Major Article

Title: Sources of variation in reciprocal herkogamy in the distyly floral syndrome of *Linum tenue* (Linaceae).

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Manuscript received _____; revision accepted _____

Running Head: Reciprocal herkogamy in *L. tenue*

Keywords: adaptive accuracy; maladaptive bias; cell length; disassortative pollen transfer; floral development; imprecision; reciprocal herkogamy

ABSTRACT

Premise of the Research: Distyly is a floral polymorphism involving reciprocal herkogamy shaped by selection for pollen transfer efficiency. The variation of the floral organs involved in pollen transfer can be individually affected by environmental and genetic sources of variance, but the organ development will be canalised to minimize reciprocal inaccuracy between anthers and stigmas as this is the focus of selection.

Methodology: We measured floral organ and cell length of both morphs of distylous *Linum tenue* (Linaceae) at different developmental stages of field- and glasshouse-grown plants. We analysed the results to measure reciprocal inaccuracy and identify sources of variance.

Pivotal results: Flowers from the field were larger than those from the glasshouse due to both environmental and genetic (population) factors. Pistil and stamen length in adult flowers correlated with flower size, but reciprocal herkogamy was mostly invariant to the size individual floral organs. The length of short floral organs showed greater maladaptive bias, while the length of tall organs showed greater imprecision. During development, the pistils of pin flowers grew at a faster rate than in thrum flowers mostly due to cell elongation, while cell division was more important for male organ height.

Conclusions: Distyly in *L. tenue* involves the interaction of multiple coordinated developmental and environmental mechanisms leading to limited but predictable patterns of variance in the expression of reciprocal herkogamy.

INTRODUCTION

Heterostyly is a breeding system characterised by the presence of typically two (distyly) or sometimes three (tristyly) reciprocally herkogamous floral morphs (Lloyd and Webb, 1992a; Barrett and Shore, 2008). Distylous species exhibit a thrum morph, with short styles and long stamens, and a pin morph, with long styles and short stamens. Reciprocal spatial displacement (reciprocal herkogamy) of floral organs functions to reduce sexual interference through the spatial separation of sexual organs within the flower (Harder and Barrett, 2006), and by promoting disassortative mating through selective pollen transfer according to the reciprocal positioning of the sexual organs (Darwin, 1877; Lloyd and Webb, 1992a; Stone and Thomson, 1994; Simón-Porcar et al., 2014; Zhou et al., 2015). A highly optimized distyly system could therefore be described as showing high reciprocal herkogamy, where the positioning of individual floral organs in each morph are highly complementary. The developmental and environmental mechanisms by which this complementarity is achieved in a distylous species case study are the focus of this paper.

Heterostyly has been observed in at least 28 plant families and thought to have evolved independently at least 20 times (Ganders, 1979; Barrett and Shore, 2008). Heterostylous species are thus a remarkable exemplar of convergent evolution (Lloyd and Webb, 1992a). Detailed studies of the morphology and development of flower form in heterostylous species have contributed to understanding the functional significance of different floral traits and their evolution starting from the seminal work of Stirling (1932). These studies have shown that the presence of other heterostyly-associated traits such as self-incompatibility and pollen size, also known as ancillary traits, contribute to the prevention of selfing through structural and physiological intramorph incompatibilities (Ganders, 1979; Dulberger, 1992; Barrett et al., 2000; Wolfe, 2001; Keller et al., 2014; Costa et al., 2017). Within-morph variation is regularly observed and can be linked to reproductive success. For example, woodland

populations of *Primula veris* show more variable floral morphs than their grassland counterparts that affects seed set of the different morphs (Brys and Jacquemyn, 2015). Nonetheless, to our knowledge no studies have examined the direct influence of environmental factors on the expression of distylous traits, although other traits that might influence reproductive success have been examined, such as plant size as part of an elevational transplant experiment of distylous *Primula nivalis* (Abdusalam and Li, 2018). Morphological studies can reveal instances of allometric relationships and developmental constraints between different floral organs, such as the case of distylous species with epipetalous flowers, where stamens are connected to the corolla, leading to non-independence between stamen height and corolla depth (Faivre, 2000, Faivre and McDade, 2001; Pérez-Barrales et al., 2014; Sá et al., 2016). Knowledge about pollinators and their relative disassortative pollination efficiency can provide further insights into the function and evolution of distylous floral traits, such as the relative outcrossing efficiency of pin versus thrum morphs or long- versus short-tongued pollinators (Armbruster et al., 2006; Pérez-Barrales and Arroyo, 2010; Santos-Gally et al., 2014; Simón-Porcar et al., 2014; Deschepper et al., 2018). Genomics advances are rapidly improving our understanding of the genetic control of distyly in the distantly related genera, *Primula* (Huu et al., 2016; Li et al., 2016) and *Turnera* (Shore et al. 2019) and, recently, *Linum* (Gutiérrez-Valencia 2022). These independently evolved systems show remarkable convergence at the level of genetic architecture. Each system seems to be controlled by a haplotype or supergene region present in hemizygous form in thrum morph individuals only containing multiple genes targeting developmental signalling pathways specific to male and female tissues (Kappel et al. 2017). This genetic architecture therefore requires careful fine-tuning of male and female floral organ development, with the development of each tissue dependent on the presence or absence of two or more genes in the thrum supergene in order to achieve a high degree of

reciprocal herkogamy in mature floral morphs. Recent advances in the morphometric analysis of distyly to quantify adaptive inaccuracy allows better identification of the developmental and functional constraints of distyly expression (Armbruster et al. 2017; Brys and Jacquemyn, 2019; Matias et al., 2020; Furtado et al., 2021).

Linum (Linaceae) has been important for the study of heterostyly ever since Darwin's pioneering work first showed the association between pollination capacity and different floral morphs (Darwin, 1862; 1877). Distyly is common in *Linum*, being present in over 40% of the ca. 180 *Linum* species in four of the five sections of the genus (McDill et al., 2009; Ruiz-Martín et al., 2018). Alongside morph-specific variation in levels of self-incompatibility, ancillary traits commonly associated with distyly have also been observed in *Linum*: including differences in the surface structure and biochemical composition of pin and thrum pollen and stigmas, and three-dimensional adjustment of reproductive organ positioning (Lewis, 1943; Rogers, 1979; Ghosh and Shivanna, 1980; Dulberger, 1981; 1987; Armbruster et al., 2006). Proteomic and transcriptomic studies of style dimorphism in *L. grandiflorum* have identified a shortlist of *S* locus gene candidates with exclusive or enhanced expression in thrum styles (Ushijima et al., 2012). One of these candidate genes, *THRUM STYLE-SPECIFIC1* (*TSS1*), also appears to be hemizygous, present only in thrum DNA and protein extracts (Ushijima et al., 2015; Gutiérrez-Valencia 2022), analogous to recent findings for the genetic control of distyly in *Primula* and *Turnera* (Kappell et al., 2017). However, distyly in *L. grandiflorum* is atypical, with only style length showing a distinct difference between floral morphs (Ushijima et al., 2015) and available material is only limited to studies of horticultural cultivars. Studies of additional *Linum* species are required since distylous *Linum* species display substantial morphological variation within and between morphs (Wolfe, 2001; Armbruster et al., 2006, Bigio et al., 2017; Ruiz-Martín et al., 2018), including within-species breeding system variation (Nicholls, 1985; 1986). These reports of within-morph

variation suggest the possibility of selection on developmental and functional constraints to optimise reciprocal herkogamy.

