

1 Original Article

2 **TITLE: The role of lateral and vertical herkogamy in the divergence of the blue- and**
3 **red-flowered lineages of *Lysimachia arvensis***

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11 **Running title:** The role of herkogamy in the isolation of colour lineages in *Lysimachia*

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1 **ABSTRACT**

2 **Background and aims**

3 Herkogamy, or anther-stigma separation, is known to reduce self-pollen deposition, but
4 little is known about the relative efficacy of different modes or conformations of
5 herkogamy. We assessed the effectiveness of vertical versus lateral herkogamy in
6 preventing or promoting self-pollen deposition in the annual herb *Lysimachia arvensis*, a
7 plant with lineages that differ in flower colour, and in which flowers first display lateral and
8 then vertical herkogamy. Because mating between the two lineages compromises fitness
9 through the production of low-quality hybrid offspring, we tested the prediction that
10 individuals sampled from sites occupied by both lineages should have flowers that promote
11 autonomous self-pollen deposition and self-fertilization as a result of selection to reduce
12 deleterious reproductive interference.

13 **Methods**

14 We characterised variation in herkogamy within and among 25 pure and mixed populations
15 of *L. arvensis* in its European range and assessed the effectiveness of lateral versus vertical
16 herkogamy in avoiding self-pollen deposition.

17 **Results**

18 Lateral herkogamy was more effective than vertical herkogamy in limiting self-pollen
19 deposition. In the case of vertical herkogamy, only approach herkogamy was effective.
20 Lineages showed consistent differences in herkogamy traits. In general, angles were
21 smaller for blue than red flowers in most populations, and blue flowers showed approach
22 herkogamy, while red flowers showed predominantly reverse herkogamy. In sympatry, the
23 red lineage showed a reduction of both herkogamy traits while for the blue lineage only
24 lateral herkogamy was reduced.

1 **Conclusions**

2 Our results demonstrate that pollen deposition is affected not only by the degree but also
3 the spatial conformation of herkogamy. They also highlight reduced herkogamy as a
4 potential mechanism for promoting reproductive assurance under pollen limitation, as well
5 as for avoiding reproductive interference between genetically divergent lineages.

6

7 **Key words:** *Anagallis*, colour polymorphism, herkogamy, *Lysimachia*, mating system,
8 reproductive assurance, reproductive isolation, selfing evolution, self-pollen deposition.

9

1

2 INTRODUCTION

3 Mating patterns in plant populations can be strongly influenced by the relative spatial
4 conformation of male and female organs within and among individuals. This applies to the
5 spatial separation of male (staminate) and female (pistillate) flowers in monoecious and
6 dioecious species (known as ‘dicliny’), but particularly to that between the anthers and
7 stigmas of bisexual flowers (‘herkogamy’). Herkogamy limits self-pollination (Brunet and
8 Eckert, 1998; Motten and Stone, 2000; Takebayasi *et al.*, 2006; reviewed in Opedal, 2018),
9 and reduced herkogamy may confer reproductive assurance through a capacity for
10 autonomous self-pollination, which can be advantageous when pollinators are rare or absent
11 (Lloyd, 1992; Eckert *et al.*, 2006). Indeed, the rate of self-fertilization is often high in self-
12 compatible populations in which anthers are close to stigmas, and the experimental
13 exclusion of pollinators has been shown to bring about the rapid evolution of reduced
14 stigma-anther separation (Roels and Kelly, 2011; Brys and Jacquemin, 2012). Selection on
15 the mating system may thus act directly and quickly through aspects of floral morphology
16 such as herkogamy (Opedal *et al.*, 2017; Toräng *et al.*, 2017).

17

18 The ways individuals achieve herkogamy and its effect on self-pollen transfer vary greatly
19 among species. This is particularly striking in heterostylous species in which anthers are
20 displaced vertically from one another (‘vertical herkogamy’), held either above (‘approach
21 herkogamy’) or below stigmas (‘reverse herkogamy’), or in enantiostylous species in which
22 anthers are displaced laterally from stigmas to the right or left (‘lateral herkogamy’). Both
23 heterostyly and enantiostyly promote disassortative mating, with pollen transferred less
24 frequently among individuals with the same than with different morphology (Lloyd and

1 Webb, 1992; Barrett *et al.*, 1996, 2000). In species with vertical herkogamy, approach
2 herkogamy is more frequent than reverse (Webb and Lloyd, 1986; Barrett, 2003), although
3 some species show continuous variation in the relative vertical positions of stigmas and
4 anthers, ranging from reverse to approach herkogamy, e.g., *Datura stramonium* (Motten
5 and Stone, 2000) and *Gilia achilleifolia* (Takebayashi *et al.*, 2006). Enantiostyly is less
6 common than heterostyly, and monomorphic lateral herkogamy is also relatively rare, e.g.,
7 *Centaureum* species (Brys *et al.*, 2014, 2016). While approach herkogamy appears to be
8 better at reducing self-pollen deposition than reverse herkogamy (Motten and Stone 2000;
9 Barrett, 2003; Takebayashi *et al.*, 2006), data on self-pollen deposition under reverse
10 herkogamy are not common (Motten and Stone, 2000; Takebayashi *et al.*, 2006). The
11 effectiveness of lateral herkogamy has been rarely reported, but a few species have reported
12 reduced self-pollination in flowers with increased herkogamy, as expected (Brys and
13 Jacquemyn, 2011, 2012). Recently, Toräng *et al.* (2017) reported a non-standard case of
14 herkogamy in *Arabis alpina*, whose flowers combine vertical herkogamy and variation in
15 anther orientation. Interestingly, both floral traits in *A. alpina* covary as a function of
16 pollinator activity, with associated effects on the degree of self-pollen deposition.

17

18 The effects of herkogamy can also vary in terms of how it changes over the course of a
19 flower's life. Such changes can involve not only alterations of the amount of self-pollen
20 deposition over time, but also the mode of self-fertilization and thus the relative costs and
21 benefits of selfing. Three types of autonomous selfing have been recognised: 'prior selfing'
22 (selfing before opportunities for outcrossing); 'competing selfing' (selfing at the same time
23 as outcrossing, such that self and outcross pollen compete directly); and 'delayed selfing'
24 (self-pollination after opportunities for outcrossing have been exhausted) (Lloyd, 1979).

1 These types incur different costs that depend on cross-pollen availability and the impact of
2 inbreeding depression (Lloyd, 1979; Harder and Routley, 2006). Increased selfing may
3 reduce opportunities for pollen export ('pollen discounting') as well as ovule availability
4 for outcrossing ('ovule discounting'; Lloyd 1992), costs that may be reduced in species
5 with delayed selfing that occurs only after outcrossing has been possible (Lloyd 1979,
6 1992; Herlihy and Eckert 2002; Harder and Routley, 2006; Goodwillie and Weber 2018).
7 Delayed selfing should be particularly favoured in species with only a single chance to
8 reproduce, such as annuals (Shivanna, 2015). In contrast, under prior and competing
9 selfing, the potential for reproductive success through outcrossing is reduced, and plants
10 incur a cost of pollen and/or ovule discounting (Holsinger *et al.*, 1984; Lloyd, 1992).

