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Intersexual flower differences in an andromonoecious species: small pollen-rich staminate flowers under resource limitation

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2	Intersexual flower differences in an andromonoecious species: small
3	pollen-rich staminate flowers under resource limitation
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14	Running head: Intersexual differences in andromonoecious Commelina communis
15	
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17	interference, pollinator attraction, resource allocation, sexual dimorphism, stabilizing
18	selection
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20	

21 Abstract

22 •	Andromonoecy, the presence of perfect and staminate flowers in the same
23	individual, has evolved repeatedly in angiosperms. The staminate flowers are
24	generally smaller than the perfect flowers in species that produce staminate
25	flowers plastically when resources are limited. The smaller staminate flowers are
26	expected to be less attractive to pollinators and have reduced size-matching with
27	pollinators than perfect flowers. We hypothesized that these potential
28	disadvantages of staminate versus perfect flowers facilitate the evolution of sex-
29	specific floral morphology, such as allometric relationship between flower size
30	and male reproductive organ, in andromonoecious species.
31 •	We compared six floral morphology traits, pollen production, pollinator visits, and
32	pollen removal from anthers between staminate and perfect flowers in several
33	natural Commelina communis populations. Nectarless and zygomorphic C.
34	communis flowers had polymorphic stamens with attracting, feeding, and
35	pollinating anthers and were visited by diverse pollinators.
36 •	Staminate flowers were significantly smaller than perfect flowers, despite a large
37	overlap in size between sexes. The lengths of pollinating stamens did not differ
38	between staminate and perfect flowers, and staminate flowers produced
39	significantly more pollen. We observed significantly more pollinator visits to
40	perfect flowers than to staminate flowers. By contrast, pollen removal from
41	pollinating stamens was significantly higher in staminate flowers than in perfect
42	flowers.
4 3 •	There is sexual dimorphism in flower morphology in C. communis. Staminate
44	flowers with smaller attraction organs, similar pollinating stamens, and higher

- 45 pollen production assured higher pollen donor success relative to perfect flowers.
- 46 Our results suggest that the morphological changes in staminate flowers enhance
- 47 pollination success, even with limited resources.
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50 INTRODUCTION

51 The diversity and evolution of sexual systems in flowering plants are central topics of 52 evolutionary biology (Darwin, 1877; Barrett, 2002a). The diverse plant sexual systems 53 involve various combinations of perfect and unisexual (staminate and pistillate) flowers 54 (Bawa and Beach, 1981; Dellaporta and Calderon-Urrea, 1993). Approximately 70% of 55 angiosperm species have only perfect flowers, and the rest have multiple sexual flower 56 types within and among individuals (Yampolsky and Yampolsky, 1922; Richards, 1997; 57 Torices et al., 2011). Various selection pressures have been proposed to explain the 58 evolution and maintenance of unisexual flowers, such as those optimizing resource 59 allocation to male and female function, facilitating outcrossing, or increasing display 60 size, although their selective roles have still not been explored fully in many species 61 with different sexual systems (Barrett, 2002a; Barrett and Hough, 2013; Goldberg et al., 62 2017).

63 Andromonoecy, the production of both perfect and staminate flowers on the 64 same individual, is thought to have evolved repeatedly and occurs in approximately 65 4,000 angiosperm species (ca. 2%) across several families (Yampolsky and Yampolsky, 66 1922; Bawa and Beach, 1981; Richards, 1997). Three mutually non-exclusive 67 hypotheses explain the conditions that favor staminate flower production in 68 andromonoecious plants. The resource reallocation hypothesis predicts that the 69 production of non-fruiting staminate flowers can save resources, which are reallocated 70 to other fitness-enhancing traits (Bertin, 1982; Spalik, 1991; Emms, 1993; Vallejo-71 Marín and Rausher, 2007a). The increased pollen donation hypothesis emphasizes the 72 superiority of staminate flowers as pollen donors compared to perfect flowers because 73 staminate flowers can produce more pollen grains and avoid pollen-pistil interference

