

1 **Heritabilities of lateral and vertical herkogamy in *Lysimachia arvensis***

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21 ABSTRACT

22

23 Herkogamy, spatial separation between stigma and anthers within a flower, is
24 important in regulating plant-mating system. We studied phenotypic variation and
25 heritability of herkogamy traits in *Lysimachia arvensis* (= *Anagallis arvensis*) that shows
26 both lateral and vertical herkogamy in the same flower, a rare strategy in flowering
27 plants. Both lateral and vertical herkogamy showed continuous variation in 15 natural
28 populations. Lateral herkogamy, measured as the angle between style and stamens,
29 ranged from 5.6 to 66.5 degrees; vertical herkogamy ranged from reverse to approach
30 herkogamy. Herkogamy traits were constant within plants but variable among plants
31 and populations. Flowers with marked lateral herkogamy showed mainly reverse
32 herkogamy, while flowers with low lateral herkogamy showed mainly approach
33 herkogamy. Both herkogamy traits showed a high degree of narrow sense heritability
34 ($h^2=0.843$ for lateral and $h^2=0.635$ for vertical herkogamy). We discuss the possibility
35 that variation in both herkogamy traits among populations of *L. arvensis* is a
36 consequence of differential selective pressures under different pollination
37 environments.

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39 Keywords: *Anagallis*, approach herkogamy, floral trait evolution, Primulaceae, reverse
40 herkogamy

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43 INTRODUCTION

44

45 Herkogamy is the spatial separation between stigma and anthers within a
46 hermaphrodite flower (Webb & Lloyd, 1986) and appears in both self-incompatible
47 and self-compatible species. In self-incompatible species, herkogamy reduces
48 interference between sexual organs, e.g., preventing stigma clogging and improving
49 pollen export (Webb & Lloyd, 1986). In self-compatible species, herkogamy may also
50 play an important role in preventing self-fertilization (Webb & Lloyd, 1986). Diverse
51 studies have shown that small differences in herkogamy result in different rates of self-
52 pollen deposition (e.g. Ritland & Ritland, 1989; Robertson et al., 1994; Karron et al.,
53 1997; Motten & Stone, 2000). Increased herkogamy can be selected in environments
54 in which cross-pollination is favoured, e.g. to prevent selfing and inbreeding
55 depression. In contrast, in environments in which pollinators or mates are limited,
56 reduced herkogamy may be favoured by selection for reproductive assurance (e.g.
57 Moeller & Geber, 2005; de Vos et al., 2012).

58

59 The precise modality of stigma-anther separation can take several forms, including
60 vertical and lateral herkogamy. Vertical herkogamy involves a vertical displacement
61 between the stigma and anthers and is by far the most common. Stigma may situate
62 above anthers such that pollinators contact it before pollen, a situation termed
63 approach herkogamy; alternatively, stigma may situate below anthers such that
64 pollinators contact it after pollen, a situation termed reverse herkogamy (Webb &
65 Lloyd, 1986). Approach herkogamy is the most frequent form of vertical herkogamy in
66 self-incompatible species while approach and reverse herkogamy are equally frequent
67 in self-compatible species (Lloyd & Webb, 1992; Opedal et al., 2017). Some plant
68 species show stylar polymorphism, with individuals presenting either approach or
69 reverse herkogamy; the genetic basis of such polymorphism has been studied in some
70 species, revealing the action of one or two major loci (Barrett et al., 2000; Ushijima et
71 al., 2012; Nowak et al., 2015). In other species, styles show continuous variation in
72 height, with flowers ranging from reverse to approach herkogamy (Motten & Stone

73 2000; Takebayashi et al., 2006). Metric traits, as the length of style or the position of
74 stamens, are usually controlled by several loci with small effects (Kulbaba & Worley,
75 2008), and covariation between floral organs is common (e.g., Conner & Sterling 1996;
76 Herrera, 2001). Such continuous covariation could constrain the evolvability of
77 herkogamy (Lande & Arnold, 1983). A much less common type of spatial separation
78 between sexual organs is lateral herkogamy, in which the style is horizontally displaced
79 from the centre of the flower, forming an angle with the stamens. Lateral herkogamy
80 has been described, e.g., in *Linum* (Ruiz-Martín et al., 2018) and *Centaureum* (Brys &
81 Jacquemyn, 2011), but as far as we are aware its genetic architecture is unknown.

82 *Lysimachia arvensis* is a self-compatible species that, unusually, shows both vertical
83 and lateral herkogamy. Flowers show lateral herkogamy during their first day of
84 opening, but their styles subsequently move to a central position, showing vertical
85 herkogamy on their second day (Jiménez-López et al., pers. obs.). The consecutive
86 expression of two types of herkogamy in the same flower is uncommon and could
87 represent a two-step barrier to self-pollination. *Lysimachia arvensis* is widely
88 distributed, occurring both in stable habitats with a favourable pollination
89 environment and in disturbed places where the pollination environment may be
90 unfavourable and/or unpredictable. If herkogamy regulates the probability of self-
91 pollen deposition, variation in herkogamy might thus reflect variation in outcrossing
92 opportunities (e.g. Takebayashi et al., 2006; Herlihy & Eckert, 2007). On the other
93 hand, variation in the expression of herkogamy might simply reflect developmental
94 instability (Dongen, 2000; Debat & David, 2001) or low trait canalization (Waddington,
95 1942; Debat & David, 2001). Either way, knowledge of its genetic architecture,
96 specifically its narrow-sense heritability, would be a useful first step towards
97 understanding the potential for herkogamy to evolve in response to selection. Our
98 study thus aimed to determine the heritability of both lateral and vertical components
99 of herkogamy in *Lysimachia arvensis*, as well as to characterize other correlations
100 between floral traits.