11

12 While traits such as reduced herkogamy that allow or promote an increased capacity for
13 selfing may evolve in response to selection for reproductive assurance when pollinators or
14 mates are scarce or absent, they can also be the outcome of selection to avoid hybridization
15 with individuals of co-occurring genetically incompatible lineages. In such situations,
16 reduced herkogamy may prevent gene flow between them, thus avoiding potentially
17 deleterious hybridization. For example, an increased capacity for selfing and reproductive
18 divergence between hybridizing populations has been previously reported in closely co-
19 occurring species of *Centaureum* (Brys *et al.*, 2014), *Mimulus* (Martin & Willis, 2007;
20 Grossenbacher & Whittall, 2011), and *Clerodendrum* (Miyake & Inoue, 2003). Variation in
21 floral morphology can thus be a function of both pollinator behaviour and the context of
22 potential interspecific mating and incipient speciation. How such processes might affect
23 selection on different modes of herkogamy has, to our knowledge, never been considered.

24

1 The Mediterranean self-compatible annual forb *Lysimachia arvensis* (L.) U. Manns and
2 Anderb. (Primulaceae) is unusual in presenting both vertical and lateral herkogamy
3 (Jiménez-López *et al.*, 2019a). Flowers show lateral herkogamy when they first open on
4 day one as a result of an angular displacement of the stamens from the pistil, but the angle
5 of separation closes on the second and third days, so that anthers end up centrally on the
6 same vertical axis as the pistil and thus potentially showing vertical herkogamy, with
7 anthers either above, next to, or below the stigma (Jiménez-López *et al.*, 2019a; Fig. 1).
8 The opportunity for self-pollen deposition in *L. arvensis* may thus not only vary among
9 individuals that differ in the relative lengths of their pistils and/or stamens, but also over
10 time. Both modes of herkogamy in *L. arvensis* have high heritability, with $h^2 = 0.843$ and
11 0.635 for lateral and vertical herkogamy, respectively (Jiménez-López *et al.*, 2019a). This
12 variation allows a direct assessment of the distribution of herkogamy within and among
13 populations, based on unmanipulated plants, as well as of its implications for self-pollen
14 deposition. As has been described for *Arabis alpina* (Toräng *et al.*, 2017), both herkogamy
15 traits in *L. arvensis* may be selected synergistically through their joint modulation of the
16 capacity for self-pollination.

17

18 *Lysimachia arvensis* also has two different flower colour morphs. Most European
19 populations comprise only plants with red flowers, but plants with blue flowers are found in
20 drier Mediterranean regions, either on their own, or mixed with red-flowered plants (Arista
21 *et al.*, 2013). In mixed Mediterranean populations, which are mostly blue-biased, both blue-
22 and red-flowered plants are visited by the same small solitary bees (Ortiz *et al.*, 2015), but
23 pollinators have a clear preference for blue flowers (Ortiz *et al.*, 2015; Jiménez-López *et*
24 *al.*, 2019b). Red-flowered plants in Mediterranean regions have a higher inbreeding

1 coefficient than blue-flowered plants, or than red-flowered plants in non-Mediterranean
2 regions. This variation corresponds partially with variation in inbreeding depression, with
3 Mediterranean populations showing higher values in red- than in blue-flowered plants
4 (Jiménez-López *et al.*, 2019c). It is not yet known, however, how patterns of herkogamy
5 vary between the two lineages, or what their interactive effects on plant mating might be.

6

7 The current taxonomy of *L. arvensis* recognises a single species (Pujadas, 1997; Manns and
8 Anderberg, 2009), but individuals with different flower colours likely belong to different
9 incipient biological species. First, they have somewhat different habitat preferences, with
10 the blue-flowered individuals performing better in hot and dry environments and red-
11 flowered individuals preferring wetter sites (Arista *et al.*, 2013). Second, variation in colour
12 in natural populations is largely discrete, with salmon-coloured F₁ progeny of parents with
13 different flower colours being uncommon (Jiménez-López *et al.*, 2019b; Table S1), even in
14 mixed populations, pointing to a degree of prezygotic isolation between the two lineages.
15 Third, although about a quarter of mixed populations show some evidence of sporadic
16 hybridization (Table S1), experimental crosses indicate that the F₂ progeny are either
17 completely sterile or have lower fruit and seed production (Jiménez-López *et al.*,
18 unpublished data). Given the clear genetic divergence (Jiménez-López *et al.*, 2019c) and
19 level of apparent isolation between the two colour morphs, we refer to them as the red and
20 blue ‘lineages’ in what follows.

21

22 Here, we use the unusual morphological variation in in *L. arvensis* to explore the
23 implications of the mode and degree of herkogamy for both reproductive assurance and the
24 avoidance of reproductive interference between genetically divergent lineages. Specifically,

1 we test the hypothesis that the reduction in herkogamy increases an individual's capacity
2 for self-fertilization, and we ask whether this capacity is promoted more by lateral or
3 vertical herkogamy. Further, because mating between the two colour morphs reduces the
4 individual reproductive success as a result of the partial sterility of their hybrid progeny, we
5 asked whether patterns of herkogamy in mixed versus pure populations are consistent with
6 selection to avoid inter-lineage pollen transfer (Levin 1971; Fishman & Wyatt, 1999).
7 Because we noted that pollinator activity is higher on flowers of the blue lineage in mixed
8 populations (Ortiz *et al.*, 2015; Jiménez-López *et al.*, 2019b), we predicted lower levels of
9 herkogamy in individuals of the red lineage in these populations.

10

11 **MATERIAL & METHODS**

12 **Study populations**

13 We measured flowers in a total of 25 populations of *L. arvensis* in both Mediterranean and
14 non-Mediterranean regions of Europe (Table S2). Populations were visited during the peak
15 of flowering, and each was categorized as belonging to the blue lineage, the red lineage or
16 was a mixed population (red- and blue-flowered plants in the same population). We
17 sampled ten populations from the blue lineage, six from the red lineage and nine mixed
18 populations, with 475 blue- and 430 red-flowered plants sampled in total. Sample sizes
19 varied among populations (from ten to 35 individuals per lineage, mean = 15 for blue-
20 flowered plants and 16 for red-flowered plants) because of variation in population size
21 (Table S2) and proportion of individuals of each lineage.

22

23 **Herkogamy traits**

1 We measured traits linked to herkogamy in *L. arvensis* to assess their role in preventing
2 self-pollination, and to assess variation across populations. All measurements were taken
3 from fresh flowers, starting on their first day of anthesis. We measured the two components
4 of herkogamy in one flower per plant using the software ImageJ, based on photographs
5 taken directly in the field or the glasshouse. To relate herkogamy to self-pollen deposition,
6 individual flowers on plants growing in the glasshouse were photographed in lateral view
7 using a tripod and a graduated scale. To characterize among-population variation in
8 herkogamy for red- and blue-flowered individuals, photographs were taken directly in the
9 field. Here, first-day flowers were cut and photographed in lateral view with a graduated
10 scale. Both herkogamy traits have a high degree of heritability and are expressed similarly
11 under glasshouse and field conditions (Jiménez-López *et al.*, 2019a). Lateral herkogamy
12 was measured as the angle between style and stamens (hereafter ‘style-stamen angle’). We
13 also measured the lengths of stamens and pistils (from the flower base to the centre of
14 anthers or pistils), and we calculated the degree of herkogamy as the difference between
15 pistil and stamen lengths (hereafter stigma-anther displacement).

16

17 **Autonomous self-pollen deposition**

18 We measured self-pollen deposition for 59 plants of both the blue and red lineages in a
19 glasshouse in which pollinators were excluded (25 blue-flowered plants from three
20 populations and 34 red-flowered plants from four populations). Plants were chosen with a
21 view to including a wide range of trait variation in our sample. To assess the effect of
22 lateral herkogamy on self-pollen deposition, we sampled a single flower from each plant at
23 the end of its first day of anthesis (1st-day flower). To assess the effect of approach or
24 reverse herkogamy on self-pollen deposition, we sampled a second flower from each plant

1 of its second day of anthesis (2nd-day flower). We stained stigmas from these flowers with
2 aniline blue (Martin, 1959), and counted the number of pollen grains on them under a
3 fluorescence microscope.

