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(Citation)
Plant Biology,24(2):259-265

(Issue Date)
2022-03

(Resource Type)
journal article

(Version)
Accepted Manuscript

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

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Running head: Intersexual differences in andromonoecious *Commelina communis*

Key-words: *Commelina*, floral allometry, flower–pollinator matching, pollen–pistil interference, pollinator attraction, resource allocation, sexual dimorphism, stabilizing selection

Abstract

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

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

INTRODUCTION

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

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

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

Smaller staminate flowers may have some disadvantages in terms of pollination success compared to perfect flowers in zoophilous andromonoecious plants. First, staminate flowers with smaller petals (or sepals) can be less attractive to pollinators and may be visited less often (Galen and Newport, 1987; Conner and Rush, 1996; Blarer et al., 2002), although pollinators do not discriminate between perfect and staminate flowers in some andromonoecious species (*Aesculus pavia*, Bertin, 1982; *Solanum carolinense*, Vallejo-Marín and Rausher, 2007a; *Passiflora incarnata*, Dai and Galloway, 2012). Second, the lower investment in stamens (filaments) may change the position of anthers in a staminate flower relative to that in a perfect flower and reduce physical matching between the anthers and pollinators, thus decreasing efficient pollen transfer to the stigmas of perfect flowers (Harder, 1990; Tong and Huang, 2018). Note that the within-flower anther position, which is generally determined by filament length, is thought to be consistent across individuals within a population under strong pollinator-mediated stabilizing selection (Cresswell, 1998; Conner et al., 2009; Ushimaru et al., 2003a; Opedal, 2019). These potential disadvantages of staminate versus perfect flowers would facilitate the evolution of sex-specific floral morphology (*i.e.*, sexual dimorphism in floral morphology) in staminate flowers of andromonoecious species, although the idea has not been tested.

In this study, we compared the sexual differences in floral traits, pollinator visits, and pollen removal success between staminate and perfect flowers in the annual andromonoecious herb *Commelina communis*. The bilaterally symmetrical (zygomorphic) flowers of *C. communis* are self-compatible but still attract diverse pollinators such as syrphid flies and bumble and honey bees with their showy blue petals and yellow stamens (Ushimaru and Hyodo, 2005; Ushimaru et al., 2007). In zygomorphic species, physical matching between flowers and pollinators is likely important for efficient pollen transfer, as it increases the precision of pollen placement (Neal et al., 1998; Wolfe and Krstolic, 1999; Ushimaru and Hyodo, 2005; Cosacov et al., 2014; Nikkeshi et al., 2015). First, we confirmed that staminate flowers were significantly smaller than perfect flowers in *C. communis*, because these flowers look very similar in size and there is no reported size difference between them (Fig. 1; Morita and Nigorikawa, 1999). Then, we addressed the following questions. Do staminate flowers receive fewer pollinator visits than perfect flowers? Is there sexual dimorphism in floral traits other than size between the two flower types? Can floral traits in staminate flowers compensate for the disadvantage in the pollination process caused by resource limitation? Finally, we discuss the selective forces acting on the morphology of staminate flowers and the maintenance of andromonoecy in flowering plants.

MATERIALS AND METHODS

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

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

Floral morphology and pollen production—To examine differences in floral organ sizes between staminate and perfect flowers, we measured floral traits in six natural populations in Hyogo and Osaka prefectures, Japan from 5 September to 1 October 2017 (Table S2). Each population was investigated for 1–3 days, during which we arbitrarily selected 4–11 perfect and staminate flowers to measure the following six floral traits using digital calipers: petal length and width with the petal between glass slides, S-anther length and width with the anther on a glass slide; and the filament lengths of medium (M-stamen) and long (L-stamen) stamens. The petals and S-anthers are visual attractants; their sizes are important for pollination success (Ushimaru et al., 2007). Filament length is important in M- and L-stamens because it influences flower–pollinator matching (Ushimaru et al., 2003a, 2007; Cosacov et al., 2014).

To quantify pollen production, we arbitrarily selected 8–9 newly opened, unvisited flowers of each flower type at 5:30–6:00 in four populations (Table S2) and 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).

Pollinator visits and pollination success**—**From 5 September to 1 October in 2017, we compared differences in pollinator visits and pollen removal from anthers with fertile 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-m^2 plots in which pollinator visits to 6–12 arbitrarily selected perfect and staminate flowers were observed for 1 h (three 20-min observations per flower) during 6:30–11:00 each observation day. The pollinators were divided into four groups: *Episyrphus balteatus*, *Bombus diversus diversus*, other bees (including small solitary bees and some honey bees), and small syrphid flies (cf. Ushimaru et al., 2014; Katsuhara and Ushimaru, 2019).

We arbitrarily selected 8–9 flowers for observation in each of the four populations to estimate pollen removal by pollinators (Table S2). The M- and L-anthers of each selected flower were collected and stored separately in 1.0 mL of 99% ethanol just before the flower closed (12:00), and the pollen grains remaining on the anthers were counted in the laboratory using the same pollen-counting method. Pollen removal from M- and L- anthers in each flower was estimated as the population mean total pollen production minus the number of pollen grains remaining on each flower for each anther type.