74 (Solomon, 1987; Elle and Meagher, 2000; Schlessman et al., 2004; Dai and Galloway, 75 2012). Finally, the pollinator attraction hypothesis holds that staminate flower 76 production increases pollinator attraction by giving rise to a larger display in a given 77 individual under resource limitation and can enhance female reproductive success 78 (Podolsky, 1992; Vallejo-Marín and Rausher, 2007b). In most cases, staminate flower 79 production in andromonoecious plants is regarded as a plastic response to resource 80 limitation or stress, as a staminate flower is less costly than a perfect flower because it 81 lacks ovules, large pistils, and fruit production (Solomon, 1985; Diggle, 1993; Granado 82 et al., 2017). This hypothetical relationship between staminate flower production and 83 resource limitation is supported by the fact that pistils and other floral organs, such as 84 the petals and sepals of staminate flowers, are typically smaller and lighter than those of 85 perfect flowers in andromonoecious species (e.g., Primack and Lloyd, 1980; 86 Schlessman, 1982; Solomon, 1986; Emms, 1993; Cuevas and Polito, 2004; Narbona et 87 al., 2005; Vallejo-Marín and Rausher, 2007a; Zhang and Tan, 2009; but see Huang, 88 2003). 89 Smaller staminate flowers may have some disadvantages in terms of pollination

90 success compared to perfect flowers in zoophilous andromonoecious plants. First, 91 staminate flowers with smaller petals (or sepals) can be less attractive to pollinators and 92 may be visited less often (Galen and Newport, 1987; Conner and Rush, 1996; Blarer et 93 al., 2002), although pollinators do not discriminate between perfect and staminate 94 flowers in some andromonoecious species (Aesculus pavia, Bertin, 1982; Solanum 95 carolinense, Vallejo-Marín and Rausher, 2007a; Passiflora incarnata, Dai and 96 Galloway, 2012). Second, the lower investment in stamens (filaments) may change the 97 position of anthers in a staminate flower relative to that in a perfect flower and reduce

98	physical matching between the anthers and pollinators, thus decreasing efficient pollen
99	transfer to the stigmas of perfect flowers (Harder, 1990; Tong and Huang, 2018). Note
100	that the within-flower anther position, which is generally determined by filament length,
101	is thought to be consistent across individuals within a population under strong
102	pollinator-mediated stabilizing selection (Cresswell, 1998; Conner et al., 2009;
103	Ushimaru et al., 2003a; Opedal, 2019). These potential disadvantages of staminate
104	versus perfect flowers would facilitate the evolution of sex-specific floral morphology
105	(<i>i.e.</i> , sexual dimorphism in floral morphology) in staminate flowers of andromonoecious
106	species, although the idea has not been tested.
107	In this study, we compared the sexual differences in floral traits, pollinator visits,
108	and pollen removal success between staminate and perfect flowers in the annual
109	andromonoecious herb Commelina communis. The bilaterally symmetrical
110	(zygomorphic) flowers of C. communis are self-compatible but still attract diverse
111	pollinators such as syrphid flies and bumble and honey bees with their showy blue
112	petals and yellow stamens (Ushimaru and Hyodo, 2005; Ushimaru et al., 2007). In
113	zygomorphic species, physical matching between flowers and pollinators is likely
114	important for efficient pollen transfer, as it increases the precision of pollen placement
115	(Neal et al., 1998; Wolfe and Krstolic, 1999; Ushimaru and Hyodo, 2005; Cosacov et
116	al., 2014; Nikkeshi et al., 2015). First, we confirmed that staminate flowers were
117	significantly smaller than perfect flowers in C. communis, because these flowers look
118	very similar in size and there is no reported size difference between them (Fig. 1; Morita
119	and Nigorikawa, 1999). Then, we addressed the following questions. Do staminate
120	flowers receive fewer pollinator visits than perfect flowers? Is there sexual dimorphism
121	in floral traits other than size between the two flower types? Can floral traits in

122 staminate flowers compensate for the disadvantage in the pollination process caused by

123 resource limitation? Finally, we discuss the selective forces acting on the morphology of

124 staminate flowers and the maintenance of andromonoecy in flowering plants.