101

102 MATERIAL AND METHODS

103 *Lysimachia arvensis* (L.) U. Manns & Anderb. (former *Anagallis arvensis* L.; Manns &
104 Anderberg, 2009) is an annual plant native to the Mediterranean Basin but widely
105 distributed around the world. The species is polymorphic in flower colour, with some
106 plants producing blue flowers and other red flowers (Arista et al., 2013). Flowers last
107 two or three days. Anther opening occurs during the first anthesis day and the stigma
108 is receptive throughout the life span of the flower. During 2015 and 2016, we sampled
109 15 natural populations of *L. arvensis* in a wide geographical range during the peak of
110 flowering (Appendix 1). In each population, we randomly collected between ten and
111 30 plants and measured herkogamy traits for two flowers per plant. Moreover, we
112 studied floral variation within maternal families growing in a common glasshouse. In
113 one population from Huelva (SW Spain; 37°17'31"N 6°22'43"W), seeds were collected
114 in 2016 from a single fruit of each of eight individuals that were separated in the
115 population by at least 5 m. Seeds were allowed to germinate in a growth chamber, and
116 the resulting plant families were raised in a glasshouse. We chose a single parent plant
117 per family and measured its two herkogamy traits. Each such parent was hand-
118 pollinated, either with pollen from another parent, or with its own pollen. Fruits
119 resulting from each female-male parent combination were separately collected, and
120 seeds were sown to obtain offspring families. Herkogamy traits were measured in
121 2017 for 6 to 16 offspring per family.

122 Herkogamy measurements were taken from fresh flowers, starting their first day of
123 anthesis, based on photographs taken directly in the glasshouse. From these images,
124 the two components of herkogamy were measured using ImageJ software. Lateral
125 herkogamy was measured as the angle between style and stamens (hereafter 'style-
126 stamen angle'; Fig. 1). To characterize approach or reverse herkogamy, we measured
127 stamen length (from flower base to anther centre) and pistil length (from flower base
128 to stigma centre; Fig. 1); approach or reverse herkogamy was then calculated as the
129 difference between pistil and stamen lengths, which would be equivalent to final
130 separation between anthers and stigma (hereafter 'stigma-anther displacement').

131 Variation in herkogamy traits among populations and plants was tested by means of
132 general linear models (GLM), with population treated as a main effect and plant

133 nested in population. Gaussian distributions were used to analyse both lateral and
134 vertical herkogamy, and analyses were conducted using GLZM module of SPSS (IBM
135 SPSS Statistic 24, USA) with Type III test. Pearson correlations between pistil and
136 stamen lengths, stigma-anther displacement and style-stamen angle were calculated
137 for the flowers measured in the field. To determine whether herkogamy traits were
138 constant within plants, we performed Pearson correlations between the herkogamy
139 values obtained in the two flowers of each plant sampled in the field. Heritability of
140 each herkogamy trait was estimated for the plants growing in glasshouse by regressing
141 values of offspring families on mean values of each trait of their parents; the slope of
142 the line of best-fit is an estimate of heritability (h^2 ; e.g. Lennartsson et al., 2000).

143

144 RESULTS

145 Flowers of *Lysimachia arvensis* display two types of herkogamy that change
146 sequentially throughout their life span. The first day of opening, all the flowers showed
147 lateral herkogamy and the anthers were longitudinally placed in relation to stamen
148 filament opening towards the inner of the flower (Fig. 1). During the second day of
149 opening, the style had moved upright, and the anthers were transversally placed in
150 relation to the stamen filament (Fig. 1). In flowers lasting three days, vertical
151 herkogamy did not change from the second day of anthesis.

152 In plants measured in the field, mean pistil length was 2.53 mm and mean stamen
153 length was 2.50 mm ($n = 600$). Style-stamen angle ranged from 5.6 to 66.5 degrees,
154 with a mean of 28.6 ($n = 600$) while stigma-anther displacement ranged from negative
155 to positive values (mean 0.03, $n = 600$; Fig. 2). These four traits were significantly
156 correlated (Fig. 3). Style-stamen angle was negatively correlated with both pistil and
157 stamen length, while stigma-anther displacement was positively correlated with both
158 traits. The two herkogamy traits were almost identical within plants, with Pearson
159 correlations for both traits between pairs of flowers of the same plant being very high:
160 $r = 0.939$ ($p < 0.0001$, $n = 300$) for style-stamen angle; $r = 0.885$ ($p < 0.0001$, $n = 300$)
161 for stigma-anther displacement. Style-stamen angle and stigma-anther displacement

162 showed significant variation among populations (Wald-chi square = 429.19, 14 df, $p <$
163 0.001 for lateral and Wald-chi square = 509.42, 14 df, $p <$ 0.001 for vertical herkogamy)
164 and plants (Wald-chi square = 9529.88, 283 df, $p <$ 0.001 for lateral and Wald-chi
165 square = 4310.46, 283 df, $p <$ 0.001 for vertical herkogamy).

166 For plants coming from a single population and grown in the glasshouse, pistil length
167 ranged from 0.90 to 2.80 mm (mean = 2.46, SD = 0.21, $n = 96$) and stamen length
168 between 2.11 – 2.72 mm (mean = 2.36, SD = 0.15, $n = 96$). Style-stamen angle ranged
169 from 12.56 – 40.71 degrees, with a mean angle of 25.38 degrees (SD = 6.89, $n = 96$)
170 while stigma-anther displacement ranged from -0.06 to 0.42 mm with a mean 0.1 mm
171 (SD = 0.1, $n = 96$). Heritabilities calculated in terms of offspring-parent regressions
172 were significant for pistil length ($F_{1, 95} = 306.42$, $p <$ 0.0001), stamen length ($F_{1, 95} =$
173 328.09, $p <$ 0.0001), style-stamen angle ($F_{1, 95} = 230.98$, $p <$ 0.0001; Fig. 4) and stigma-
174 anther displacement ($F_{1, 95} = 63.68$, $p <$ 0.0001; Fig. 4). Narrow-sense heritability (h^2)
175 was high for all the traits: 0.875 for pistil length, 0.882 for stamen length, 0.843 for
176 lateral herkogamy and 0.635 for vertical herkogamy.