In this study, we identify and characterise sources of variation in reciprocal herkogamy in the distyly floral syndrome of *L. tenue*. In order to separate floral traits and other population and environmental influences that contribute to the expression of distyly, we measured floral organ length in open flowers both in the field and the glasshouse, in developing flower buds and measured floral organ cell lengths in plants grown in the glasshouse. To test how within and between variation in floral organs and populations might affect the function of distyly, we use these data also to calculate morph ratios and reciprocal accuracy. Further, we measured developing flowers to determine when and where floral morph differences occur. Finally, we examined floral organ cell length to determine their contribution to morph differences. We complemented these data with observations of pollinator behaviour in the field to verify their ability to contact anthers and stigmas of pin and thrum flowers with different parts of the body and promote pollen transfer between morphs.

MATERIALS AND METHODS

Species description

Linum tenue is a locally frequent annual forb of grassland that occupies meadows, olive groves, and orchards. The species, growing 30 to 150 cm tall, is distylous with actinomorphic yellow flowers with five-fold symmetry including five sepals, petals, stamens and carpels (fused at the ovary into a single pistil). Sepals and petals form a corolla with basal nectaries, internal short reproductive organs, and slightly protruding tall reproductive organs (Fernández-Galiano Fernández et al., 1987). The native range of *L. tenue* extends through southwest Iberia and northwest Africa, although recent phylogenetic studies suggest the lineages on each continent are separate species (Ruiz-Martín et al., 2018).

Plant sampling

To capture any potential between population variation in distyly expression, this study used samples from 21 natural populations each consisting of 100s to 1000s of individuals across the region of Andalusia, Spain (Figure 1G, Table A1). Either flowers or seeds were collected from 10-80 separately sampled maternal plants from each population, with at least one-meter distance among sampled individuals to avoid pseudoreplication. Flower collection involved one newly open flower per individual plant that was preserved in separate 1.5 mL screw-top microcentrifuge tubes of 70% ethanol. Seed collection involved several ripe fruit capsules per plant placed in separate glassine envelopes. Later, in the glasshouse, one seed per maternal plant was germinated in individual 10 cm diameter pots of two thirds John Innes no. 2 compost (ICL, Ipswich, UK) and one third Perlite (LBS horticultural supplies, Colne UK) grown to flowering in greenhouses at the Department of Biosciences, Durham University, UK, under semi-controlled growth conditions of 20°C for 16 hours of day length and 15°C for 8 hours of darkness. Upon flowering, approximately all individuals from each population were visually classified as pin or thrum floral morphs prior to more detailed floral organ measurement.

Open flower floral organ length and herkogamy

In order to quantify the contribution of different floral traits and other conditions to within and between morph variation, measurements of floral organ length and herkogamy of field and glasshouse open flowers were made from field collected flowers stored in 70% ethanol or one to three freshly opened flowers per glasshouse grown individual. Flowers were dissected whorl-by-whorl under a dissecting microscope and the vertical length of the sepals and petals, petal width, stamens, and their component filaments and anthers, pistils and their component styles, stigmas, and ovaries were measured from the base of the ovary, shown in Figure 1, using a combination of Vernier callipers and analysis of photos using ImageJ software

(Rasband, 2017). Only ovaries, styles, and filaments were measured for field collected flowers as other floral organs tended to be shed when stored in alcohol. The digital photographs of dissected flowers were made against a 1 mm ruled graph paper background using a Leica M80 light microscope (Leica Biosystems, Nussloch, Germany) set at 7.5 times magnification connected to a computer. Multiple investigators contributed different measures at different times, so to account for observer differences in the glasshouse data, a constant was added to the measures of each morph type for each observer, in order to have the same mean as the samples measured using ImageJ, which were considered to be the most accurate measures.

Linear mixed effect models on the data for fresh flowers from the glasshouse were used to test floral organ lengths and herkogamy (difference in height between pistils and stamens) for the fixed effects of flower morph (pin or thrum), flower size measured as petal length, and their interaction, while controlling for the random effects of sample individual nested within sample population. These analyses were conducted on Ln(value +1) transformed data as recommended for studies to identify allometric effects (Armbruster and Wege, 2019). The lmerTest R package (Kuznetsova et al., 2017) was used to fit models and assess the significance of main effects using the restricted maximum likelihood approach. The significances of random effects were assessed by analysis of variance comparisons of nested models dropping individual effects first, followed by population effects. The proportions of variances (coefficient of determination, r^2) explained by the models were calculated using the MuMIn R package (Bartoń, 2017) that evaluates both marginal r^2 for fixed effects only and conditional r^2 for both fixed and random effects.

Morph frequency and adaptive inaccuracy

To evaluate the potential functional consequences of population-level morph variation, chi-squared (χ^2) tests were used to assess population deviations from the expected 1:1 morph

ratio under complete disassortative mating. Inaccuracy in the reciprocal placement of tall and short organs was estimated using the adaptive inaccuracy method developed by Armbruster et al. (2017). We use their terminology to describe measures and results. The disassortative pollination function of distylous flowers predicts that the optimal position of tall pin stigmas (S) is equal to the position of tall thrum anthers (A). Similarly, the optimum position of short thrum stigmas (s) is equal to the position of short pin anthers (a). Hence, it is possible to estimate population-level adaptive inaccuracy by studying the contribution of differences between the means of tall organs and short organs (maladaptive bias), and the variance of organ position (imprecision) compared to expected perfect matching between anthers and stigmas of pin and thrum flowers. The measures were done on height of filaments and styles (Figure 1), excluding anthers and stigmas as anthers had been mostly shed for the wild-sampled flowers, for each individual with measures done for nine or more flowers of each morph type per population. First, the mean and variance of tall and short male and female organs were calculated. Tall organ maladaptive bias was calculated as the square of the difference between male and female tall organ means, while tall organ adaptive inaccuracy was calculated as the tall organ maladaptive bias plus the imprecision of each tall organ. The equivalent calculations were performed for short organ measures. To facilitate comparisons between tall and short organs and the relative contributions of maladaptive bias and imprecision in each of these organs, all measures were then expressed as percentages of total measures . Total adaptive inaccuracy was calculated as the sum of tall and short adaptive inaccuracies and presented as absolute mm2 values. To evaluate the relative magnitude of adaptive inaccuracy in terms of flower size, standardised adaptive inaccuracy was calculated as a percentage of the total tall and short organ mean lengths (see Armbruster et al., 2009 for detailed description of the mathematical equations).

Confidence intervals were obtained by bootstrapping using a custom script (GH. Bolstad, personal communication).

Floral organ length of developing flowers

To determine when and where floral morph differences develop, one to three developing flowers of various bud sizes, representing different developmental stages from approximately ten to one days prior to opening, were collected from each glasshouse-grown individual to describe the morphological development of stamens and pistils in pin and thrum flowers. Flowers were dissected and photos of floral organs were taken a Leica M80 light microscope (Leica Biosystems, Nussloch, Germany) set at 7.5 times magnification connected to a computer and measured using ImageJ, as described for the open flowers. To identify differences in flower development, linear mixed effect models were used to test floral organ lengths and herkogamy for the fixed effects of developing bud morph type, bud developmental stage (measured as petal length), and their interaction, while controlling for the random effects of sample individual nested in sample population, using an analogous approach to the open flower analysis.