125

126 MATERIALS AND METHODS

127 Study species—Commelina communis L. (Commelinaceae) grows near rice fields and 128 roadsides in temperate Northeast Asia. One plant has many inflorescences, each usually 129 bearing one to five flowers, with each flower opening from sunrise to noon. The first 130 flower in each inflorescence is usually perfect and those opening later can be staminate 131 depending on conditions (Morita and Nigorikawa, 1999). Fruit production (or 132 fertilization success) by early perfect flower(s) within a given inflorescence makes later 133 flowers staminate, suggesting the resource conditions of each inflorescence influence 134 the sex expression of later flowers (Morita and Nigorikawa, 1999; Katsuhara et al., in 135 prep.). Both flower types are zygomorphic with two showy blue petals and have two 136 long, one medium, and three short stamens, which have different functions. The anthers 137 of long and medium stamens (L- and M-anthers, respectively) produce fertile pollen, 138 which mainly contributes to pollination and serves as a reward for pollinators, 139 respectively (Ushimaru et al., 2007). The anthers of short stamens (S-anthers) produce 140 some sterile pollen and function as advertising for pollinators (Ushimaru et al., 2007). 141 The long stamens provide a landing site for pollinators and the medium and short 142 stamens function as floral guides (Ushimaru et al., 2007). Perfect flowers have four 143 fertile ovules and a style of similar length to that of the long stamens, whereas staminate 144 flowers have atrophied ovules.

145	Diverse insect groups including several syrphid flies and social and solitary bees
146	frequently visit and forage or collect pollen from flowers of C. communis, which can
147	also reproduce through bud pollination and delayed self-pollination (Morita and
148	Nigorikawa, 1999; Katsuhara and Ushimaru, 2019). The existence of staminate flowers
149	and the relatively high pollen-to-ovule ratio (1000–3000) in perfect flowers suggest the
150	importance of outcrossing in this species (Cruden, 1977; Morita and Nigorikawa, 1999;
151	Ushimaru et al., 2014). In addition, anther polymorphism (heteranthery) likely promotes
152	outcrossing by controlling pollinator orientation in Commelina flowers (Ushimaru et al.,
153	2007; Solís-Montero and Vallejo-Marín, 2017).

154

155 *Floral morphology and pollen production*—To examine differences in floral organ 156 sizes between staminate and perfect flowers, we measured floral traits in six natural 157 populations in Hyogo and Osaka prefectures, Japan from 5 September to 1 October 158 2017 (Table S2). Each population was investigated for 1–3 days, during which we 159 arbitrarily selected 4–11 perfect and staminate flowers to measure the following six 160 floral traits using digital calipers: petal length and width with the petal between glass 161 slides, S-anther length and width with the anther on a glass slide; and the filament 162 lengths of medium (M-stamen) and long (L-stamen) stamens. The petals and S-anthers 163 are visual attractants; their sizes are important for pollination success (Ushimaru et al., 164 2007). Filament length is important in M- and L-stamens because it influences flower-165 pollinator matching (Ushimaru et al., 2003a, 2007; Cosacov et al., 2014). 166 To quantify pollen production, we arbitrarily selected 8–9 newly opened, 167 unvisited flowers of each flower type at 5:30-6:00 in four populations (Table S2) and 168 collected the M- and L-anthers from each. Each anther type of each flower was stored

separately in 1.0 mL of 99% ethanol. We estimated the total number of pollen grains per
anther type by counting the pollen grains in three 10.0-µL droplets per sample under a
microscope (Ushimaru et al., 2014).

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173 Pollinator visits and pollination success—From 5 September to 1 October in 2017, we 174 compared differences in pollinator visits and pollen removal from anthers with fertile 175 pollen grains between perfect and staminate flowers over 1–3 days in each of seven natural populations (Table S2). In each population, we set three 1×1 -m² plots in which 176 177 pollinator visits to 6-12 arbitrarily selected perfect and staminate flowers were observed 178 for 1 h (three 20-min observations per flower) during 6:30–11:00 each observation day. 179 The pollinators were divided into four groups: Episyrphus balteatus, Bombus diversus 180 diversus, other bees (including small solitary bees and some honey bees), and small 181 syrphid flies (cf. Ushimaru et al., 2014; Katsuhara and Ushimaru, 2019). 182 We arbitrarily selected 8–9 flowers for observation in each of the four 183 populations to estimate pollen removal by pollinators (Table S2). The M- and L-anthers 184 of each selected flower were collected and stored separately in 1.0 mL of 99% ethanol 185 just before the flower closed (12:00), and the pollen grains remaining on the anthers 186 were counted in the laboratory using the same pollen-counting method. Pollen removal 187 from M- and L- anthers in each flower was estimated as the population mean total 188 pollen production minus the number of pollen grains remaining on each flower for each 189 anther type.