4

5 **Data analysis**

6 To assess herkogamy trait variation among populations and between the two lineages, we
7 performed two-way ANOVAs, with population specified as a random effect and colour as a
8 fixed effect and populations nested within colour. To test whether the two types of
9 herkogamy differed between pure and mixed populations, and whether these differences
10 were associated with colour, we performed a multivariate analyses of variance (MANOVA)
11 using both herkogamy measurements, with colour, population type (pure/mixed), and
12 population size as fixed effects. Subsequently, two-way ANOVAs were carried out for each
13 herkogamy trait to determine whether co-occurrence of lineages affected floral
14 morphology. Here, colour, population type (pure or mixed) and their interaction were used
15 as explanatory variables in the model; population size was excluded from the model, as it
16 was not significant in the previous MANOVA.

17 To assess the importance of lateral herkogamy in preventing self-pollen deposition, we
18 found that cubic regression provided the best fit to our data, and we report our results
19 accordingly. Self-pollen deposition on stigmas of 2nd-day flowers was estimated as the sum
20 of pollen depositions during first and second day of anthesis. To ascertain the importance of
21 stigma-anther displacement in preventing self-pollen deposition, we selected 2nd-day
22 flowers whose angle during the previous day was greater than 20 degrees. This selection
23 was based on the results of the cubic regression obtained for 1st-day flowers, which showed
24 that self-pollen deposition is practically zero when lateral herkogamy is over 20 degrees

1 (see Results). Our observations indicated a different relationship between self-pollen
2 deposition and herkogamy for flowers displaying approach versus reverse herkogamy. We
3 thus separated the corresponding data into two classes, with measurements of 2nd-day
4 flowers obtained from reverse and approach herkogamy, respectively. While data from
5 reverse herkogamy (displacement < 0) did not show any significant relationship between
6 herkogamy and self-pollen deposition, we fitted a significant negative exponential
7 regression between the two variables for flowers with approach herkogamy (displacement >
8 0).

9
10 Finally, we determined whether self-pollen deposition among red- and blue-flowered plants
11 growing in pure and mixed populations depended on the two herkogamy traits. Specifically,
12 we inferred ‘predicted self-pollen deposition’ through the use of previously obtained
13 regression curves relating self-pollen deposition to the degree of herkogamy for each
14 herkogamy mode, for both pure and mixed populations. We used GLMs to test for
15 differences in predicted self-pollen deposition as a function of the stigma-anther angle and
16 displacement, flower colour, and type of population, assuming a Poisson error distribution
17 and a log link function.

18

19 **RESULTS**

20 **Variation in herkogamy traits among populations and lineages**

21 All traits related to herkogamy showed high variation among populations, with the lateral
22 style-stamen angle being the most variable (coefficient of variation 40.7%; Table S3).

23 Lateral style-stamen angle varied significantly between the blue and red lineages ($F =$
24 513.47 , 1 df, $p < 0.0001$) and among populations nested into lineages ($F = 22.140$, 24df, $p <$

1 0.0001; Figs. 2a, 2c, 2e). In general, angles were smaller for blue than red flowers in most
2 populations (Fig. 2e). Flowers with angles > 20 degrees were found in 59% and 96% of
3 blue- and red-flowered plants, respectively (Fig. 2a). Vertical stigma-anther displacement
4 also varied significantly between lineages ($F = 763.37$, 1 df, $p < 0.0001$), and among
5 populations nested into lineages ($F = 17.34$, 2 df, $p < 0.0001$; Figs. 2b, 2d, 2f). In most
6 populations, blue flowers were approach herkogamous, while red flowers were reverse- or
7 non-herkogamous, or had stigmas and anthers at the same level (Fig. 2f). Indeed, blue
8 flowers showed reverse herkogamy in only two populations, and red flowers showed
9 approach herkogamy in only one population (Fig. 2f). Overall, approach herkogamy was
10 found in 83.8% of blue flowers, but in only 17.7% of red ones. Most floral traits were
11 correlated for both colour lineages (Table 1). In blue-flowered plants, lateral style-stamen
12 angle correlated significantly with vertical stigma-anther displacement ($R^2 = 0.255$; Table
13 1), i.e., the larger the style-stamen angle, the greater the degree of approach herkogamy. In
14 red-flowered plants, however, that correlation was negative, i.e., the larger the style-stamen
15 angle, the greater the degree of reverse herkogamy, though this association was no longer
16 significant after Bonferroni correction ($R^2 = -0.114$; Table 1).

17

18 MANOVA results showed marked and significant differences in flower morphology
19 between blue- and red-flowered plants in pure versus mixed populations, with a significant
20 interaction between these two factors (Table 2). Flower morphology was not affected by
21 population size, but it was particularly strongly associated with flower colour (Table 2).
22 Interestingly, flowers of both the blue and red lineages had a smaller lateral style-stamen
23 angle in mixed than in pure populations, but stigma-anther displacement only differed in
24 the red lineage. In mixed populations, the stigmas of the red lineage were situated at almost

1 the same level as the anthers, whereas in pure populations they showed marked reverse
2 herkogamy (Table 2, Fig. 3b).

3

4 **Association between herkogamy traits and autonomous self-pollination**

5 Self-pollen deposition varied non-linearly with both measures of herkogamy, with lateral
6 herkogamy particularly effective at reducing self-pollen deposition. Specifically, self-pollen
7 deposition decreased from 15 pollen grains for 1st-day flowers with angles of about 10
8 degrees to effectively zero for those flowers with angles > 20 degrees (Fig. 4a). Vertical
9 stigma-anther displacement for 2nd-day flowers was only effective in preventing self-pollen
10 deposition when the stigma was above the anthers, i.e., in approach-herkogamous flowers
11 (Fig. 4b). In contrast, self-pollen deposition was highly variable for flowers displaying
12 reverse herkogamy, with no dependence on stigma-anther separation (Fig. 4b).

13

14 **DISCUSSION**

15 **Mating effects of lateral versus vertical herkogamy**

16 We found that although both types of herkogamy reduced autonomous self-pollination in
17 *Lysimachia arvensis*, lateral herkogamy was more effective, with no self-pollen deposition
18 observed for flowers with a lateral displacement angle > 20 degrees. This results suggests
19 that these flowers thus had little capacity for autonomous self-pollination on their first day
20 of opening. The marked lateral separation between style and anthers in these flowers likely
21 also limits self-pollination even when their pollinators, largely small bees (Ortiz *et al.*,
22 2015), are collecting pollen. By contrast, on the second day, after movement of the style
23 into a perpendicular position, autonomous self-pollination was more likely, particularly in
24 flowers in which the stigma was at the same level as the anthers, or in reverse-herkogamous

1 flowers.