Filament and style cell length

To examine the expression of distyly at a cellular level, whole filaments and styles were separated from freshly harvested newly open flowers from glasshouse-grown individuals. Floral organs were mounted on microscope slides, stained with 0.05 % toluidine blue solution, and viewed at 100x to 400 times magnification using a differential interference contrast Leica DMI2500 microscope (Leica Biosystems, Nussloch, Germany) with an eyepiece graticule and photographed with a Panasonic GP-US932HAE camera (Panasonic UK, Bracknell, UK). To account for localised differences in cell length at different positions along the organs, each style and stamen filament was classified into five approximately equal-length regions, R1 to R5, counting from base to tip. Using ImageJ software (Rasband,

2017), up to 20 clearly visible cells were chosen from each image and measured along their longest axis to the nearest 0.1 μm.

Linear mixed effect models were used to test the potential effects influencing the dependent variable, floral organ cell length. The fixed effects were organ type (pistil or stamen), flower morph (pin or thrum), and organ region treated as an ordinal variable going from the base to the tip. There were five levels of region so models with higher order linear, quadratic, cubic, and quartic relationships were fitted for this variable. The random effects were sample individual nested in sample population as for the floral organ length analyses. The significance of random effects and both marginal and conditional r^2 coefficients were calculated as for the floral organ length analyses.

Pollinator observations

Observation of insect visits were conducted in two populations (Ronda km 10, Ronda km 17), on patches with 10-20 plants with open flowers in intervals of ca. 10 minutes, changing of patch after two intervals. During the observations, a record was made of the reward collected (nectar or pollen) and if insects contacted anthers and stigmas. Insects were identified to the lowest possible taxonomic level. All observations were made between 10:00 and 17:00.

RESULTS

Open flower floral organ length and herkogamy

The results of the mixed model analysis showed that the length of pistils and herkogamy of open flowers could be predicted by morph type but not the stamen length (Table 1). Morph type was also a significant predictor of the lengths of ovaries plus styles, but not for filament, stigma, anther, sepal or petal width (Table A2). Moreover, flower size as measured by petal length also had a significantly positive effect on pistil and stamen length, with shallow slopes of 0.309 and 0.317, respectively, indicative of non-linear allometry between these floral

organs. Despite these paired allometric relationships, the compound herkogamy measure was hardly affected by flower size (slope of 0.008). There was no evidence for an interaction effect between flower size and morph type in the expression of pistil and stamen length or herkogamy. Individual nested in population was shown to be a significant random effect in all tests, while the random effect of population was also significant in the test of pistil length and pistil-stamen herkogamy.

Morph frequency and adaptive inaccuracy

Population morph ratio (the percentage of pin flowers) of wild-measured populations ranged between 51.3% and 59%, and in all cases χ^2 analyses showed that populations did not depart significantly from the 1:1 ratio (Table 2). The male and female organs of field-sampled pin and thrum flowers were longer than glasshouse grown flowers in general, with the exception of the field sample from the population mva that showed similar floral organ lengths to glasshouse material (Table 2). The contribution of maladaptive bias and imprecision to adaptive inaccuracy of tall and short organs is summarised in Table 3. In comparison with glasshouse measures, absolute adaptive inaccuracy was greater for field measures, although standardised adaptive accuracy was smaller for field measures. For both field and glasshouse flowers, measures of maladaptive bias were generally greater for short organs compared to tall organs. In contrast, tall organs consistently showed greater imprecision than small organs, with the least imprecision being shown by short pistils.

Floral organ length of developing flowers

The youngest buds measured were less than 4 mm long and approximately 10 days from flowering while the longest buds were just less than 10 mm long and had fully developed petals that were about one day from opening, based on observations of other tagged but non-harvested flower buds. The results of the mixed model analysis showed that, during development, morph type was an important predictor of the lengths of pistils, stamens (Table

4), ovaries plus styles, stigmas, and pistil-stamen herkogamy but not the lengths of filaments, anthers, sepals, bud width, or ovary plus style-filament herkogamy (Table A3). Petal length showed significant positive relationships in all tests, indicative of overall floral organ growth during development. There was a significant interaction effect between morph type and petal length for developing pistils and stamens, and pistil-stamen herkogamy, indicating differences in the rate of growth and development of organ type in each morph. Both pistils and stamens lengthened during flower development (petal lengthening), but they did so at different rates and to different extents in different morph types (Figure 2). Growth rates were different between tall and short organs with thrum stamens having a steeper slope than pin stamens and pin pistils having a steeper slope than thrum pistils (Figure 2). The pin pistil showed the fastest growth rate and largest variance compared to the other organs, possibly reflective of less canalization and more developmental noise. The mixed model analysis results (Table 3) indicated that for most floral organs, the random effects of individual nested in population and population made significant contributions to reproductive organ length also.

Filament and style cell length

A summary of the results of mixed model analysis of floral organ cell length are presented in Table 5. The random effects of both individual nested in population and population were highly significant. Cell lengths were subject to significant interaction effects between morph, organ, and region. Significant positive linear and negative quadratic ordinal region effects were detected. These interacting effects on cell length are visualised in Figure 3. Pin styles and filaments of both morphs had shorter cells, about 50 μm long, in region 1 at the base of each organ that increased to a constant limit of about 125 μm by region 3. In contrast, cell lengths stayed consistently short at about 50 μm across all regions of thrum-morph styles. Therefore, cell length seemed to contribute to differences in style length between the two

morphs but not for the filaments and differences in the filament length between each morph must be achieved by alternative growth mechanisms, such as differences in cell division.

Pollinator observations

A total of 4h. and 8h. of observations were accumulated in Ronda km. 10 and Ronda km 17 respectively. At each site, the insect visitors foraged also outside the zone of observation so it was not possible to accurately record the number of visitors as opposed to the number of visits. In Ronda km 10, flowers were visited equally by *Usia* cf. *pusilla* and *Bombylius* cf. *major* (Bombiliidae), with 17 visits by each visitor during the period of observation. In Ronda km 17 visits were paid mostly by *Usia* cf. *pusilla*, and less frequently by small a Halictidae (cf. *Lasioglossum*), with 16 and 3 visits respectively. An example *Usia* visit is shown in Figure 4 (republished with permission from *Plant Biology*). In all cases, insects collected nectar, but displayed different behaviour. Both *Usia* and cf. *Lasioglossum* landed on the petals and crawled down to the bottom of the flower towards the nectaries to collect nectar, visiting all five nectaries. In all cases, short organs (i.e., pin anthers and thrum stigmas) were contacted more often than tall organs (i.e., pin stigmas and thrum anthers) with the dorsal part of the body. Specifically, these insects placed pin pollen on the head and the thorax, and thrum pollen on the abdomen, and less often on the thorax. In all cases, the contact with the floral organ was done with the dorsal part of the body, and not the ventral side. Because both *Usia* and cf. *Lassioglossum* moved inside the flower to harvest nectar from all nectaries, thrum stigmas were often contact along the body surface, from head to thorax, and sometimes the lower abdomen. In contrast, pin stigmas contacted the abdomen at lower rates that thrum stigmas. *Bombylius* visited by hovering in front of the flowers, but visits were fast and it was not possible to retrieve detailed information regarding the contact with anthers or stigmas.