177

178 DISCUSSION

179 *Lysimachia arvensis* displays two types of herkogamy that show continuous variation.
180 The first day of opening, all the flowers observed showed lateral herkogamy, although
181 in some cases the angle between the style and the stamens was as little as 5.6
182 degrees. However, in the second day of opening, the style had moved upright, and
183 flowers showed wide and continuous variation in vertical herkogamy, ranging from
184 reverse to approach herkogamy, and including flowers with their stigma and anthers at
185 the same level. Both herkogamy traits showed a high variation among plants, as has
186 been found in other species (e.g., Luijten et al., 1999; Lennartsson et al., 2000; Herlihy
187 & Eckert, 2007). Although we do not yet know the role of approach and reverse
188 herkogamy in self-pollination in *L. arvensis*, flowers without vertical herkogamy are
189 certainly able to self-pollinate autonomously (Jiménez-López, unpub. data). Thus,
190 during their first day of opening, flowers of *L. arvensis* are more likely to be

191 outcrossed, but those that do not show vertical herkogamy may show delayed self-
192 pollination. Such delayed selfing is likely to be adaptive in an annual plant by
193 conferring reproductive assurance in the absence of mates or pollinators (Kalisz &
194 Vogler, 2003; Kalisz et al., 2004).

195 The lengths of pistil and stamens in *L. arvensis* were strongly correlated, i.e., flowers
196 with longer pistils also had longer stamens. Both sexual organs also showed a positive
197 correlation with vertical herkogamy, although variation in the stigma-anther
198 displacement depended mainly on variation in pistil length. As indicated by the
199 coefficients of determination (r^2), the length of the pistil was responsible for 51.2% of
200 variation in stigma-anther displacement (Fig. 3B), while the length of the stamen was
201 responsible for only 2.9% (Fig. 3D). That is, vertical herkogamy is mainly a consequence
202 of variation in pistil length, and this may suggest that the correlation between sexual
203 organs does not constrain the evolution of this trait in response to selective pressures
204 (Ushimaru & Nakata, 2002). A similar result has been found for other taxa with
205 continuous variation in vertical herkogamy, such as *Mimulus* (Kleunen & Ritland,
206 2004), *Aquilegia* (Herlihy & Eckert, 2007) and *Polemonium* (Kulbaba & Worley, 2008),
207 as well as in species with stylar polymorphisms (e.g., Barrett et al., 2000). In contrast
208 with vertical herkogamy, sexual organs showed a negative correlation with lateral
209 herkogamy. Although the style-stamen angle does not necessarily depend on the
210 lengths of the sexual organs, in *L. arvensis* this does appear to be the case. This implies
211 that flowers with marked lateral herkogamy show mainly reverse vertical herkogamy,
212 whereas flowers with low lateral herkogamy show mainly approach vertical
213 herkogamy, as shown by the negative correlation between both herkogamy traits.

214 *Lysimachia arvensis* only offers pollen as a reward, and bees grab the anthers to collect
215 pollen, landing either directly on the anthers or on the petals and going quickly to the
216 anthers. Thus, in flowers of *L. arvensis* with lateral herkogamy pollinators would
217 contact the anthers before the stigma in most visits, which makes lateral herkogamy
218 functionally similar to reverse vertical herkogamy. It would be interesting to determine
219 the relative role of each herkogamy trait in preventing self-pollination, and whether
220 they act synergistically.

221 Herkogamy traits were very similar within plants, as suggested by the strong
222 correlations between the two measured flowers of each plant. This indicates that
223 variation in herkogamy traits is unlikely to be a consequence of developmental
224 instability (Dongen, 2000; Debat & David, 2001) and suggests strong broad-sense
225 heritability. In fact, regressions of progeny on parent traits showed a high degree of
226 narrow-sense heritability for both lateral and vertical herkogamy. Heritability of
227 vertical herkogamy has been shown in numerous species, ranging from 0.30 to 0.85
228 (e.g., Luijten et al., 1999; Lennartsson et al., 2000; Kleunen & Ritland, 2004; Kulbaba &
229 Worley, 2008): our estimate ($h^2=0.635$) is towards the high end of variation
230 documented among species studied to date. Heritability of the lateral herkogamy
231 ($h^2=0.843$) was even higher than that of vertical herkogamy. Heritability estimates for
232 lateral herkogamy are much less frequent in the literature and, to our knowledge, our
233 estimate seems to be the first.

234 In summary, our results indicate that both herkogamy traits in *L. arvensis* are
235 phenotypically variable and have a high degree of heritability. If we assume that
236 herkogamy affects self-pollen deposition as happens in other studied species (e.g.
237 Barrett & Shore, 1987; Kalisz & Vogler, 2003; Kalisz et al., 2004; Takebayashi et al.,
238 2006), differences in pollination environment can select for different herkogamy traits.
239 *Lysimachia arvensis* is a widely distributed species that occurs mainly in ruderal
240 environments where pollinator availability is very variable. In fact, marked differences
241 in pollinator attendance has been previously reported, with some populations of
242 Southern Spain receiving very few visits (Gibbs & Talavera, 2001), others being visited
243 by solitary bees (Ortiz et al., 2015) or other populations from Germany receiving visits
244 from *Bombus terrestris* (Raine & Chittka, 2007). Thus, the variation in herkogamy traits
245 found among populations could be a consequence of differential selective pressures by
246 pollinators. Differences in pollinator attendance between colour morphs have also
247 been reported, with the blue flowered morph receiving more visits than the red
248 flowered morph (Ortiz et al., 2015). Although we have not measured differences in
249 herkogamy traits between colour morphs, it is tempting to speculate a lower
250 herkogamy expression in the less visited morph to increase reproductive assurance.

251 Either way, knowing how each herkogamy trait prevents self-pollen deposition in *L.*
252 *arvensis* would be crucial to understand their evolution.

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- 362

363 **Figure 1.** Schematic representation of flowers of *Lysimachia arvensis* on the first and
364 the second day of anthesis, showing the traits measured to characterize herkogamy. A,
365 lateral herkogamy; B, approach herkogamy; C, reverse herkogamy.

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367

368 **Figure 2.** (A) Frequency distribution of style-stamen angle (degrees) in the first day of
369 flower opening and (B) the stigma-anther displacement in the second day of flower
370 opening in *Lysimachia arvensis* from 15 natural populations.