2

3 Although we did not evaluate the effect of herkogamy on the mating system, it is likely that
4 its influence on self-pollen deposition affects selfing rates in wild populations. Covariation
5 between herkogamy and the mating system has been reported for other species with either
6 lateral (Brys and Jackemin, 2011) or vertical herkogamy (Motten and Stone, 2000;
7 Takebayashi *et al.*, 2006). More specifically, differences in the role of approach and reverse
8 herkogamy in controlling self-pollen deposition have been suggested previously (Webb and
9 Lloyd, 1986; Barrett and Shore, 1987; Barrett, 2003). In species with stigmas placed always
10 above the anthers, outcrossing typically increases with herkogamy (Brunet and Eckert
11 1998; Herlihy and Eckert, 2007; but see Medrano *et al.*, 2005). However, the role of
12 herkogamy when it ranges from reverse to approach has been less studied and would not
13 necessarily reduce selfing (Kulbaba and Worley, 2008). Our results for *L. arvensis* are
14 similar to those for *Datura stramonium* (Motten and Stone, 2000) and *Gilia achilleifolia*
15 (Takebayashi *et al.*, 2006), in which outcrossing was favoured only when the stigma was
16 held above the anthers, but not when it was below them. In *L. arvensis*, flowers with
17 stigmas at the same level as the anthers or below them received up to 35 pollen grains by
18 autonomous self-pollination. A stigmatic pollen load of two to six grains per ovule has been
19 shown to ensure full seed-set ranges in other species (Cruden, 1977; Shore and Barrett,
20 1984; Aizen and Harder, 2007), although pollen quality can also limit seed-set (Aizen and
21 Harder, 2007). If the same requirement applies to *L. arvensis*, in which flowers have
22 between 17 and 30 ovules (Arista *et al.*, 2013; Ortiz *et al.*, 2015), our results suggest that as
23 many as half of the ovules could be fertilized as a result of delayed autonomous selfing
24 facilitated by reverse herkogamy on the second day of anthesis (Lloyd and Shoen, 1992),

1 even though seed-set may not be complete.

2

3 **Differences in herkogamy between populations and flower colour**

4 The blue and red lineages of *L. arvensis* differed for both herkogamy traits across the
5 sampled populations. On the one hand, red flowers showed strong lateral herkogamy on the
6 first day, with style-stamen angles generally > 20 degrees, but largely either no herkogamy
7 or reverse herkogamy on the second day. These results strongly implicate a strategy of
8 delayed selfing in red-flowered plants (when pollinators are abundant, they are likely
9 largely outcrossed on the first day of opening, but they may self-pollinate on their second
10 day). On the other hand, flowers of the blue lineage showed much greater variation in the
11 degree of lateral herkogamy on their first day of opening, with flowers in about half of the
12 studied plants and populations having stigma-stamen angles < 20 degrees, therefore being
13 susceptible to some autonomous self-pollination, whereas most blue flowers showed
14 substantial approach herkogamy on the second day of anthesis. While blue-flowered plants
15 with angles of lateral herkogamy > 20 degrees thus appear to be incapable of autonomous
16 self-pollination on both days of opening, those with angles < 20 degrees (and which display
17 lower approach herkogamy too) likely self-pollinate throughout anthesis, via competing
18 selfing (Lloyd 1979; Leclerc-Potvin and Ritland, 1994). The mating system of the blue-
19 flowered lineage is thus likely to be more variable than that of the red-flowered lineage.

20

21 We do not know the evolutionary basis or history of the floral and likely mating-system
22 differences between the red and blue lineages. However, the observed variation in
23 herkogamy traits particularly of the blue-flowered lineage of *L. arvensis* hints at possible
24 interpopulation differences in the ecological context of mating. For instance, some

1 populations of the blue lineage may be subject to more frequent cross-pollen limitation and
2 thus selection for reproductive assurance, which might not be an important force in other
3 populations. Variation in herkogamy might also reflect spatial variability in the expression
4 of inbreeding depression (e.g., Pujol *et al.*, 2009). Models that incorporate ecological
5 factors such as cross-pollen availability, gamete discounting (Johnston *et al.*, 2008) or
6 temporal variability in the expression of inbreeding depression (Cheptou and Schoen, 2002)
7 help to account for mixed mating in plants, which is otherwise difficult to explain (Lande
8 and Schemske, 1985; Jarne and Charlesworth, 1993; Holsinger, 1996), and it is possible
9 that such variation plays a role in maintaining mixed mating in *L. arvensis*.

10

11 The lower levels of herkogamy that we found for the red lineage in mixed populations of *L.*
12 *arvensis* may reflect either selection for reproductive assurance or selection to avoid mating
13 with the blue morphs in these populations (or both factors). Given that the red lineage of *L.*
14 *arvensis* tends to receive fewer pollinator visits than the blue lineage in mixed populations
15 (Ortiz *et al.*, 2015; Jiménez-López *et al.*, 2019b), its lower herkogamy may be a response to
16 stronger selection for reproductive assurance. However, we also found lower lateral
17 herkogamy for individuals of the blue lineage in mixed populations. This finding suggests
18 that the blue lineage may have a greater capacity for selfing in mixed populations, perhaps
19 reflecting selection to avoid the deleterious effects of hybridization with the red lineage.
20 Evolution towards a greater capacity for selfing has been interpreted as a mechanism to
21 avoid reproductive interference between a number of other co-occurring species or
22 divergent lineages in a number studies, e.g., *Phlox* (Levin, 1985), *Arenaria* (Fishman and
23 Wyatt, 1999), *Ipomoea* (Smith & Rausher, 2008), *Centaureium* (Brys *et al.*, 2014), and
24 *Mimulus* (Martin and Willis, 2007; Grossenbacher and Whittall, 2011). Such reductions in

1 gene flow between two lineages via enhanced prezygotic isolation should ultimately allow
2 further genetic divergence and the evolution of postzygotic isolation (Coyne and Orr,
3 2004), and may have been a component contributing to the current pattern of divergence
4 between the red and blue lineages of *L. arvensis*.

5
6 If the observed variation in floral behaviour displayed by the two *L. arvensis* lineages
7 affects the mating system in the manner we have suggested, we might expect to find
8 associated variation in inbreeding coefficients among populations. This association remains
9 to be studied in detail, but those data that do exist are not entirely consistent with
10 expectations. Of the various combinations of lateral versus vertical herkogamy, only those
11 that involve low lateral and approach herkogamy implies the promotion of predominant
12 prior or competing selfing; this pattern was observed mainly for the blue lineage in mixed
13 populations with the red. Accordingly, the decrease of approach herkogamy in the blue
14 lineage in mixed populations was associated with a significant increase in the predicted
15 self-pollen deposition, and estimates of inbreeding depression in populations of the blue
16 lineage are low (0.19 to 0.36; Jiménez-López *et al.*, 2019c), as might be expected for a
17 largely selfing lineage (Jiménez-López *et al.*, 2019c). In contrast, the decrease in
18 herkogamy observed for the red lineage did not correspond to greater predicted self-pollen
19 deposition. Moreover, the red lineage showed relatively high coefficients of inbreeding in
20 mixed populations (Jiménez-López *et al.*, 2019c), pointing to likely higher selfing rates in
21 these populations in particular, and consistent with the possibility that selection for reduced
22 interference between lineages has mainly affected the red lineage. Of course, the high
23 values of inbreeding depression estimated for Mediterranean populations of the red lineage
24 (0.61 to 0.65; Jiménez-López *et al.*, 2019c) should mitigate any advantage of reduced

1 reproductive interference due to selfing in these populations (Delmas *et al.*, 2014).

2

3 In conclusion, our study has uncovered an unusual combination of dynamic lateral and
4 vertical modes of herkogamy in an annual plant with two genetically divergent lineages
5 with different floral colours and that experience different levels of attractiveness to
6 pollinators. The conformation, extent and timing of herkogamy is partially consistent with
7 expectations for populations that vary in their susceptibility to pollinator limitation. At the
8 same time, the extent to which these patterns vary between pure and mixed populations is
9 consistent with selection, on one of the two interacting lineages, with selection to avoid
10 reproductive interference between them via increased self-fertilization. Future work should
11 establish the details of within- versus between lineage mating in mixed population, as well
12 as its fitness implications and thus the direction and strength of selection on the floral
13 morphology and behaviour. Certainly, the morphological and genetic divergence between
14 the red and blue lineages of *L. arvensis* offers scope to test several general hypotheses
15 concerning selection on traits that affect patterns of plant mating within populations and
16 gene flow between diverging lineages.