DISCUSSION

Altogether, the morphological and cytometric findings in this study confirm that the breeding system of *L. tenue* is based upon reciprocal herkogamy between pin and thrum flowers that likely promotes disassortative mating between different floral morphs. Expression of pin and thrum floral traits is influenced by both environmental conditions and genetic background, but within-morph herkogamy is robust to these influences. Detailed measurements of floral reproductive organ and cell length identified that the floral traits that primarily distinguish the two morphs are pistil length and the direction of within-morph herkogamy, but these floral traits make differing contributions to the expression and function of distyly. Finally, the cell length results support the hypothesis that distinct developmental pathways in each floral organ and in each morph contribute to the overall distyly floral syndrome in *L. tenue*.

Morphological measures of open flowers showed that female organ height differences contributed more to reciprocal herkogamy within each morph type than male organ height (Tables 1 and S2). The lengths of other floral organs that are not directly involved in reciprocal herkogamy did not show significant differences between morphs, suggesting that they are not important contributors to the distyly floral syndrome in *L. tenue*. The expression of distyly, and herkogamy in particular, is relatively robust to environmental influences. To our knowledge, no previous study of distyly has combined both field and glasshouse measures. Adaptive inaccuracy measured in the field can indicate the influence of environmental variation on the development of reproductive organs, the reciprocal placement of tall and short organs and the magnitude of reciprocity (Armbruster et al., 2009 b; Jacquemyn et al., 2018). The same measures of glasshouse grown plants focuses more on the genetic contribution of floral variation to adaptive inaccuracy after controlling for environmental variation. In general, we found that larger flowers in both the field and

glasshouse produced longer reproductive organs, but that the degree of herkogamy between male and female organs was conserved (Tables 1, 2 and 3). This result highlights the importance of within-flower herkogamy to the distyly floral syndrome and the tight control of expression of this key trait. There was a trend for wild-sampled flowers to be larger than glasshouse-sampled flowers (Table 2). It might be that there is substantial environmentally induced variation in flower size depending on growing conditions, although all the measures reported here are within the range given for the species in the region where populations were surveyed (Flora Vascular de Andalucía Occidental, Fernández-Galiano Fernández et al., 1987). Alternatively, it might be that storage in ethanol of field-collected flowers might have affected the measures, although ethanol storage is a popular method of storing and fixing plant samples and is unlikely to have major effects. However different populations were measured under field and glasshouse conditions, so it was not possible to fully quantify the extent to which flower size is environmentally controlled. This issue should be investigated in future studies by studying more directly comparable samples under field and glasshouse conditions.

Individual- and population-level variation in reproductive organ height and herkogamy was found for *L. tenue* (Table 2), in common with other heterostylous species (Richards and Koptur, 1993; Eckert and Barrett, 1994; Faivre and McDade, 2001; De Vos et al., 2012; Brys and Jacquemyn, 2015). The presence of genetic variation in the expression of floral morphology associated with distyly could be an important source of standing variation to permit rapid and flexible breeding system responses to a changeable pollination environment (De Vos et al., 2012; Kissling and Barrett, 2013; Brys and Jacquemyn, 2015; Jiang et al., 2017; Simón-Porcar 2018). Differences in distyly expression between populations might be driven by spatial variation in pollination efficiency or the presence of other plant species that might compete for shared pollinators (Kálmán et al., 2007; Keller et al., 2012; Kissling and

Barrett, 2013). For example, fine-tuning of reciprocal pistil-stamen length differences might contribute to avoidance of interspecific hybridization as observed in a morphological survey of three *Primula* species (Primulaceae) (Keller et al., 2014). More extensive studies to explicitly examine individual- and population-level differences in distyly in *L. tenue* would help to better understand the fitness consequences of variation in distyly expression under local pollination conditions that could include pollinators and co-flowering species.

Male and female floral organ height of pin and thrum morphs contribute differently to reciprocal herkogamy. Our measures of adaptive inaccuracy provide insights into the functional constraints on heterostyly in *L. tenue* by considering different tall and short organ maladaptive bias and imprecision contributions to adaptive inaccuracy separately. Greater maladaptive bias was found for short reproductive organs, particularly the difference in the average position of short stigmas and tall anthers (Table 3). The maladaptive bias in short floral organ heights was due to thrum pistils generally being shorter than pin stamens (Table 2). Thrum pistils and their cell lengths also showed less imprecision than pin stamens, suggesting tighter inhibitory control of growth in this organ and morph. These findings match with a study of *L. grandiflorum* by Ushijima et al. (2015) that thrum style cells were generally shorter compared to other reproductive organs. Therefore, it is possible that limited cell elongation in this tissue leads to less imprecision in whole organ length. Only one out of the three previously studied *Primula* species, *P. veris*, also showed a greater maladaptive bias in short organs than tall organs in the reanalysis presented by Armbruster et al., (2017), highlighting that sources of maladaptive bias and imprecision are labile features of distyly that can differ between species.

Our adaptive inaccuracy results were broadly within the range of other studies that have measured adaptive inaccuracy in other distylous species that revealed greater imprecision in tall reproductive organs than short organs (Armbruster et al. 2017; Jacquemyn et al. 2018;

Brys and Jacquemyn, 2020; Matías et al., 2020; Furtado et al., 2021). Imprecision in any floral organ is expected as consequence of developmental variation and or relatively weak canalization (Armbruster et al., 2009 a, b; Armbruster and Wege, 2019). Part of the imprecision detected in this study was probably associated with genetic factors since the data was collected under a shared glasshouse environment, but also developmental noise (as larger cells displayed larger variance) and variance increased at later stages of development (Figures 3 and 4). These patterns could be related to the function of distyly. Pollinator movement within flowers, variation in flower morphology, the fit of the pollinator/pollinating organ within flowers, or grooming behaviour can all influence the distribution of pin and thrum pollen on the pollinator´s body, in turn affecting assortative and disassortative pollen transfer patterns (Thompson and Dommée, 2000; Lau and Bosque, 2003; Massinga et al., 2005; Pérez-Barrales et al., 2006; 2014; Pérez-Barrales and Arroyo 2010, Holmquist et al., 2012; Keller et al., 2014; Zhu et al., 2015; Furtado et al., 2021). For example, lower imprecision in short anther placement would result in a less scattered distribution of pin pollen on the bodies of insects compared to thrum pollen, potentially leading to an improved disassortative pollination between low organs compared to tall organs (Lau and Bosque, 2003; Massinga et al., 2005; Jacquemyn et al. 2018). Maladaptive b ias and developmental imprecision have been shown to be related to the efficiency of disassortative pollen transfer (Brys and Jacquemyn, 2020; Matias et al., 2020; Furtado et al., 2021) but selection for accuracy in reciprocal herkogamy in *Linum* remains to be tested. Such tests will require data on pollen flow and mating patterns, but also detailed observations of pollinator behaviour and distribution of pin and thrum pollen on the body.

The pollinator observations of this study found that small *Usia* flies and cf. *Lasioglossum* bees that enter the corolla to feed on nectar tend to make most contact with short reproductive organs and less often with tall organs, which in turn could bias disassortative pollen transfer.