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372

373 **Figure 3.** Pearson correlations between floral traits of 15 populations of *Lysimachia*
374 *arvensis* in the W Europe and N Africa. (A) Pistil/Stamen length, (B) Pistil
375 length/Stigma-anther displacement, (C) Pistil length/Style-stamen angle, (D) Stamen
376 length/Stigma-anther displacement (E) Stamen length/Style-stamen angle and (F)
377 Style-stamen angle/stigma-anther displacement. Sample sizes are 600 in all cases. All
378 correlations were significant at $p < 0.0001$.

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380

381 **Figure 4.** Relationships between the style-stamen angle (A) and the anther-stigma
382 displacement (B) of parent plants of *Lysimachia arvensis* and their offspring derived
383 from hand pollinations. Herkogamy values of parent plants were calculated as the
384 mean value between both parents (i.e., mid-parent value).

Appendix 1. Sampled populations of *Lysimachia arvensis* with sample sizes and geographic coordinates.

Locality	Sample size	Coordinates
Portugal. Alentejo. Sines	10	37°57'11"N / 8°55'51"W
Portugal. Algarve. Aljezur. Monte Clerigo	27	37°18'35.3"N / 8°48'37.9"W
Portugal. Algarve. Carrapateira	29	37°11'47.4"N 8°54'27.4"W
Spain. Tarragona. Mont-Roig del Camp	22	42°04'43.3"N / 2°09'07.7"E
Spain. Sevilla. Dos Hermanas	30	37°21'09.8"N / 5°56'23.2"W
Spain. Sevilla. Alamillo	30	37°24'46.9"N / 5°59'48.8"W
Spain. Cádiz. Zahara de la Sierra	18	36°49'30.2"N / 5°22'44.9"W
Spain. Cádiz. Zahara de los Atunes	15	36°7'7.0"N / 5°49'58.8"W
Spain. Huelva. Aracena 1	20	37°54'13.3"N / 6°34'08.2"W
Spain. Huelva. Aracena 2	15	37°54'44.8"N / 6°34'08.0"W
Spain. Alicante. Albaida	11	38°48'38.2"N / 0°30'0.0"W
Spain. Alicante. Sierra de Bernia	13	38°40'19.4"N / 0°2'45.8"W
France. Corsica. Solenzara	30	41°51'19.5"N / 9°21'43.67"E
Switzerland. Chéserey. Les Rouges	15	46°24'11.6"N / 6°09'34.8"E
Tunisia. Tabarka	15	36°57'46"N / 8°44'51"E

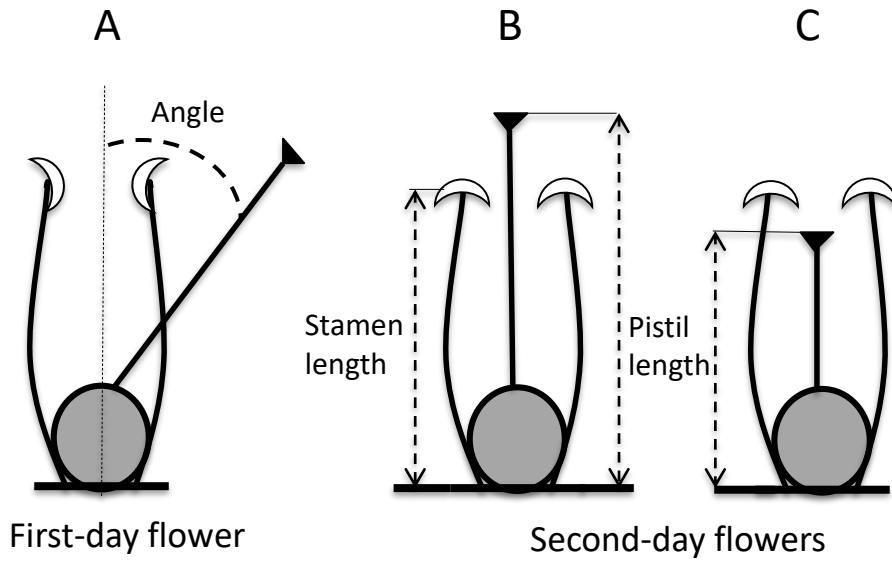


Figure 1.