17

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4

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- 3

1 **Captions of figures**

2

3 Figure 1. Flowers of the blue and red lineage of *Lysimachia arvensis* during the first (a, c)
4 and second anthesis day (b, d).

5

6 Figure 2. Frequency distribution of style-stamen angle in flowers in 1st day of anthesis (a)
7 and stigma-anther displacement in flowers in 2nd day of anthesis (b). Differences between
8 blue and red plants in the style-stamen angle (c) and in stigma-anther displacement (d).
9 Medians, quartiles and ranges of the overall data are shown. Means and standard errors of
10 the style-stamen angle (e) and in stigma-anther displacement (f) in each sampled
11 population. In a, b, e and f, blue- and red-flowered plants are denoted in black and grey,
12 respectively. Sample sizes: 475 blue flowers from 19 populations and 430 red flowers from
13 16 populations.

14

15 Figure 3. Style-stamen angle (a) and stigma-anther displacement (b) of blue and red
16 lineages of *L. arvensis* in pure and mixed populations. Median, quartiles, maximum and
17 minimum are shown. Different letters indicate significant differences for the trait between
18 pure and mixed populations of each lineage.

19

20 Figure 4. Relationship between (a) style-stamen angle and self-pollen deposition in flowers
21 on the 1st day of anthesis, and (b) stigma-anther displacement and self-pollen deposition in
22 flowers on the 2nd day of anthesis in *L. arvensis*.

23

24

1 Table 1. Correlations between all the measured floral traits from the blue (above the
 2 diagonal) and the red (below the diagonal) lineages of *Lysimachia arvensis*. * correlations
 3 are significant at $p < 0.01$ after Bonferroni correction. Mean and standard deviation for blue
 4 flowers: 2.76 ± 0.3 style length, 2.6 ± 0.2 stamen length, 22.59 ± 9.3 style-stamen angle,
 5 0.16 ± 0.19 stigma-anther displacement; $n = 475$. For red flowers: 2.31 ± 0.2 style length,
 6 2.41 ± 0.2 stamen length, 34.43 ± 10.4 Style-stamen angle, -0.1 ± 0.15 stigma-anther
 7 displacement; $n = 430$.

8

	Style length (mm)	Stamen length (mm)	Stigma-anther displacement (mm)	Style-stamen angle (degree)
Style length (mm)	-	0.755*	0.551*	0.220*
Stamen length (mm)	0.789*	-	-0.1	0.069
Stigma-anther displacement (mm)	0.371*	-0.279*	-	0.255*
Style-stamen angle (degree)	-0.385*	-0.322*	-0.114	-

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Table 2. (a) MANOVA between two herkogamy traits: style-stamen angle and stigma-anther displacement as dependent variables and lineage (red or blue), population type (pure or mixed) and their interaction as independent variables. (b) Two-way ANOVAS between each of the two herkogamy traits and lineage, population type and their interaction.

Effect	λ Wilk	df	F	P
(a) MANOVA				
Intercept	0.112	2	3564.99	<0.000
Lineage	0.477	2	490.71	<0.000
Population type	0.963	2	17.19	<0.000
Population size	0.992	4	1.91	0.106
Lineage x Population type	0.966	2	15.96	<0.000
(b) Two-way ANOVAs				
Style-stamen angle	MS	df	F	P
Intercept	734087.15	1	7806.53	<0.000
Lineage	32604.8	1	346.73	<0.000
Population type	2894.69	1	30.78	<0.000
Lineage x Population type	16.867	1	0.18	0.672

Error	94.035	901		
Stigma-anther displacement	MS	df	F	P
Intercept	0.531	1	18.96	<0.000
Lineage	16.485	1	589.02	<0.000
Population type	0.589	1	21.05	<0.000
Lineage x Population type	1.234	1	44.10	<0.000
Error	0.028	901		