This is relevant to reproductive success as *L. tenue* is dependent on insect pollination for cross-pollination (authors pers. obs.). It is currently unclear whether more superficial visits by larger *Bombylius* flies alter this pollination dynamic. *Usia* flies are common pollinators in other *Linum* species (Johnson and Dafni, 1998; Armbruster et al., 2006; Lebel et al., 2018), with reports of an insect feeding behaviour similar to that observed in *L. tenue*. In the heterostylous *L. suffruticosum*, a species with three-dimensional arrangement of anthers and stigmas, *Usia* flies promote disassortative pollen transfer, as they contact upper and lower organs with the dorsal and ventral parts of the body, allowing an apparent complete separation of the thrum (dorsal) and pin (ventral) pollen (Armbruster et al., 2006). Future research integrating the morphological variation with pollination studies in *Linum* could offer new insights to understand selection on the arrangement of anthers and stigmas to favour disassortative pollen transfer.

Male and female floral organs of pin and thrum morphs were found to show differences in their development and cell lengths. Developing floral organs of each morph showed consistent differences in growth rates, primarily driven by the different pistil growth rates, from a relatively early stage from one week to one day prior to flower opening (Figure 2). These developmental differences are probably completed after flower opening, as previously noted for related *L. grandiflorum* (Ushijima et al. 2015). Analysis of floral morph development and cell lengths identified at least two distinct mechanisms to achieve reproductive organ height differences. Developing thrum flowers showed enhanced growth of the tall thrum stamens during floral development (Figure 2). Since the cell lengths of thrum filaments in mature flowers are not significantly different from the cell lengths of pin filaments (Figure 3), this additional length has probably been achieved through increasing cell number by extra cell division in thrum filaments. The second developmental mechanism to achieve morph differences is reduced cell elongation in short thrum styles compared to pin

styles, which had similar cell lengths to pin and thrum filaments (Figure 3). Therefore, there appears to be at least two different developmental mechanisms to control height in each floral morph. These observations of organ-specific developmental mechanisms support the model for a genetic control of distyly by a haplotype consisting of multiple physically linked genes, each contributing to a distinct floral trait (Lewis and Jones, 1992). The recently sequenced *Linum* tenue thrum specific S locus contains nine genes (Gutiérrez-Valencia et al. 2022), one of which is homologous to previously identified *LgTSS1* (Ushijima et al. 2015). This gene shows similarity with *A. thaliana Vascular-related Unknown Protein 1* (*VUP1*) that is associated with suppression of cell expansion during development. Another *Linum tenue* candidate is *WD Repeat-containing protein 44* (*LtWDR-44*) that functions in hormone signalling and development (Gutiérrez-Valencia et al. 2022). The same study performed a differential expression analysis of *L. tenue* floral tissues that found that both pistils and stamens were enriched for cell wall-related genes. Distyly in *Primula* (Primulaceae) is relatively well understood since the identification and sequencing of the thrum-specific *S* locus region containing at least five expressed genes in *P. vulgaris* (Li et al., 2016; Cocker et al. 2018). Pin and thrum floral tissues show extensive expression differences concordant with differences in timing of development (Burrows and McCubbin 2018). Further, functional studies of two of the thrum-specific *S* locus genes, CYP^{T}, a brassinosteroid hormone inactivator and GLO^{T}, a paralogue of the B function floral homeotic gene, have shown that they influence the height of pistils and stamens, respectively, (Nowak et al., 2015; Huu et al., 2016; Li et al., 2016; Huu et al. 2022). In distylous *Turnera subulata* (Passifloraceae), three thrum-specific genes have been identified; *TsSPH1,* potentially involved in extracellular self-incompatibility signalling, and *TsYUC6,* potentially involved in auxin hormone synthesis, are both expressed exclusively in the male organs, and *TsBAHD,* a brassinosteroid hormone inactivator, is expressed only in the pistils (Shore et al., 2019; Matzke et al., 2020). In

distylous *Fagopyrum esculentum* (Polygonaceae), a thrum specific *S-Locus Early Flowering 3* (*S-ELF3*) gene has putative floral development functions (Yasui et al., 2012). A second polygalacturonase gene (*FePG1*) has also been found to be specifically expressed in short thrum styles, most likely via regulation from the S locus as it is not physically linked to the S locus itself (Takeshimi et al. 2022). This family of genes are pectin hydrolytic enzymes that alter cell wall properties and function in pollen development and floral development. Across these distylous plant families with independently evolved distyly, many of the responsible genes appear to have similar functions, particularly with regards to developmental signalling and control of cell elongation. Gene expression and growth hormone treatment experiments are underway to confirm the developmental control of the distyly phenotype in *L. tenue*.

CONCLUSIONS

Detailed morphological and developmental analysis of reproductive organ height in distylous *L. tenue* has revealed how reciprocal herkogamy is achieved through the interaction of distinct responses in male and female floral organs. Morph-specific differences are driven by arrested cell elongation in short thrum pistils and contrasting enhanced cell division in long thrum filaments. Reproductive organ growth rates differ most between pin and thrum pistils, highlighting the importance of pistil length differences for the expression of distyly. In terms of adaptive inaccuracy, short reproductive organs show a greater bias (mismatch) in organ heights than tall organs, while tall organs show greater imprecision (variance) in organ height. In particular, thrum pistils show the least variance but are consistently shorter than their matching pin stamens. Lastly, both genetic and environmental variation influences the expression of distyly. These fine-scale morphological and developmental observations raise many further questions about the evolutionary and functional constraints of distyly in this and other species. Further understanding will require detailed ecologically and phylogenetically

informed studies of *S* locus genetics, floral morphology, and pollination biology in more *Linum* species and other distylous groups.

ACKNOWLEDGEMENTS

The authors thank James Gardiner, Georgina Keggin, and Jasmine Staples who contributed results as part of their student projects at Durham and Portsmouth. The authors also thank Tim Hawkins, Joanne Louise Robson and Patrick Duckney for their guidance with microscopy work, and Scott Armbruster and Øystein Opedal for useful comments. This work was supported by: a Durham Doctoral Scholarship [to AF], research development funding from the Universities of Durham and Portsmouth [to ACB and RPB, respectively] a N8 Research Partnership Agrifood Resilience Programme Pump Priming Grant [to AF and ACB], the Percy Sladen Memorial Fund of the The Linnean Society [to RPB], and a new investigator research grant from The Royal Society [to RPB].

AUTHOR CONTRIBUTIONS

AF, RPB, and ACB conceived and designed the study. All authors contributed to acquisition of data and its analysis. AF, ACB, RPB drafted the manuscript with input from all authors.

DATA AVAILABILITY

Datasets consisting of open flower measures, developing flower measures, and cell measures will be deposited in the DRYAD archive in advance of publication.

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Table 1. Analysis of *Linum tenue* open flower measurements.