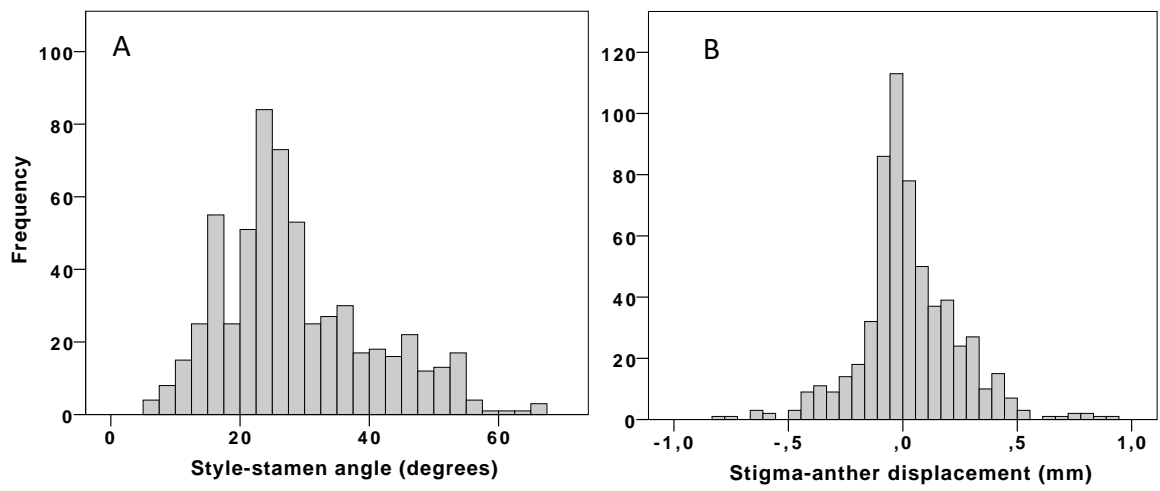


Figure 2

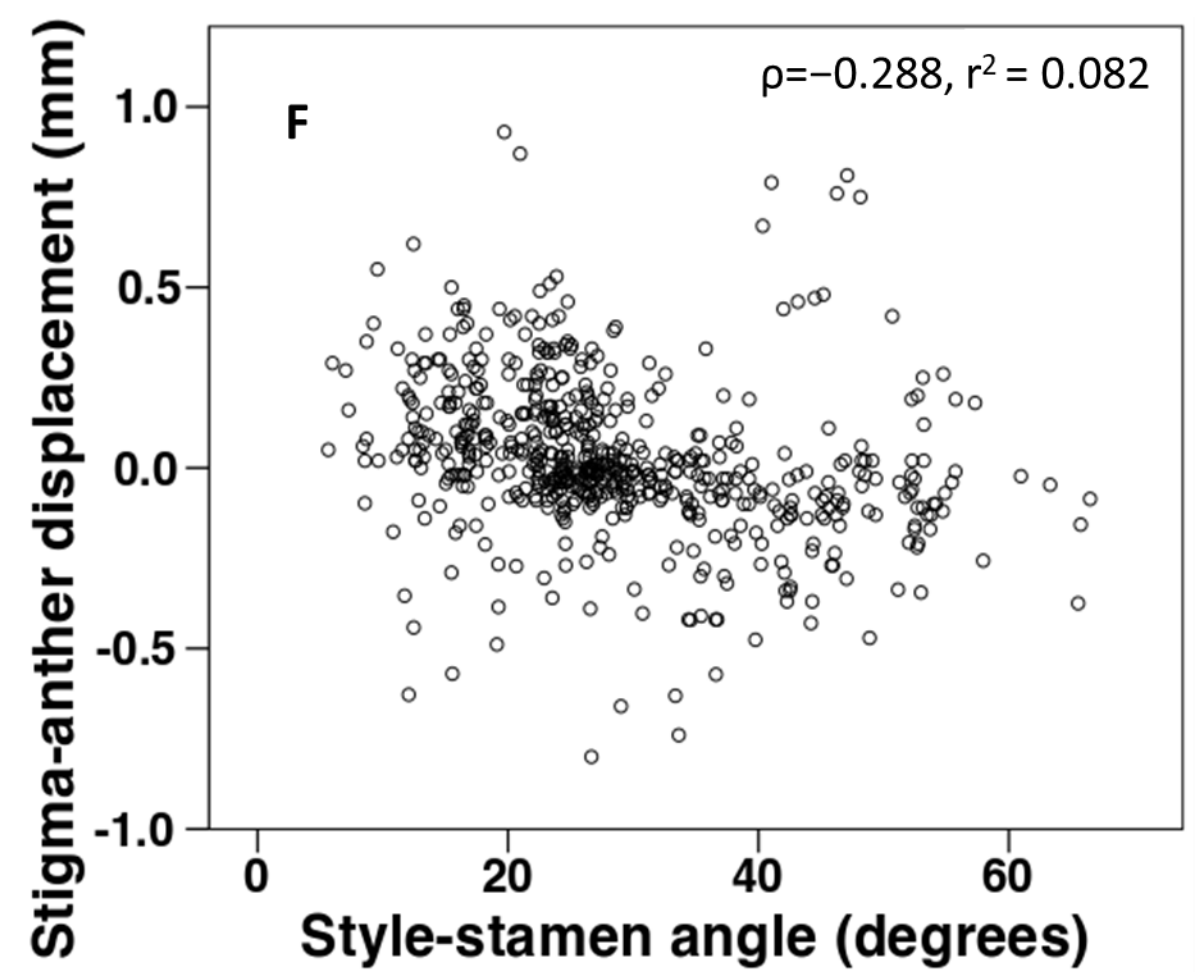