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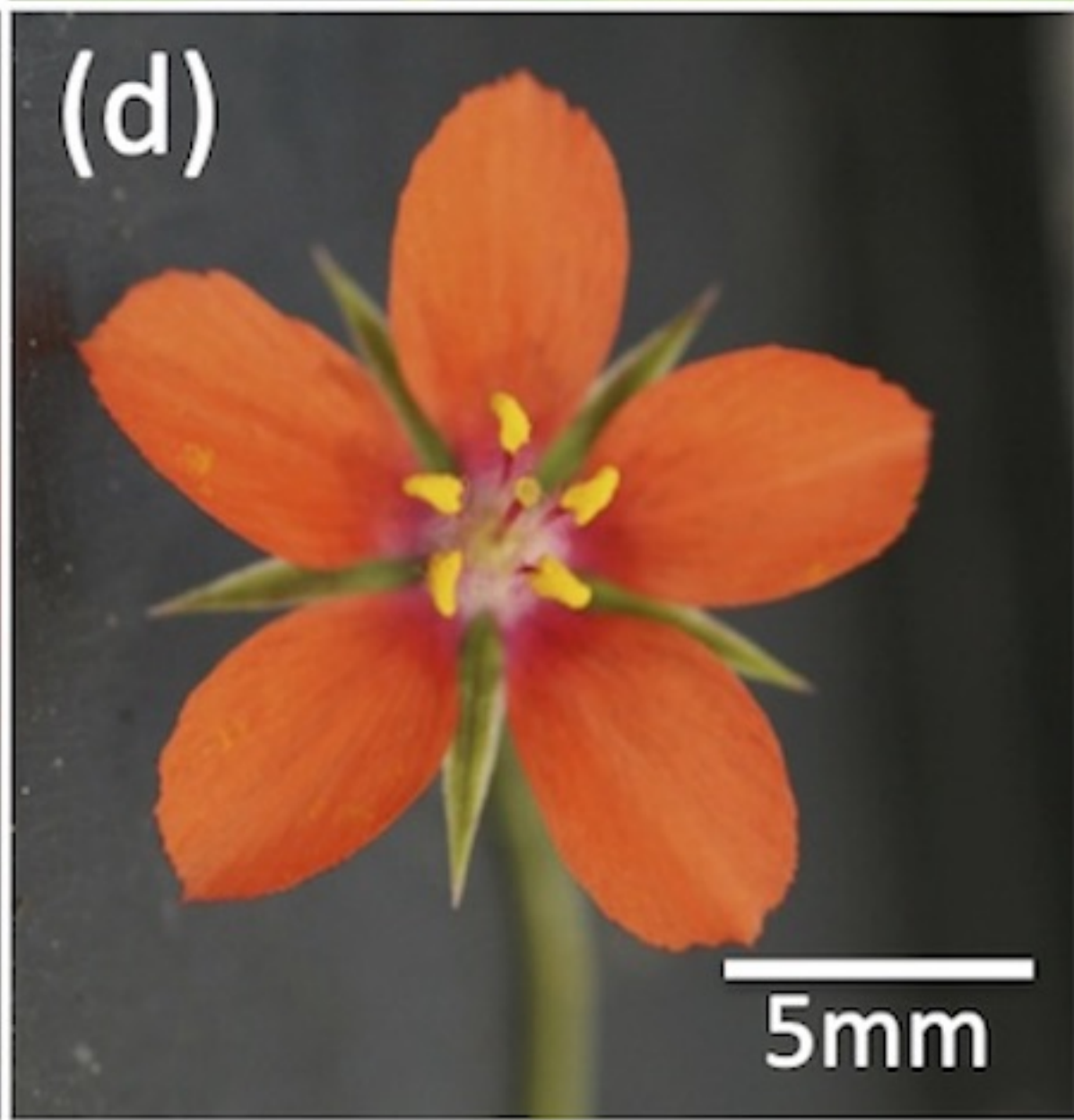