191 Data analyses

192 Floral traits—First, we conducted principal component analysis (PCA) using the six 193 floral parameters (petal length and width, S-anther length and width, and M-stamen and 194 L-stamen lengths) to summarize the floral morphology of each flower type. We 195 compared the mean value of each floral trait separately between staminate and perfect 196 flowers because the six floral traits have different functions and might be influenced 197 differently by resource limitation. Then, we constructed a linear mixed model (LMM), 198 including the first principal component axis (PC1, which was positively correlated with 199 all floral measurements and regarded as an index of flower size; Table S3) as the 200 response variable, flower type (perfect = 0, staminate = 1) and days after 1 September as 201 the explanatory variables, and population identity as a random term to examine sexual 202 differences in flower size. If staminate flowers are produced under resource limitation in 203 C. communis, we expect that staminate flowers would be smaller overall than perfect 204 flowers that bloomed the same day. Second, to compare each floral measurement and 205 pollen production between perfect and staminate flowers, we constructed six LMMs, 206 each of which included one of the six floral measurements as the response variable and 207 population identity and observation date as random terms, and two LMMs that included 208 the pollen number for the M- and L anthers as the response variable and population 209 identity as a random term. All LMMs incorporated flower type as the explanatory 210 variable. Finally, to compare the size variation in each trait between flower types, we 211 calculated the coefficient of variation (CV) and conducted an F-test for each 212 measurement. We compared the size variation of each floral trait separately between 213 staminate and perfect flowers because resource limitation might influence the variation 214 in the six traits differently. We expect that organs related to pollinator attraction would

215 be more variable than those associated with flower–pollinator matching in staminate

216 flowers, which are often produced under different levels of resource limitation

217 (Ushimaru and Nakata 2001; Ushimaru et al. 2003a).

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219 Pollination success—To compare pollinator visitation frequency between perfect and 220 staminate flowers, we constructed a generalized LMM (Poisson error and log link) that 221 incorporated the total number of visits per hour per flower as the response variable, 222 flower type as the explanatory variable, and population identity and observation date as 223 independent random terms. We also constructed LMMs that included pollen removal 224 from M- or L- anthers and flower type as the response and explanatory variables, 225 respectively, with population identity and observation date as random terms. 226 We examined the significance of each explanatory variable in the models using 227 the Wald test. We performed all analyses using the glmmADMB package in R software (ver. 4.0.2; R Core Team, 2020; Fournier et al., 2012). 228 229 230 RESULTS 231 Floral traits—In the PCA with floral measurements, PC1 explained 77.9% of the total 232 variance, whereas the second axis (PC2) explained 9.5% and was negatively correlated 233 with all measurements, except for M- and L-stamen lengths (Table S3). The PCA 234 scatterplot did not show a clear difference between perfect and staminate flowers, 235 possibly because floral organs other than the pistils appear not to differ in size or

morphology between the sexes prima facie (Figs. 1 and 2). However, the LMM analysis

revealed that the PC1 loadings were significantly lower in staminate flowers than in

238 perfect flowers when we considered a significant decrease in the variable with

239 observation days in the analysis (Fig. 2, Table S4).

240 The LMM analyses of floral traits revealed that five measurements (petal length 241 and width, S-anther length and width, and M-stamen length) were significantly smaller 242 in staminate versus perfect flowers (Fig. 3, Table S4). The L-stamen length did not 243 differ between staminate and perfect flowers (Fig. 3, Table S4). By contrast, staminate 244 flowers produced significantly more pollen on both M- and L-anthers than did perfect 245 flowers (Fig. 3, Table S4). Note that our preliminary experiment revealed that pollen 246 ability (pollen fertility and pollen tube growth) did not differ between perfect and 247 staminate flowers (Table S1).

Compared to perfect flowers, staminate flowers had larger CVs for the six floral measurements and smaller CVs for pollen production on M- and L-anthers (Table 1), although significant differences were only found for petal and S-anther lengths (Table 1). The CV of L-stamen length was the smallest among all traits in staminate flowers, and the value was comparable with that of perfect flowers.