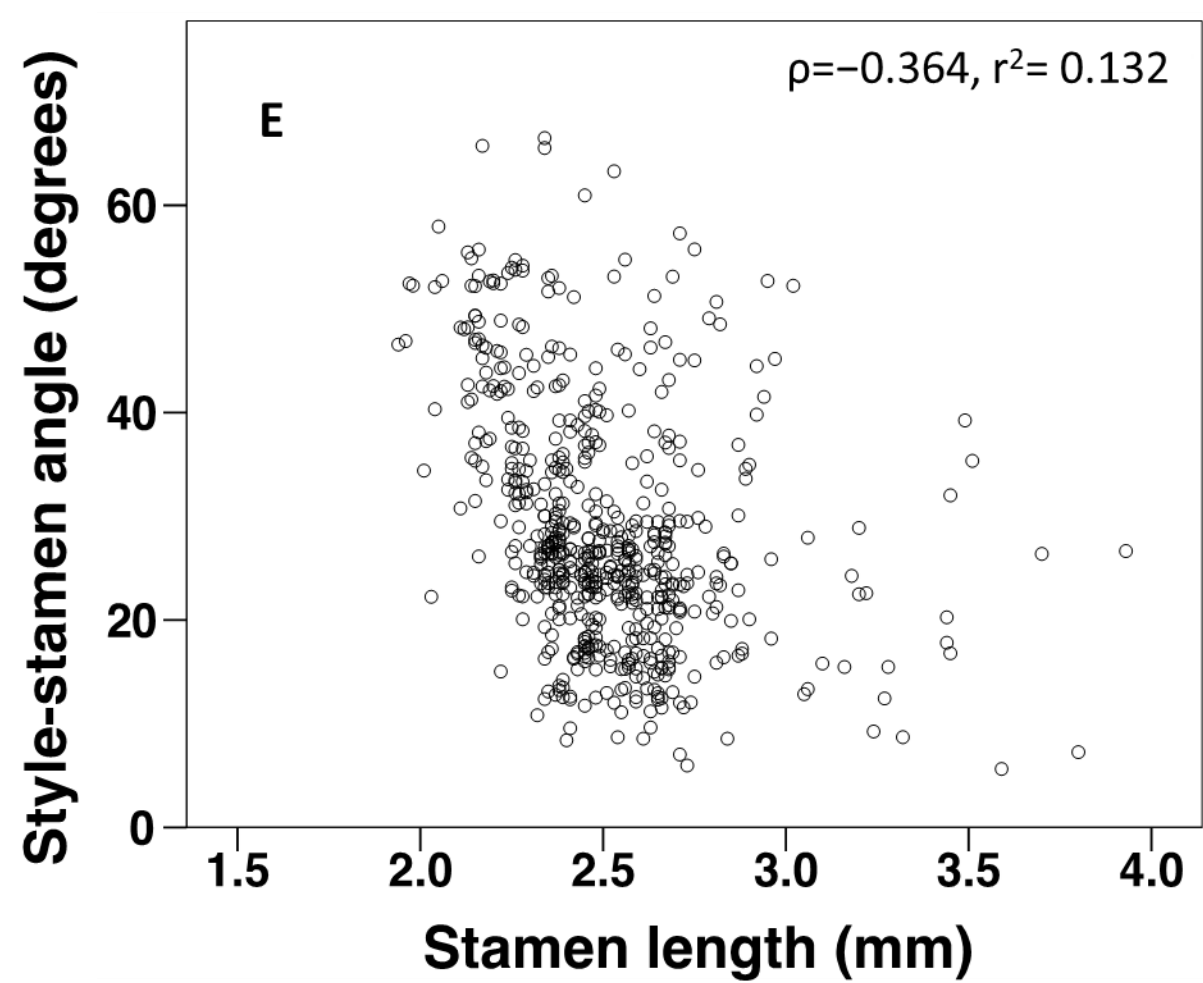
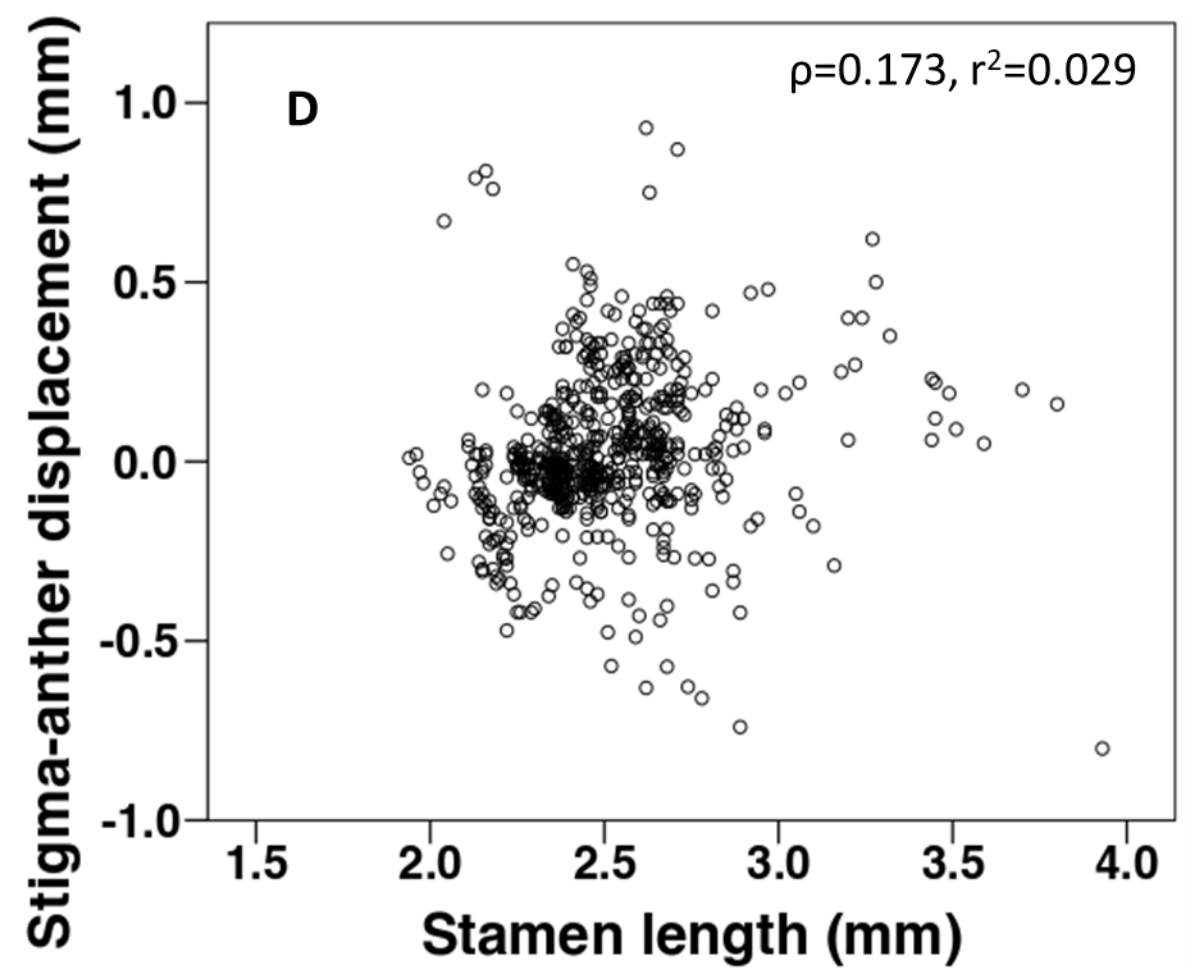
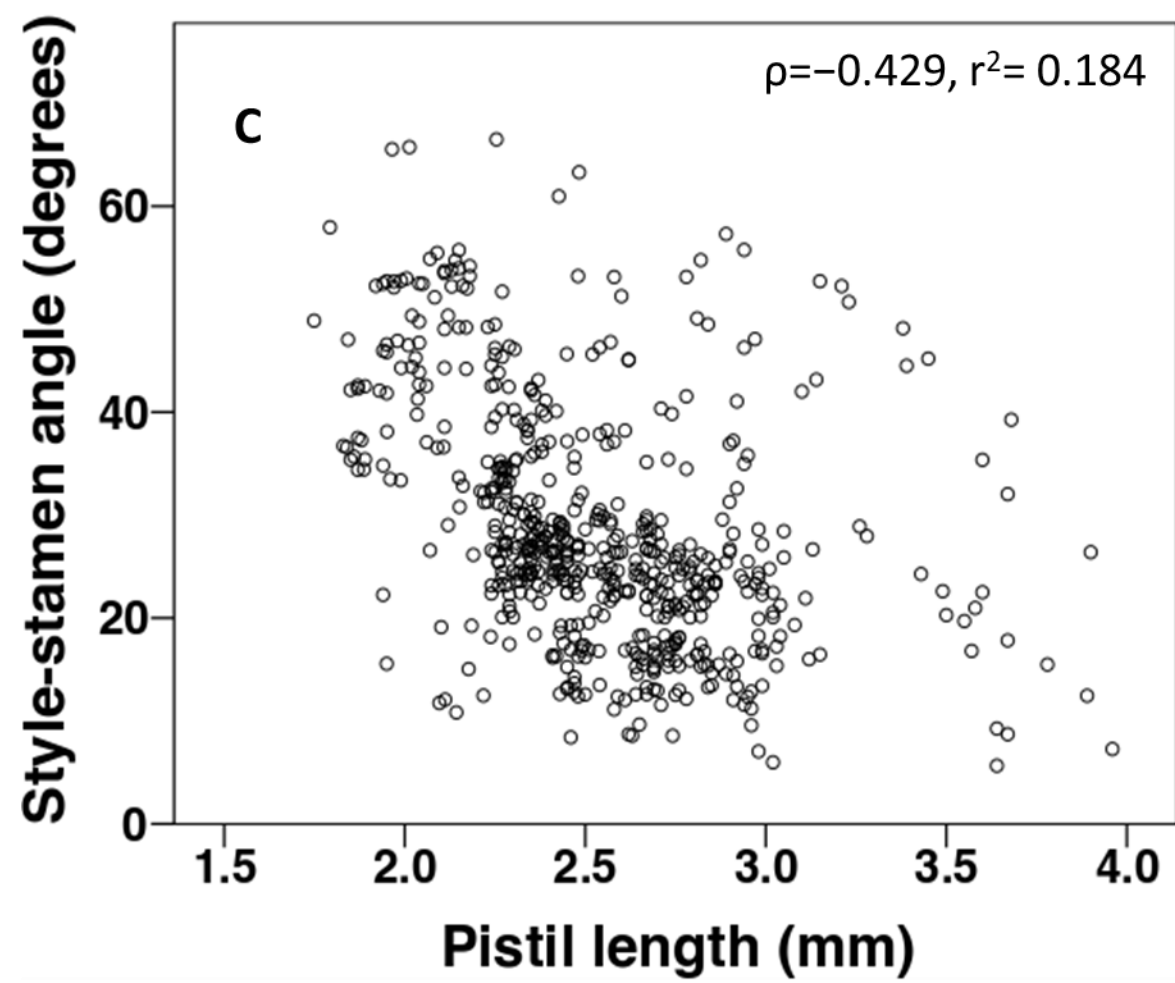