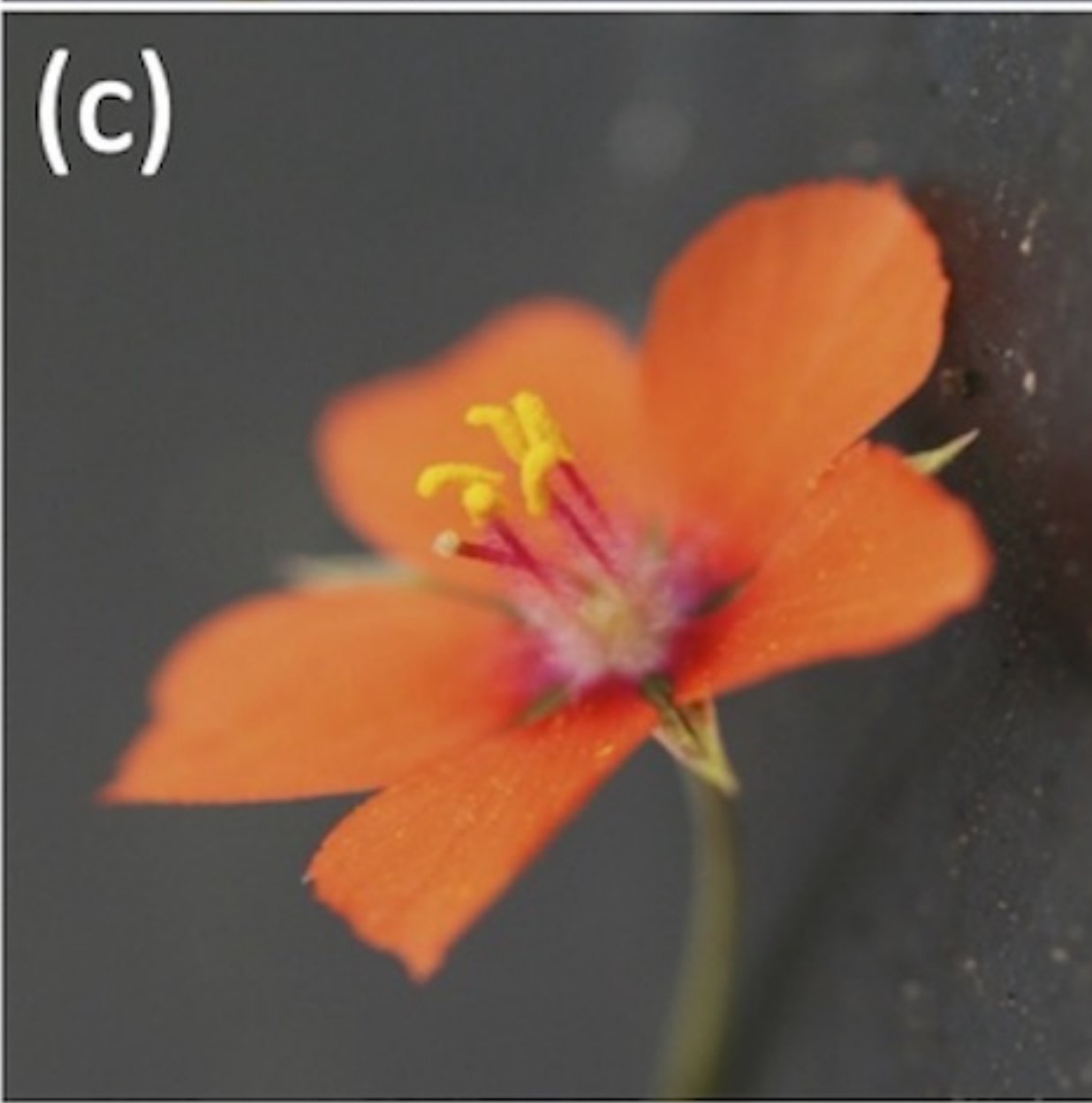
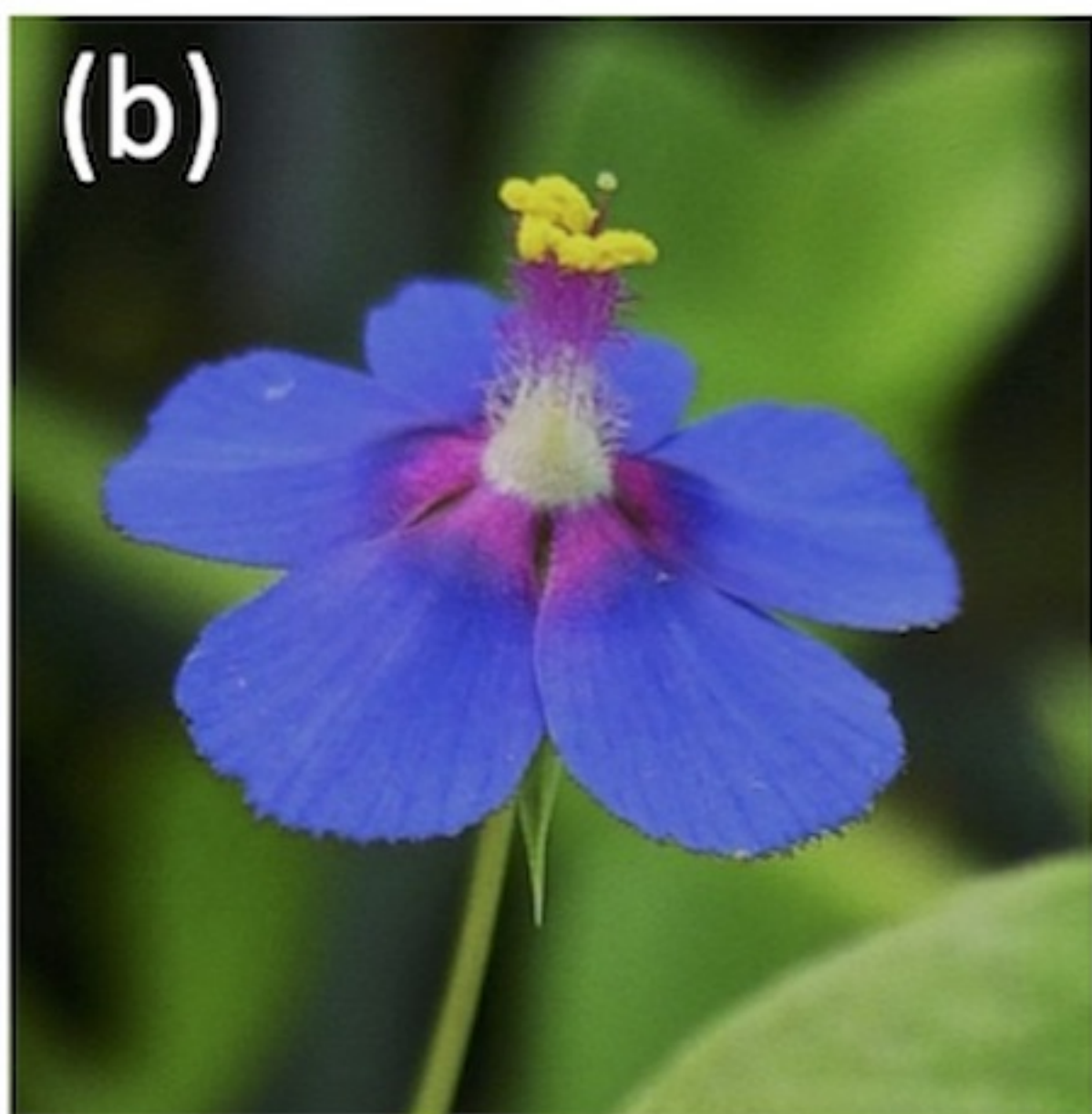
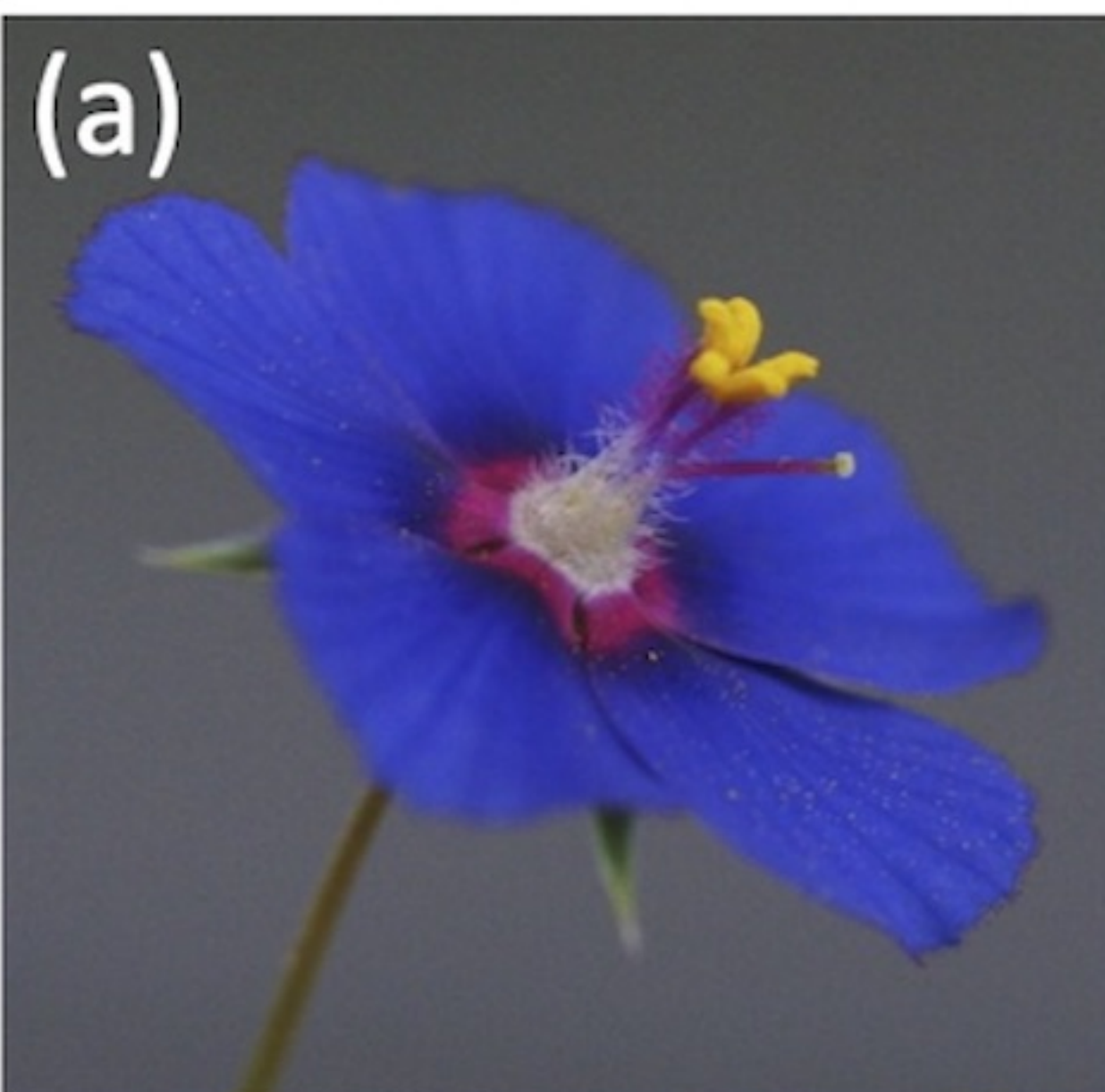