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Pollination success—We observed 90 pollinator visits to 118 perfect flowers and 48 to 108 staminate flowers (44 and 14 *Episyrphus balteatus*, 3 and 1 *Bombus diversus*, 14 and 11 other bee, and 29 and 22 other small syrphid fly visits, respectively). The pollinator composition did not differ between flower types (Fisher's exact test, p =0.11). Pollinator visits per flower per hour was significant less frequent for staminate flowers (mean 0.76 for perfect flowers and 0.44 for staminate flowers; Fig. 4, Table S4).

260 Pollen removal from the L-anthers was significantly higher in staminate flowers,

whereas pollen removal from the M-anthers did not differ significantly between flowertypes (Fig. 4, Table S4).

263

264 **DISCUSSION**

265 Our results demonstrated that although there was a large overlap between flower types, 266 staminate flowers were significantly smaller than perfect flowers upon comparison on 267 the same observation dates at the same site. This suggests that staminate flowers were 268 under resource limitation. Staminate flowers were significantly more pollinator limited 269 compared to perfect flowers, likely because of their reduced attractiveness due to the 270 relatively smaller petals (Fig. S1). By contrast, the lengths of pollinating L-stamens in 271 staminate flowers were comparable to those in perfect flowers and were the most stable 272 trait. Therefore, the L-stamen length is under strong stabilizing selection to enable 273 precise pollen placement on pollinator bodies corresponding to the stigma position of 274 perfect flowers (Ushimaru et al., 2003a,b). This intersexual difference in the allometric 275 relationship between flower size and L-stamen length may ensure pollen donor success 276 in staminate flowers under resource limitation and likely contributes to the maintenance 277 of andromonoecy in C. communis.

We found that pollen removal from the L-anthers was higher in staminate flowers than in perfect flowers, although the staminate flowers received fewer pollinator visits (ca. 60% of the visits to perfect flowers). The first possible explanation for this discrepancy is that reduced pollen–pistil interference promotes pollen removal per visit in staminate flowers (Lloyd and Webb, 1986; Fetscher, 2001; Barrett, 2002b), as reported for andromonoecious *Solanum carolinense* (Elle and Meagher, 2000). However, further examination of this is required because pollen–pistil interference was 285 not found in another study of S. carolinense (Vallejo-Marín and Rausher, 2007a). As a 286 second possibility, increased pollen foraging from L-anthers by syrphid flies could have 287 resulted in greater pollen removal from staminate flowers (Ushimaru and Hyodo, 2005; 288 Ushimaru et al., 2007). However, this is unlikely because the relative abundance of 289 syrphid flies and their foraging behavior did not differ between flower types. A future 290 study of pistil-removed perfect flowers and detailed observations of pollinator behaviors 291 are needed to elucidate the advantages/disadvantages of staminate flowers in terms of 292 pollination success.

293 The lowest size variation in L-stamens among all the measurements of staminate 294 flowers is consistent with a study of eight natural C. communis populations that 295 demonstrated that this trait was conserved within and among populations (Ushimaru et 296 al., 2003a). By contrast, the attraction-related organs (petals and S-anthers) of staminate 297 flowers exhibited significantly larger size variation compared to perfect flowers. These 298 results suggest that the size of attraction-related organs is flexible and likely responds 299 plastically to resource conditions. These findings support the hypothesis that pollinators 300 impose stronger stabilizing selection on traits related to flower-pollinator matching than 301 on those involved in attraction (Cresswell, 1998; Worley et al., 2000; Ushimaru et al., 302 2003a). Staminate flowers may preferentially allocate available resources to pollination-303 related organs and then invest the surplus in attraction-related traits under resource-304 limited conditions. Elucidating the developmental process of staminate flowers in C. 305 communis and other and romonoecious species will improve our understanding of the 306 evolution and maintenance of andromonoecy in flowering plants. 307 We found that the M- and L-anthers of staminate flowers produced slightly, but 308 significantly, more pollen grains than those of perfect flowers (ca. 1.17- and 1.27-fold