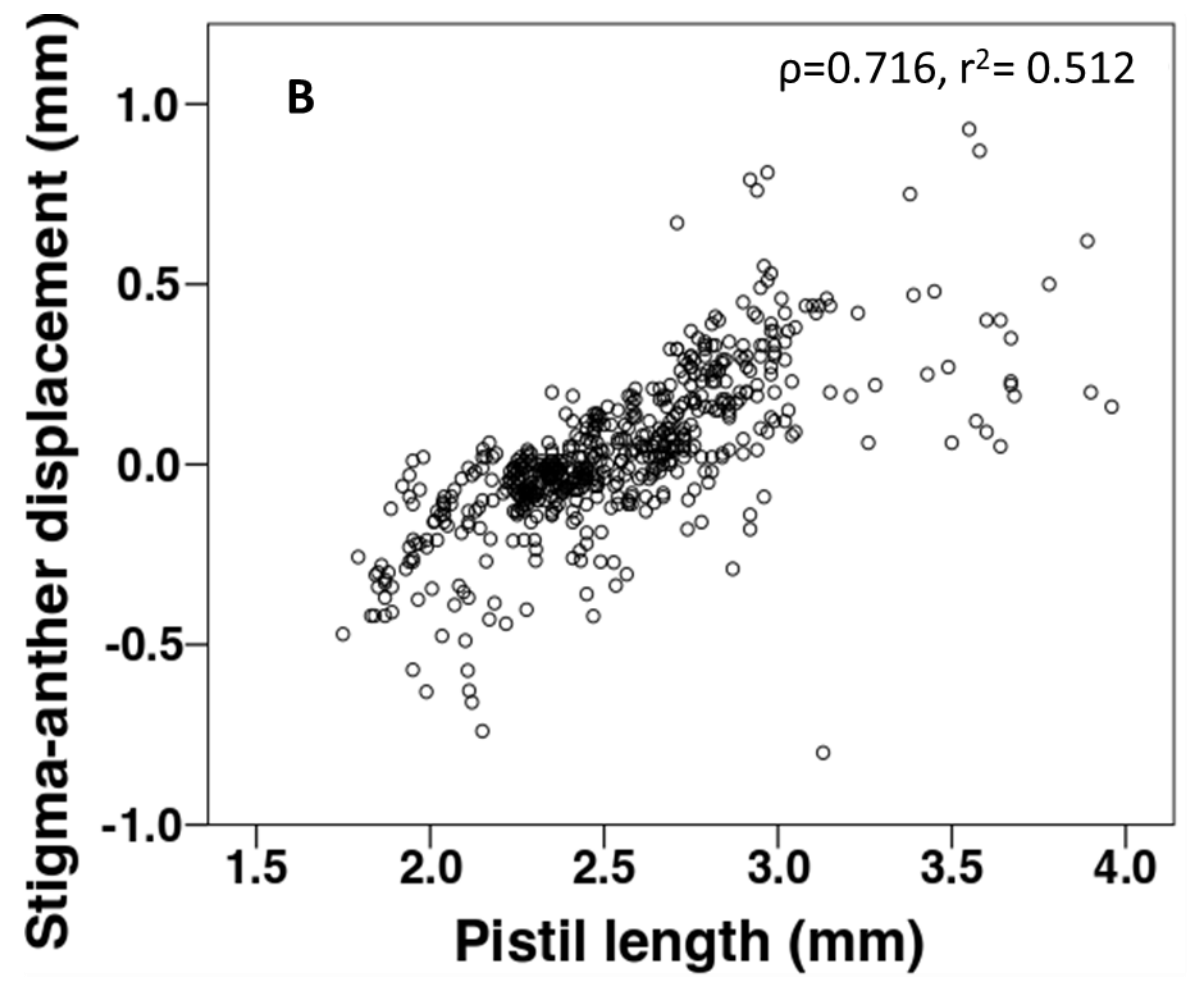
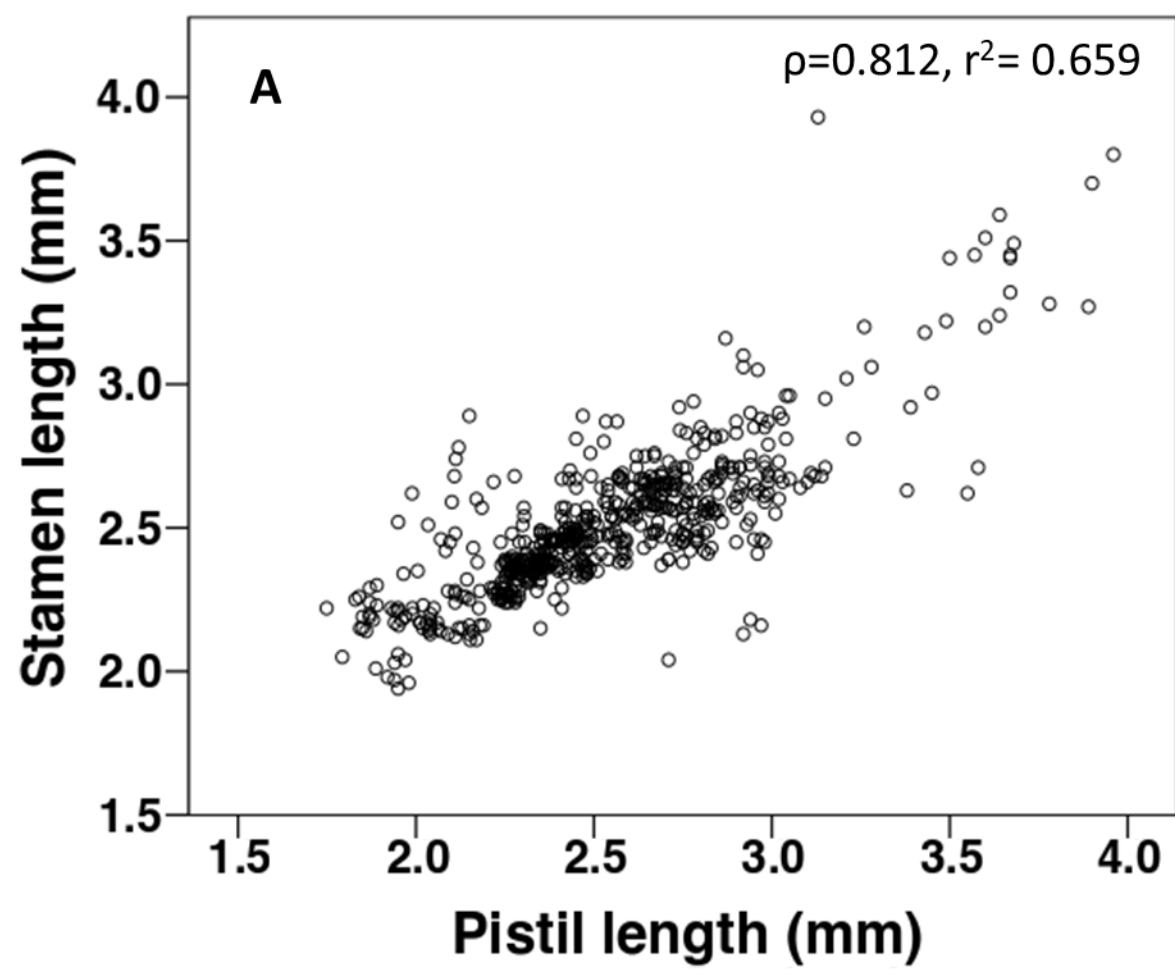