Response (sample size)	Pistil length (407)	Stamen length (317)	Herkogamy (pistil-stamen) (317)
Random effects (DF, variance, SD)			
individual x population	165, 0.003, 0.051***	130, 0.002, 0.042***	130, 0.003, 0.052***
population	16, <0.001, <0.001*	16, <0.001, 0.012**	16, <0.001, <0.001
Fixed effects (estimate, SE)			
intercept	1.299, 0.088***	0.995, 0.114***	0.261, 0.112*
morph	-0.520, 0.147***	0.127, 0.163	-0.626, 0.160***
petal length	0.309, 0.033***	0.317, 0.042***	0.008, 0.042
morph x petal length	0.004, 0.054	0.063, 0.060	-0.067, 0.059
R^2			
conditional	0.962	0.926	0.988
marginal	0.924	0.866	0.972

Notes: Mixed model summary results showing relationships of pistil length, stamen length and herkogamy against flower size (petal length) and floral morph (pin or thrum) while controlling for the random effects of individual and population. Flowers were measured from glasshouse-

grown plants only. Mixed models were performed on non-transformed data using the lmer REML fit function of the R lmerTest. The significance of mixed effects were evaluated using t-tests with Satterthwaite degrees of freedom approximations, while the significance of random effects were evaluated by sequentially dropping random effects from the model and comparing the prior model using the anova function with likelihood ratio tests. Asterisks indicate the significance of each explanatory variable (***,**,* for P<0.001, <0.01, <0.05 respectively). R^2 values were calculated using the r.squaredGLMM function of the R MuMIn package, and are conditional for the full mixed model or marginal for fixed effects only.

Table 2. Summary of *Linum tenue* open flower organ lengths and frequencies per population and per morph.

Popn. (seed source)	Popn. Type	Sample size		Mean length of tall organs (mm)		Mean length of short organs (mm)		Mean organ length (mm)	Variance of tall and short organs (mm^2)				Popn. Pin morph %	Chi-square
		P	T	S	A	s	a		Var (S)	Var (A)	Var (s)	Var (a)		
mva	F	39	37	7.231	7.131	3.900	4.406	5.671	0.258	0.244	0.107	0.126	51.32	0.053 (n.s.)
pdp	F	45	34	11.390	12.004	5.836	7.028	9.084	0.867	1.052	0.212	0.564	56.96	1.532 (n.s.
r10	F	42	37	12.150	12.185	6.187	7.435	9.509	0.891	0.831	0.347	0.658	53.16	0.317 (n.s.)
r17	F	40	37	12.766	12.962	6.492	7.704	9.991	0.415	1.323	0.205	0.505	51.95	0.117 (n.s.)
sdn	F	36	25	12.000	11.406	6.125	6.869	9.165	0.985	1.940	0.302	0.696	59.02	1.984 (n.s.)
ara	G	23	11	6.763	6.417	3.464	4.677	5.468	0.391	0.143	0.113	0.292	-	-
bur	G	9	17	6.181	7.184	3.626	4.646	5.408	0.446	0.529	0.126	0.216	-	-
cbt	G	10	10	6.359	6.981	3.466	4.604	5.352	0.334	0.189	0.040	0.083	-	-
ebo	G	10	9	7.110	6.772	3.486	4.550	5.498	0.137	0.395	0.056	0.118	-	-
hin	G	9	14	6.368	7.041	3.427	3.946	5.204	0.295	0.321	0.073	0.141	-	-
lum	G	9	15	6.316	6.772	3.595	4.101	5.193	0.175	0.366	0.094	0.062	-	-

mon	G	14	21	5.926	6.785	3.543	3.948	5.073	0.305	0.664	0.172	0.234	-	-
snv	G	10	14	6.151	6.897	3.538	4.415	5.245	0.304	0.317	0.073	0.030	-	-
svt	G	16	17	6.463	7.134	3.752	4.362	5.428	0.469	0.225	0.147	0.120	-	-

Notes: Population seed source codes are mapped in Figure 1. The population types F and G refer to field- and glasshouse-harvested flowers respectively. Sample sizes are numbers of individuals of pin (P) and thrum (T) flowers respectively. S refers to pin flower ovary and style length, A refers to thrum flower filament length, s refers to thrum flower ovary and style length, and a refers to pin flower filament length. Mean and variance values were used to calculate maladaptive bias and adaptive inaccuracy values presented in Table 3. The chi square statistic tests for equal morph frequencies (1 degree of freedom, no significant departures from 1:1 ratio). Sample morph ratios were not tested for glasshouse measures because sampling was non-random with respect to morph type.

Table 3. Summary of *Linum tenue* per population adaptive inaccuracy, imprecision, and bias of tall and short floral organ length.

Population	Popn. type	Organ type	Inaccuracy %	Maladaptive bias squared %	Variance filament %	Variance ovary plus style %	Total inaccuracy mm^2	Mean squared standardised total inaccuracy
mva	F	Tall	51 (42, 61)	1 (0, 8)	24 (14, 35)	26 (16, 35)	1.00 (0.75, 1.30)	3.1 (2.1, 4.2)
		Short	49 (39, 58)	26 (14, 38)	13 (8, 18)	11 (6, 17)		
pdp	F	Tall	51 (40, 63)	8 (1, 22)	23 (15, 32)	19 (13, 26)	4.49 (3.63, 5.43)	5.4 (3.8, 7.3)
		Short	49 (37, 60)	32 (21, 43)	13 (8, 17)	5 (2, 8)		
r10	F	Tall	40 (28, 53)	0 (0, 5)	19 (11, 27)	21 (12, 30)	4.29 (3.28, 5.41)	4.7 (3.1, 6.4)
		Short	60 (47, 72)	36 (23, 51)	15 (10, 20)	8 (4, 12)		
r17	F	Tall	45 (34, 57)	1 (0, 9)	33 (21, 45)	11 (6, 16)	3.95 (3.17, 4.85)	4.0 (2.9, 5.4)
		Short	55 (43, 66)	37 (24, 51)	13 (7, 19)	5 (3, 7)		
sdn	F	Tall	68 (53, 77)	8 (0, 24)	40 (17, 54)	20 (10, 37)	4.85 (2.74, 8.18)	5.8 (2.8, 10.3)
		Short	32 (23, 47)	11 (4, 24)	14 (6, 28)	6 (3, 9)		
mean	F	Tall	52	4	29	18	3.72	4.6
		Short	48	28	14	6		
ara	G	Tall	26 (18, 33)	5 (0, 14)	6 (2, 10)	15 (8, 23)	2.53 (0.78, 1.81)	8.5 (2.8, 6.1)

		Short	74 (67, 82)	58 (46, 70)	12 (6, 17)	4 (1, 10)		
bur	G	Tall	59 (33, 80)	30 (5, 63)	16 (5, 30)	13 (4, 19)	3.36 (1.22, 2.14)	11.5 (4.2, 7.7)
		Short	41 (20, 67)	31 (14, 57)	6 (1, 9)	4 (1, 7)		
cbt	G	Tall	39 (14, 65)	17 (2, 41)	8 (3, 12)	14 (2, 24)	2.33 (0.93, 1.49)	8.1 (3.3, 5.7)
		Short	61 (35, 86)	56 (30, 82)	4 (0, 5)	2 (1, 3)		
ebo	G	Tall	33 (20, 46)	6 (0, 25)	20 (3, 35)	7 (0, 16)	1.95 (0.63, 1.35)	6.5 (2.2, 4.6)
		Short	67 (54, 80)	58 (46, 71)	6 (2, 10)	3 (0, 6)		
hin	G	Tall	69 (45, 88)	29 (4, 68)	21 (5, 35)	19 (5, 29)	1.55 (0.51, 1.08)	5.7 (1.9, 4.2)
		Short	31 (12, 55)	17 (3, 46)	9 (2, 15)	5 (1, 8)		
lum	G	Tall	65 (45, 80)	18 (0, 50)	32 (11, 46)	15 (1, 36)	1.16 (0.35, 0.85)	4.3 (1.4, 3.2)
		Short	35 (20, 55)	22 (7, 45)	5 (1, 9)	8 (1, 13)		
mon	G	Tall	75 (57, 89)	32 (8, 61)	29 (14, 42)	13 (3, 26)	2.28 (0.76, 1.57)	8.9 (3.1, 6.1)
		Short	25 (11, 43)	7 (0, 25)	10 (4, 16)	8 (3, 12)		
snv	G	Tall	57 (33, 75)	27 (7, 52)	15 (4, 27)	15 (6, 21)	2.05 (0.7, 1.43)	7.5 (2.6, 5.3)
		Short	43 (25, 67)	38 (21, 61)	1 (0, 3)	4 (1, 6)		
svt	G	Tall	64 (37, 83)	25 (5, 50)	13 (4, 22)	26 (8, 39)	1.78 (0.56, 1.3)	6 (1.9, 4.8)
		Short	36 (17, 63)	21 (7, 47)	7 (3, 12)	8 (3, 14)		