Figure 1

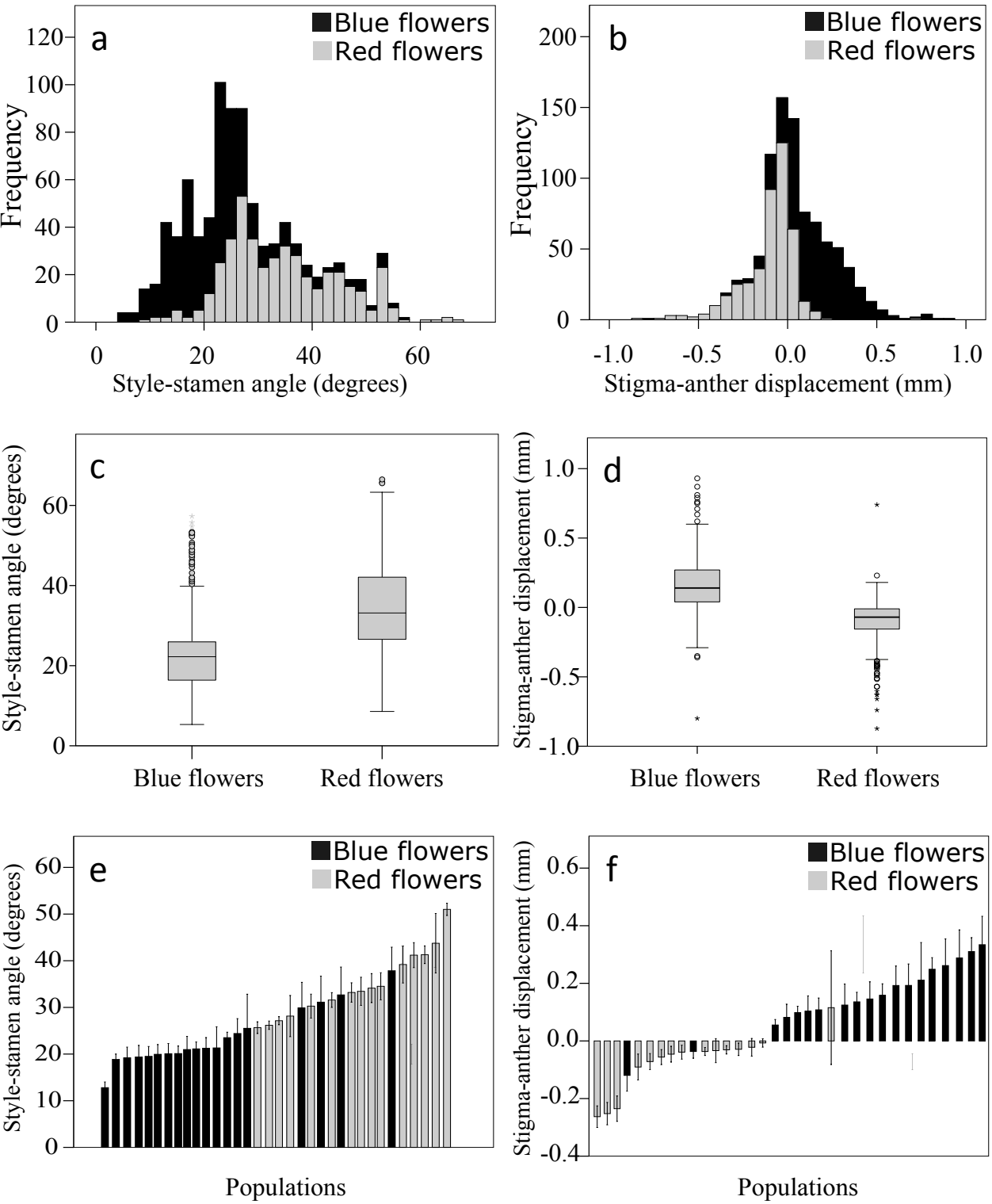


Figure 2

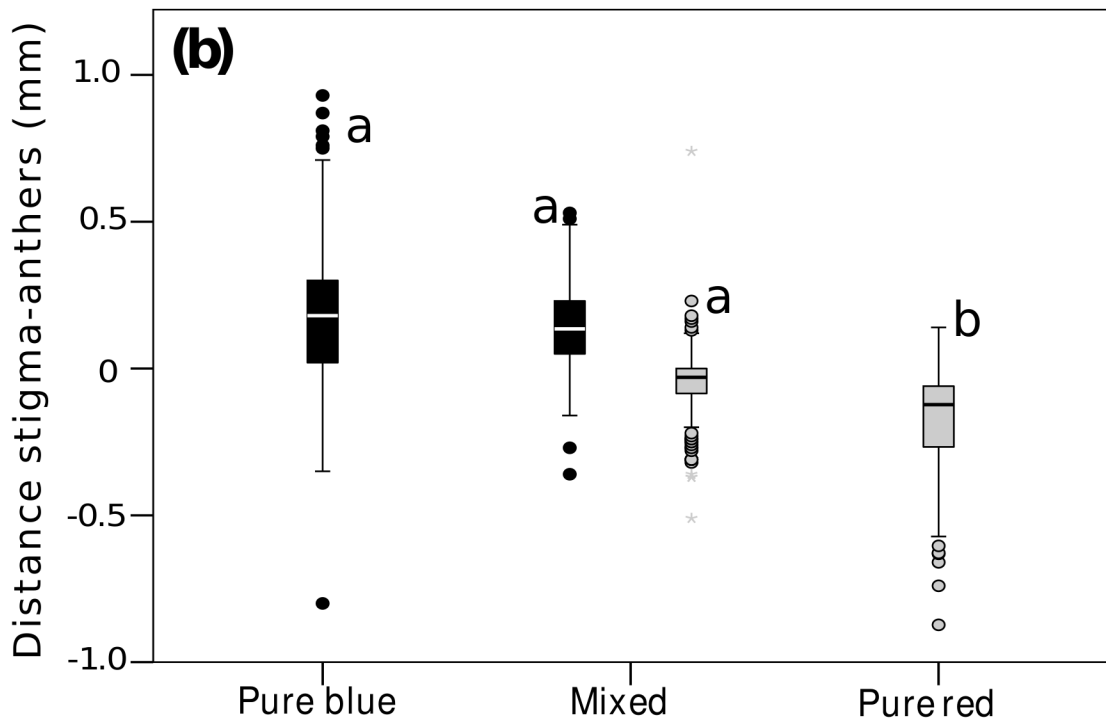
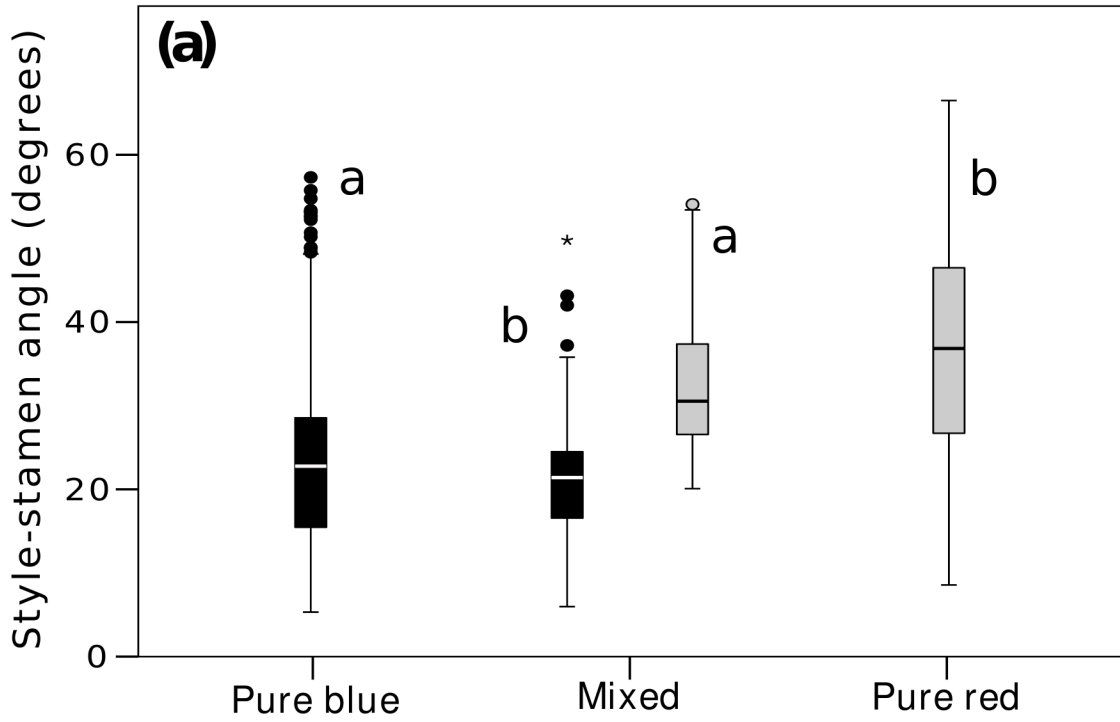


Figure 3

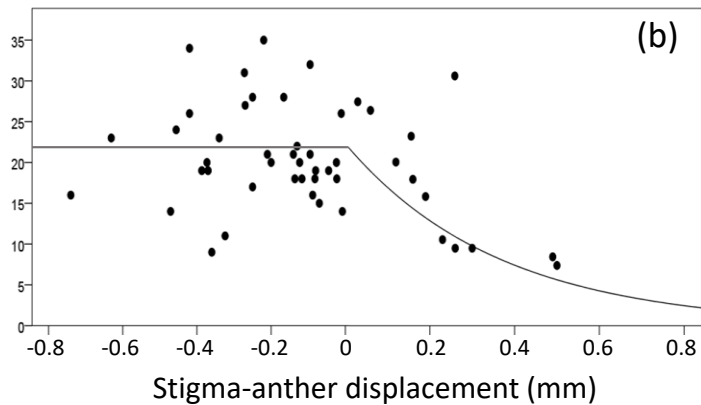
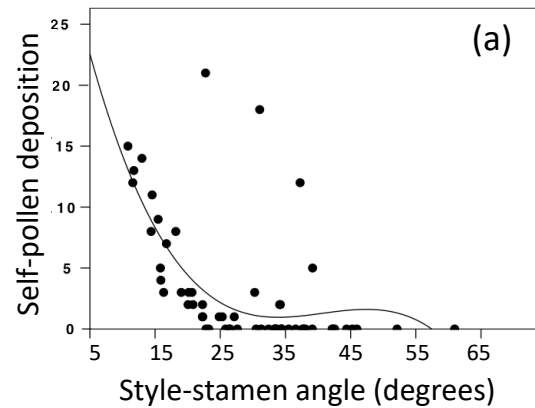


Figure 4