309	compared to perfect flowers, respectively), and pollen fertility did not differ between
310	flower types (Table S1). The findings support the increased pollen donation hypothesis.
311	Patterns of sexual dimorphism in pollen production vary among andromonoecious
312	species—compared to perfect flowers, staminate flowers of Sagittaria
313	guyanensis subsp. lappula produce more pollen (Huang et al., 2000), those of Solanum
314	carolinense (Solomon, 1986) and Olea europaea produce similar amounts of pollen
315	(Cuevas and Polito, 2004), and those of Anthriscus sylvestris produce less pollen
316	(Spalik and Woodell, 1994). There was no common trend (Vallejo-Marín and Rausher,
317	2007a). In C. communis, increased pollen production in staminate flowers likely
318	compensates for fewer pollinator visits compared to perfect flowers to assure pollen
319	donation success. Although we compared only pollen removal between flower types, it
320	is necessary to examine the relationship between paternity success and pollen
321	production with molecular markers in andromonoecious species exhibiting sexual
322	dimorphism in pollen production.
323	In conclusion, we found cryptic sexual dimorphism in flower morphology in
324	andromonoecious C. communis. The results from pollinator observations and the
325	assessment of pollen removal success suggest that staminate flowers with smaller
326	attraction organs compared to perfect flowers have similar pollinating stamens and
327	higher rates of pollen production to ensure higher chances of pollen donor success.
328	Because this is a case study of a single species, more andromonoecious species should
329	be examined to generalize our findings.
330	

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340 AUTHOR CONTRIBUTIONS

- 341 All authors conceived the ideas and designed the methodology;
- 342 KM collected and analyzed the data;
- 343 KM and KRK led the writing of the manuscript;
- 344 All authors contributed critically to the drafts and gave final approval for publication.

345

346 DATA AVAILABILITY

- 347 We are going to archive our data in the Dryad Digital Repository.
- 348

349 SUPPORTING INFORMATION

- 350 Additional supporting information may be found online in the Supporting Information
- 351 section at the end of the article.

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507 Figure legends

508 Fig. 1. Perfect (left) and staminate (right) flowers of Commelina communis. Both were

509 the second-opening flowers of each inflorescence on the same individual. The

510 photograph was taken in the middle of the flowering season.

511 Fig. 2. Scatterplot of a principal component analysis of floral traits (a), and the

512 relationship between principal component 1 (PC1) and the observation date for each

513 flower type (b). PC1 explained 77.9% of the variation, was positively correlated with all

514 measurements, and was taken to represent flower size (Table S3). Red circles, perfect

515 flowers; blue circles, staminate flowers. Red and blue lines in (b) indicate the estimated

516 mean values for perfect and staminate flowers, respectively, based on the results from

517 generalized linear mixed model analysis. Boxplots in (b) indicate PC1 values for perfect

518 (P) and staminate (S) flowers, and asterisks indicate a significant difference (***

519 p < 0.001).

520 Fig. 3. Boxplots of floral traits of perfect (P) and staminate (S) flowers: petal length (a)

and width (b), S-anther length (c) and width (d), M-stamen (e) and L-stamen (f) lengths,

522 and the number of pollen grains on an M-anther (g) and L-anther (h). The Wald test

result for each comparison is indicated as follows: *** p < 0.001; ** p < 0.01; *

524 p < 0.05; ns, not significant.

525 Fig. 4. Pollinator visitation frequency (a) and pollen removal from M-anthers (b) and L-

526 anthers (c) in perfect (P) and staminate (S) flowers. The Wald test result for each

527 comparison is indicated as follows: *** p < 0.001; ** p < 0.01; ns, not significant.

528 Pollinator visitation data are presented as the means and standard errors (bars) in (a),

529 and the medians are indicated with bold lines in the boxplots in (b) and (c).

- 531 Table 1. Coefficients of variation (CVs) and variation test (F-test) results for each floral
- 532 trait. P, perfect flowers; S, staminate flowers; S-, M-, L-anther, anther on a short,

	CV (%)		Variation test	
Trait	Р	S	F value	р
Petal length	10.0	13.8	1.93	0.001
Petal width	14.1	16.3	1.33	0.09
S-anther length	10.8	17.6	2.66	< 0.001
S-anther width	10.6	12.4	1.38	0.070
M-stamen length	14.3	15.4	1.17	0.235
L-stamen length	10.6	10.9	1.07	0.383
No. of M-anther pollen	51.4	41.8	1.51	0.127
No. of L-anther pollen	46.7	43.5	1.15	0.348

533 medium, or long stamen, respectively. Boldface indicates significant effects (p < 0.05).









