamy is a population-level floral polymorphism, and therefore cannot reduce the amount of self-pollination any more or less than other monomorphic floral strategies with the same degree of herkogamy (Ganders, 1979; Lloyd and Webb, 1992b), contrary to what Mulcahy and Caporello (1970) concluded. A more rigorous assessment of the function of heterostyly requires the emasculating of flowers and comparison of the relative amounts of intermorph vs. intramorph pollen on stigmas (e.g., Ganders, 1974; Barrett and Glover, 1985). Our results provided evidence for a significant component of self-pollination in all three style morphs that if not excluded by emasculation would confound evaluation of the efficacy of heterostyly in promoting disassortative pollination. Indeed, when the confounding effects of self-pollination were excluded by emasculation in distylous *Jepsonia heterandra* (Ganders, 1974), *Palicourea padifolia* (Ree, 1997), *Persicaria japonica* (Nishihira and Washitani, 1998), and tristylous *Pontederia cordata* (Barrett and Glover, 1985), reciprocal herkogamy was shown to be successful in promoting intermorph compatible pollination supporting Darwin's cross-promotion hypothesis.

Geitonogamous pollination is a unique mode of self-pollination because it involves the same mechanisms used for cross-pollination, but confers no reproductive assurance and results in complete pollen and seed discounting (Lloyd, 1992). Heterostyly promotes cross-pollination through pollen segregation on the bodies of pollinators, and thus pollen grains remain on a pollinator's body until transferred to stigmas of a reciprocal style morph. In this way, heterostyly increases pollen carryover (Feinsinger and Busby, 1987; Harder and Barrett, 1996; Matsumura and Washitani, 2002), and in species with multiflowered inflorescences probably functions to limit geitonogamy and pollen discounting (Harder and Barrett, 1996). Our study supports the hypothesis that

heterostyly may function to limit geitonogamy, as also suggested by Mulcahy and Caporello (1970). We found that levels of between flower self-pollination were the lowest of the three components of intramorph pollination in monomorphic arrays.

The levels of geitonogamous pollination that we obtained for *L. salicaria* were roughly similar to previous estimates for distylous *Gaertnera vaginata* (Pailler et al., 2002). They were consistently low across each of the three style morphs indicating that their particular morphologies limit self-pollination between flowers on an inflorescence. However, it is possible that by trimming inflorescences to 15–20 flowers in our experimental arrays, we may have reduced geitonogamous self-pollination to lower levels than would occur in natural populations. In the most comprehensive study investigating the relative contributions of intra- and between-flower selfing rates in a tristylous species, Eckert (2000) found that geitonogamy was the prevailing mode of selfing in *Decodon verticillatus*. However, in this highly clonal species floral display involves numerous ramets per clone, which together with the local foraging of pollinators and the mass flowering habit of the species may lead to particularly high levels of geitonogamy.

In conclusion, our manipulative experiments involved garden arrays raising the question to what extent our findings apply to natural populations of *L. salicaria*. The pollination process in heterostylous species is affected by diverse factors including floral design (Harder and Barrett, 1993; Stone and Thomson, 1994), variation in stigma-anther separation (Baena-Díaz et al., 2012; Keller et al., 2014), floral display size (Harder and Barrett, 1995), geographical variation in environmental factors (Hodgins and Barrett, 2008; Cunha et al., 2014), pollinator diversity (Wolfe and Barrett, 1988; Stone, 1996), population size (Waites and Ågren, 2004), and the patterns of style morph distribution within populations (Levin, 1968; Lughadha and Parnell, 1989). In our study, we focused on the influence of sex-organ height on pollen transfer and capture. Because most of the pollination in our arrays was mediated by a single solitary bee, *Anthidium manicatum*, this enabled us to isolate the influence of features of floral morphology (e.g., sex-organ height) on the pollination process. The variation in pollen transfer and capture that we measured among sex-organ levels probably differs in natural populations of *L. salicaria*, because the diversity of insect visitors to flowers is often greater than in our experimental arrays. Bumblebees, solitary bees, honey bees, wasps, butterflies, and syrphid flies have all been observed foraging on flowers of *L. salicaria* in native European populations (Waites and Ågren, 2004; J. Costa, personal observations) and introduced North American populations (Brown et al., 2002; King and Sargent, 2012). These insect groups vary in body size and foraging behavior and the patterns of pollen transfer and deposition that we report in our experiments are therefore likely to be more complex in natural populations.

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