mean	G	Tall	53	21	17	15	2.11	7.4
		Short	47	36	7	5		

Notes: Population seed source codes are mapped in Figure 1. The population types F and G refer to field- and glasshouse-harvested flowers respectively. For organ type, the female organ is the sum of ovary length and style length, and the male organ is the stamen filament. The 95 % confidence intervals of estimates are presented in parentheses. The inaccuracies of the tall and short organs are presented as a percentage of the total inaccuracy so that they sum to 100% (rounding to the nearest integer might cause some small discrepancies). The components of total inaccuracy, including maladaptive bias squared (square of the departure of the trait mean from the optimum), variance (=imprecision) of the filaments and ovaries plus styles, are presented as percentages summing to the total of their respective tall or short organ type so that the sum of all six values sum to 100%. Total inaccuracy is presented both as absolute mm^2 values and as a percentage of the mean squared. Mean estimates across populations were calculated from population estimates in mm^2 units before converting to percentages.

Table 4. Summary of *Linum tenue* developing flower measurements analysis.

Response (sample size)	pistil length (115)	stamen length (115)	herkogamy (pistil-stamen) (115)
Random effects (DF, variance, SD)			
individual x population	56, 0.002, 0.042**	56, 0.001, 0.033*	56, 0.003, 0.056***
population	9, 0.001, 0.027***	9, <0.001, 0.011	9, 0.001, 0.031***
Fixed effects (estimate, SE)			
intercept	-0.676, 0.073***	0.245, 0.063***	-0.927, 0.08***
morph	0.746, 0.100***	-0.263, 0.086**	1.041, 0.110***
petal length	1.064, 0.038***	0.515, 0.033***	0.553, 0.042***
morph x petal length	-0.525, 0.053***	0.155, 0.045**	-0.697, 0.058***
R^2			
conditional	0.945	0.900	0.906
marginal	0.892	0.839	0.775

Notes: Mixed model summary results showing relationships of pistil length, stamen length and herkogamy against developmental stage (petal length) and floral morph (pin or thrum) while controlling for the random effects of individual and population. Flowers were measured from glasshouse-grown plants only. Mixed models were performed on non-transformed data using the lmer REML fit function of the R lmerTest. The

significance of mixed effects were evaluated using t-tests with Satterthwaite degrees of freedom approximations, while the significance of random effects were evaluated by sequentially dropping random effects from the model and comparing the prior model using the anova function with likelihood ratio tests. Asterisks indicate the significance of each explanatory variable (***,**,* for P<0.001, <0.01, <0.05 respectively). R^2 values were calculated using the r.squaredGLMM function of the R MuMIn package, and are conditional for the full mixed model or marginal for fixed effects only.

Table 5. Summary of *Linum tenue* floral organ cell measurements analysis.

Response (sample size)	cell length (1563)
Random effects (DF, variance, SD)	
individual x population	9, 0.060, 0.245***
population	2, <0.001, <0.001***
Fixed effects (estimate, SE)	
intercept	4.391, 0.111***
organ	0.074, 0.031*
morph	0.051, 0.167
region-linear	0.519, 0.044***
region-quadratic	-0.385, 0.045***
organ:morph	-0.811,0.045***

organ:region-linear	0.030, 0.066
organ:region-quadratic	0.109, 0.068
morph:region-linear	0.227, 0.067***
morph:region-quadratic	-0.193, 0.068***
organ:morph:region-linear	-0.263, 0.100***
organ:morph:region-quadratic	0.454, 0.100***
r2	
conditional	0.579
marginal	0.444

Notes: Summary of mixed model results showing relationships of cell length against region of organ (1 to 5 counting from base to tip) and floral morph type (pin or thrum) while controlling for the random effects of individual and population. Flowers were measured from glasshouse-grown plants only. Mixed models were performed on non-transformed data using the lmer REML fit function of the R lmerTest. The fixed effect, region, was treated as an ordinal factor with five levels, permitting tests of linear (L), quadratic (Q), cubic (C) and quartic (^4) models of this factor. Only linear and quadratic model results are shown as higher order results were insignificant. The significance of mixed effects were

evaluated using t-tests with Satterthwaite degrees of freedom approximations, while the significance of random effects were evaluated by sequentially dropping random effects from the model and comparing the prior model using the anova function with likelihood ratio tests. Asterisks indicate the significance of each explanatory variable (***,**,* for P<0.001, <0.01, <0.05 respectively). R^2 values were calculated using the r.squaredGLMM function of the R MuMIn package, and are conditional for the full mixed model or marginal for fixed effects only.

FIGURES

Fig. 1. Dissected *Linum tenue* flowers showing floral organ measures and map of sample populations. (A) Thrum morph with petals and sepals removed, (B) Thrum morph with sepals, petals, and stamens removed, (C) Thrum morph with petals removed, (D) Pin morph with sepals and petals removed, (E) Pin morph with sepals, petals, and stamens removed, (F) a removed petal. The lengths measured are: i = filament, ii = anther, iii = stigma, iv = ovary, v = style, vi = pistil, vii = sepal, viii = petal height, ix = petal width. (G) Map of sampled region. The inset shows the sampled region within the context of Europe. Populations were sampled during the summers of 2013, 2014, and 2015 by ACB, AF, and RPB.

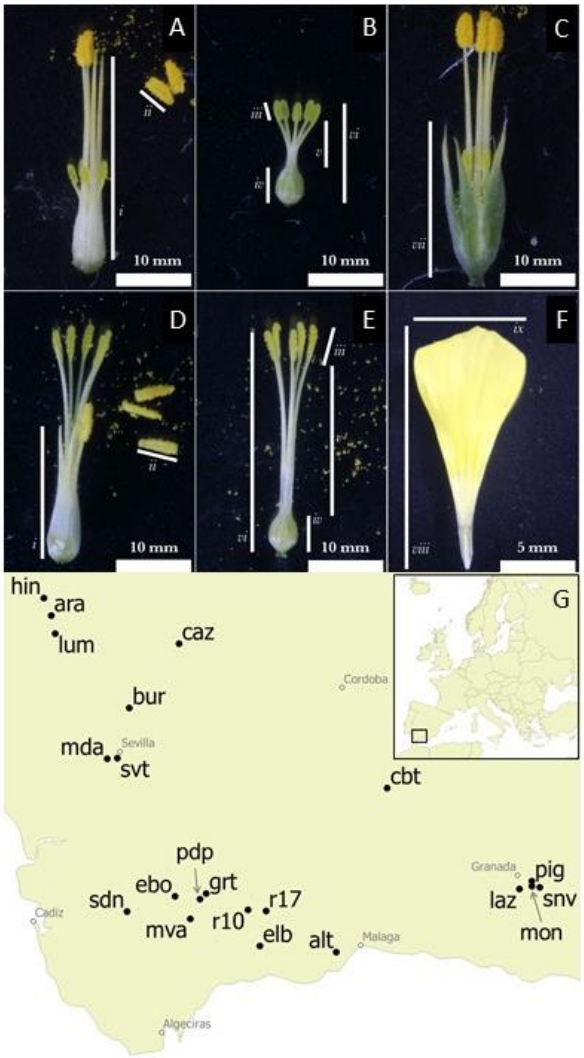

Fig. 2. Relationships between *Linum tenue* floral organ length, developmental stage (petal length), and floral morph in developing flower buds. Lines indicate best fit linear models between the plotted variables. Values are the estimated slopes of each of the best fit mixed models. psdiff indicates pistil-stamen difference or herkogamy.