1 **Table S1**. Pollen fertility and pollen tube growth in perfect (P) and staminate (S)

2 flowers.

3

		Р	S
Pollen	fertility		
Pro	oportion of mature fruit	88% (21/24)	70% (19/27)
Pollen	tube growth		
Pro to t	oportion of styles with pollen growth the ovary (3 h after pollination)	39% (7/18)	58% (11/19)
Pro to t	oportion of styles with pollen growth the ovary (6 h after pollination)	93% (14/15)	100% (14/14)

Method: In 2017, we performed two types of hand-pollination to examine differences 4 5 in pollen fertility and pollen tube growth between perfect and staminate flowers in Commelina communis. To examine pollen fertility, we hand-pollinated the stigmas of 6 7 perfect flowers with pollen grains collected from perfect and staminate flowers and 8 examined the fruit set after anthesis in a population near Ogo01 (see Table S2). The 9 petals of experimentally pollinated perfect flowers were removed to eliminate pollinator 10 visitation. We conducted the experiment over 3 days and obtained fruit set data from 24 11 and 27 flowers pollinated using pollen from perfect and staminate flowers, respectively. To examine pollen tube growth, we hand-pollinated petal-removed perfect 12 13 flowers with pollen from each flower type in a similar way and collected and stored 14 their pistils in 99% ethanol for 3 or 6 h after pollen application. Then, we rehydrated the 15 samples with 0.5 N sodium hydroxide solution for 15 min at 60°C and stained them with 0.1% aniline blue solution for 24 h. After the treatment, we put samples on a glass 16 17 slide, crushed them with a glass cover, and observed pollen tube growth to the ovary 18 within the style via fluorescence microscopy. These experiments were conducted over

19 1–3 days at Ogo01 and in a population close to Ogo01 (Table S2). We observed 33
20 pollinated pistils of each flower type (perfect versus staminate).

21 For pollen fertility, we constructed a generalized linear mixed model (GLMM; 22 binomial error and logit link), including fruit set, pollen source (perfect/staminate), and 23 observation date as the response variable, explanatory variable, and random term, 24 respectively. For pollen tube growth, we constructed a GLMM (binomial error and logit 25 link) in which the proportion of styles with pollen growth to the ovary was the response 26 variable, the source of pollen (perfect/staminate) and time after pollen application were 27 the explanatory variables, and observation date and population identity were random 28 terms. We examined the significance of each explanatory variable in the models using 29 the Wald test. We performed all analyses using R, with the glmmADMB package. 30 Results: According to the GLMM analyses, pollen source had no significant effect on 31 fruit set (p = 0.11). There were no significant differences in the proportions of styles with pollen growth to the ovary between the sources of pollen (p = 0.12), whereas the 32 proportion increased significantly with time after pollen application (p = 0.006). 33

- 34 **Table S2**. Characteristics of the sampled populations: population identity, location, sample size for each observation, and pollen production
- 35 on M- and L-anthers.

Prefecture Population	lat	long	Pollinator observations (days)	Pollinator observations (P/S flowers)	Pollen count after pollination (P/S flowers sampled)	Froral trait mesurements (days spent)	Floral trait measurements (P/S flowers sampled)	Pollen count before pollination (P/S flowers sampled)	Pollen on M-anthers before pollination (P/S flowers)	Pollen on L-anthers before pollination (P/S flowers)
Hyogo					(175 nowers sumpled)	(duys spend)		(175 nowers sumpled)		(175 Howers)
Yamada	34.7636	135.1302	2	20/19	8/8	2	18/16	8/8	1866.7 / 2237.5	3120.8 / 3679.2
Ogo01	34.8168	135.1708	1	12/8	na	1	12/8	na	na	na
Ogo02	34.8139	135.1918	2	18/18	na	2	13/16	na	na	na
Ogo03	34.8170	135.1940	1	10/10	9/9	1	9/9	8/8	1658.3 / 2229.2	3354.2 / 4708.3
Rokko	34.7372	135.2346	1	8/6	na	na	na	na	na	na
Osaka										
Turumi	34.7116	135.5799	2	21/19	8/8	2	14/16	9/8	2559.3 / 2145.8	5333.3 / 7050.0
Yodogawa	34.7312	135.5337	3	29/28	8/8	3	23/20	8/8	1304.2 / 1687.5	3362.5 / 3766.7