Figure 3

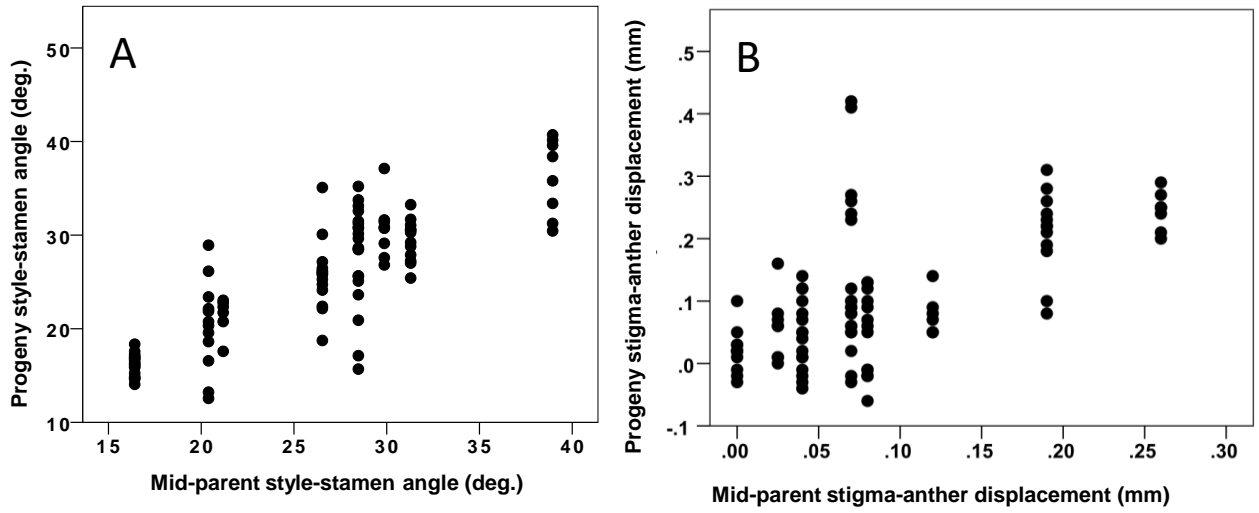


Figure 4