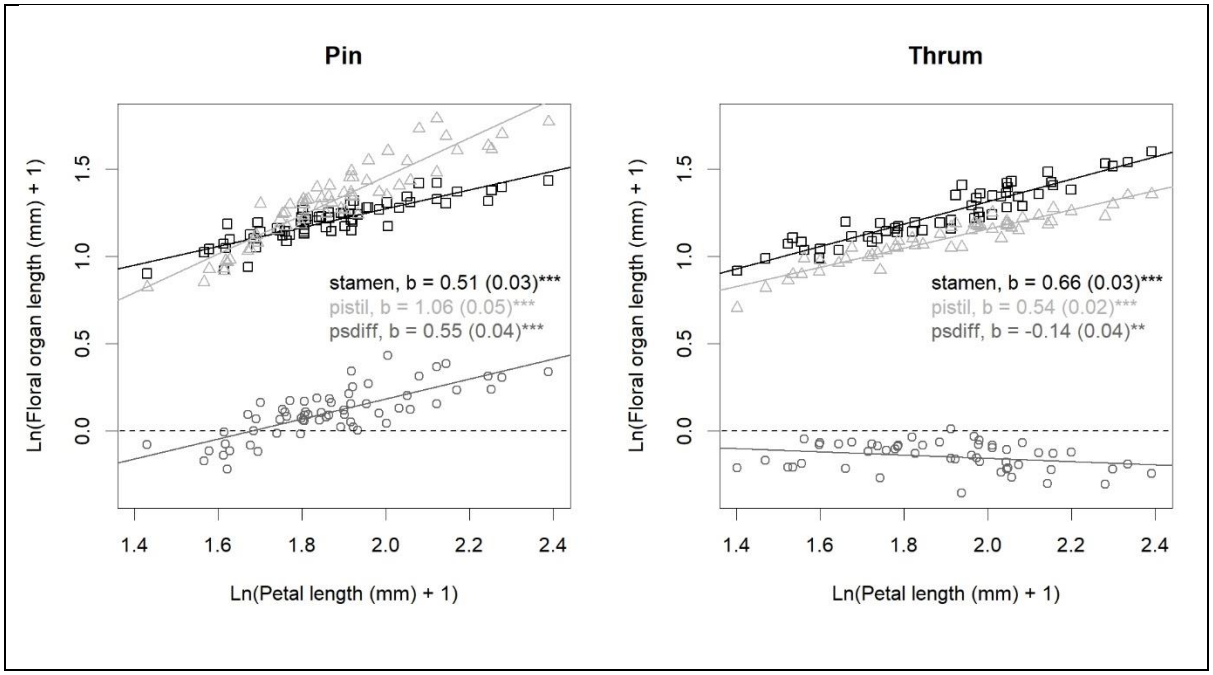

Fig. 3. Cell length means and standard deviations for *Linum tenue* floral morph, organ type, and region of organ. Organ regions divide the total length of each organ into five, starting from the base (R1) to the tip of the organ (R5).

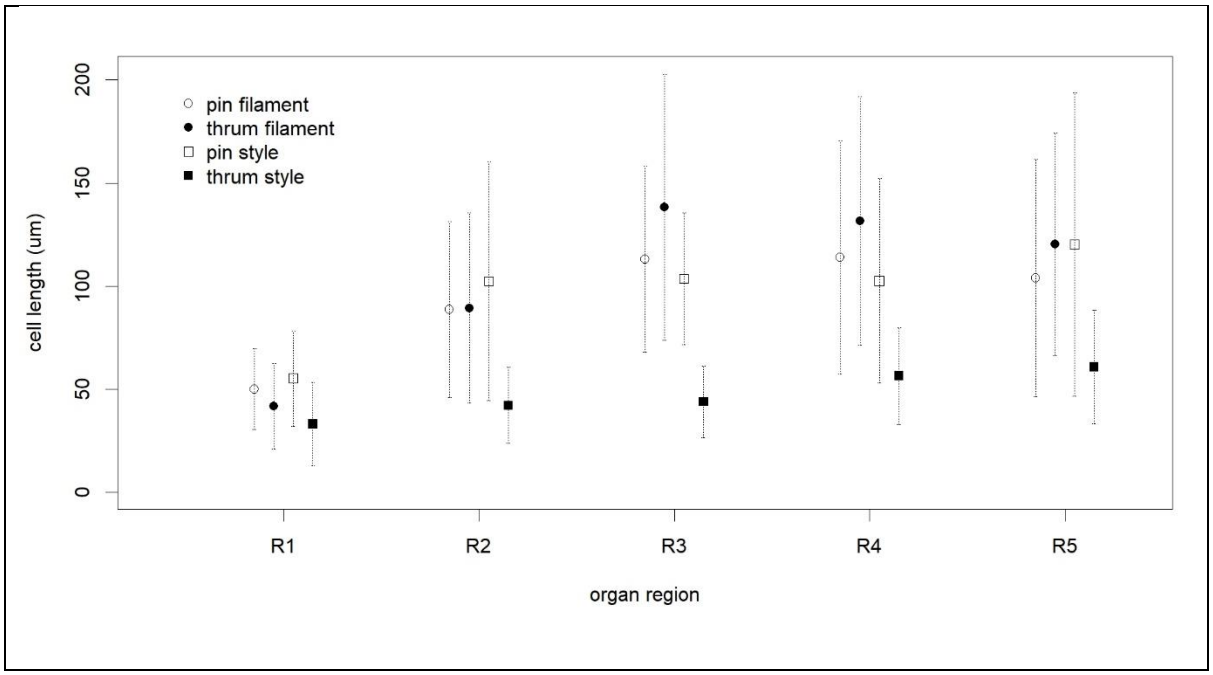

Fig. 4. *Usia cf. pusilla* pollinator visit to a *Linum tenue* thrum flower.

Appendixes

Table A1. Location and sample size data for *Linum tenue* sample populations.

Table A2. Analysis of *Linum* tenue open flower measurements showing relationships of multiple floral traits against flower size (petal length) and floral morph (pin or thrum) while controlling for the random effects of individual and population.

Table A3. Analysis of *Linum tenue* developing flower measurements showing relationships between multiple floral traits against developmental stage (petal length) and floral morph (pin or thrum) while controlling for the random effects of individual and populations.