36 na, not applicable

37 **Table S3**. Results of principal component analysis (PCA). The values show the PC axis

Trait	PC1 (77.9%)	PC2 (9.5%)	
Petal length	0.59	-0.46	
Petal width	0.62	-0.23	
S-anther length	0.03	-0.07	
S-anther width	0.05	-0.05	
M-stamen length	0.32	0.41	
L-stamen length	0.39	0.75	

38 loadings obtained for each floral trait.

39

41 **Table S4**. Results of generalized linear mixed model analyses. The Wald test results are

Response variable / Explanatory variable	Estimated coefficient	Standard error	z-value	P	
Flower size					
PC1 (Flower size) /					
Intercept	3.02	0.527	5.73	< 0.001	***
Flower type - staminate	-0.14	0.025	-5.64	< 0.001	***
Days after September first	-1.07	0.244	-4.37	< 0.001	***
Floral traits					
Petal length /					
Intercept	12.98	0.416	31.18	< 0.001	***
Flower type - staminate	-0.99	0.175	-5.64	< 0.001	***
Petal width /					
Intercept	10.83	0.474	22.86	< 0.001	***
Flower type - staminate	-0.57	0.18	-3.17	0.002	**
S-anther length /					
Intercept	2.12	0.0484	43.78	< 0.001	***
Flower type - staminate	-0.13	0.0424	-3.07	0.002	**
S-anther width /					
Intercept	2.50	0.0478	52.22	< 0.001	***
Flower type - staminate	-0.10	0.0397	-2.55	0.011	*
M-stamen length /					
Intercept	6.93	0.271	25.6	< 0.001	***
Flower type - staminate	-0.57	0.113	-5	< 0.001	***
L-stamen length /					
Intercept	11.12	0.344	32.35	< 0.001	***
Flower type - staminate	0.20	0.134	1.52	0.13	
No. of M-anther pollen /					
Intercept	1858	164	11.29	< 0.001	***
Flower type - staminate	218	100	2.17	0.03	*
No. of L-anther pollen /					
Intercept	3783	557	6.79	< 0.001	***
Flower type - staminate	1018	100	10.17	< 0.001	***
Pollination success					
No. of pollinator visits /					
Intercept	-0.45	0.275	-1.63	0.104	
Flower type - staminate	-0.51	0.179	-2.86	0.004	**
Pollen removal of M-anther /					
Intercept	877	147.7	5.93	< 0.001	***
Flower type - staminate	181	99.3	1.82	0.069	
Pollen removal of L-anther /					
Intercept	1091	600	1.82	0.069	***
Flower type - staminate	982	101	9.74	< 0.001	***

42 indicated as follows: * P < 0.05; ** P < 0.01; *** P < 0.001.



44

45 Fig. S1. Relationships between pollinator visits and petal length in perfect and
46 staminate flowers. Red circles, perfect flowers; blue circles, staminate flowers; black
47 line, mean value based on GLMM result.

49 Method: To test whether the smaller petals of staminate versus perfect flowers explain 50 the reduced rate of pollinator visitation to staminate flowers, we performed a GLMM 51 analysis (Poisson error and log link) that incorporated the total visits per hour per flower 52 as the response variable, flower type (perfect/staminate), petal length, and their 53 interaction as explanatory variables, and population identity and observation date as 54 independent random terms. We used 113/118 perfect and 104/108 staminate flowers for

55	which floral traits were measured and pollinator visits were observed, respectively. We
56	examined the significance of each explanatory variable in the models using the Wald
57	test. We performed all analyses using R, with the glmmADMB package.
58	
59	Results: From the GLMM analysis, pollinator visitation increased significantly with
60	petal length ($p = 0.02$), whereas flower type and the interaction between flower type and
61	petal length were not significant ($p = 0.14$ and 0.08, respectively). These results suggest
62	that staminate flowers receive fewer pollinator visits because staminate flowers have

63 smaller blue petals compared to perfect flowers.