Data analyses

Floral traits—First, we conducted principal component analysis (PCA) using the six floral parameters (petal length and width, S-anther length and width, and M-stamen and L-stamen lengths) to summarize the floral morphology of each flower type. We compared the mean value of each floral trait separately between staminate and perfect flowers because the six floral traits have different functions and might be influenced differently by resource limitation. Then, we constructed a linear mixed model (LMM), including the first principal component axis (PC1, which was positively correlated with all floral measurements and regarded as an index of flower size; Table S3) as the response variable, flower type (perfect = 0, staminate = 1) and days after 1 September as the explanatory variables, and population identity as a random term to examine sexual differences in flower size. If staminate flowers are produced under resource limitation in *C. communis*, we expect that staminate flowers would be smaller overall than perfect flowers that bloomed the same day. Second, to compare each floral measurement and pollen production between perfect and staminate flowers, we constructed six LMMs, each of which included one of the six floral measurements as the response variable and population identity and observation date as random terms, and two LMMs that included the pollen number for the M- and L anthers as the response variable and population identity as a random term. All LMMs incorporated flower type as the explanatory variable. Finally, to compare the size variation in each trait between flower types, we calculated the coefficient of variation (CV) and conducted an F-test for each measurement. We compared the size variation of each floral trait separately between staminate and perfect flowers because resource limitation might influence the variation in the six traits differently. We expect that organs related to pollinator attraction would be more variable than those associated with flower–pollinator matching in staminate flowers, which are often produced under different levels of resource limitation (Ushimaru and Nakata 2001; Ushimaru et al. 2003a).

Pollination success—To compare pollinator visitation frequency between perfect and staminate flowers, we constructed a generalized LMM (Poisson error and log link) that incorporated the total number of visits per hour per flower as the response variable, flower type as the explanatory variable, and population identity and observation date as independent random terms. We also constructed LMMs that included pollen removal from M- or L- anthers and flower type as the response and explanatory variables, respectively, with population identity and observation date as random terms.

We examined the significance of each explanatory variable in the models using 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).

RESULTS

Floral traits—In the PCA with floral measurements, PC1 explained 77.9% of the total variance, whereas the second axis (PC2) explained 9.5% and was negatively correlated with all measurements, except for M- and L-stamen lengths (Table S3). The PCA scatterplot did not show a clear difference between perfect and staminate flowers, 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 perfect flowers when we considered a significant decrease in the variable with observation days in the analysis (Fig. 2, Table S4).

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

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). Pollen removal from the L-anthers was significantly higher in staminate flowers, whereas pollen removal from the M-anthers did not differ significantly between flower types (Fig. 4, Table S4).

DISCUSSION

Our results demonstrated that although there was a large overlap between flower types, staminate flowers were significantly smaller than perfect flowers upon comparison on the same observation dates at the same site. This suggests that staminate flowers were under resource limitation. Staminate flowers were significantly more pollinator limited compared to perfect flowers, likely because of their reduced attractiveness due to the relatively smaller petals (Fig. S1). By contrast, the lengths of pollinating L-stamens in staminate flowers were comparable to those in perfect flowers and were the most stable trait. Therefore, the L-stamen length is under strong stabilizing selection to enable precise pollen placement on pollinator bodies corresponding to the stigma position of perfect flowers (Ushimaru et al., 2003a,b). This intersexual difference in the allometric relationship between flower size and L-stamen length may ensure pollen donor success in staminate flowers under resource limitation and likely contributes to the maintenance 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 not found in another study of *S. carolinense* (Vallejo-Marín and Rausher, 2007a). As a second possibility, increased pollen foraging from L-anthers by syrphid flies could have resulted in greater pollen removal from staminate flowers (Ushimaru and Hyodo, 2005; Ushimaru et al., 2007). However, this is unlikely because the relative abundance of syrphid flies and their foraging behavior did not differ between flower types. A future study of pistil-removed perfect flowers and detailed observations of pollinator behaviors are needed to elucidate the advantages/disadvantages of staminate flowers in terms of pollination success.

The lowest size variation in L-stamens among all the measurements of staminate flowers is consistent with a study of eight natural *C. communis* populations that demonstrated that this trait was conserved within and among populations (Ushimaru et al., 2003a). By contrast, the attraction-related organs (petals and S-anthers) of staminate flowers exhibited significantly larger size variation compared to perfect flowers. These results suggest that the size of attraction-related organs is flexible and likely responds plastically to resource conditions. These findings support the hypothesis that pollinators impose stronger stabilizing selection on traits related to flower–pollinator matching than on those involved in attraction (Cresswell, 1998; Worley et al., 2000; Ushimaru et al., 2003a). Staminate flowers may preferentially allocate available resources to pollination-related organs and then invest the surplus in attraction-related traits under resource-limited conditions. Elucidating the developmental process of staminate flowers in *C. communis* and other andromonoecious species will improve our understanding of the evolution and maintenance of andromonoecy in flowering plants.

We found that the M- and L-anthers of staminate flowers produced slightly, but significantly, more pollen grains than those of perfect flowers (ca. 1.17- and 1.27-fold compared to perfect flowers, respectively), and pollen fertility did not differ between flower types (Table S1). The findings support the increased pollen donation hypothesis. Patterns of sexual dimorphism in pollen production vary among andromonoecious species—compared to perfect flowers, staminate flowers of *Sagittaria guyanensis* subsp. *lappula* produce more pollen (Huang et al., 2000), those of *Solanum carolinense* (Solomon, 1986) and *Olea europaea* produce similar amounts of pollen (Cuevas and Polito, 2004), and those of *Anthriscus sylvestris* produce less pollen (Spalik and Woodell, 1994). There was no common trend (Vallejo-Marín and Rausher, 2007a). In *C. communis*, increased pollen production in staminate flowers likely compensates for fewer pollinator visits compared to perfect flowers to assure pollen donation success. Although we compared only pollen removal between flower types, it is necessary to examine the relationship between paternity success and pollen production with molecular markers in andromonoecious species exhibiting sexual dimorphism in pollen production.

In conclusion, we found cryptic sexual dimorphism in flower morphology in andromonoecious *C. communis*. The results from pollinator observations and the assessment of pollen removal success suggest that staminate flowers with smaller attraction organs compared to perfect flowers have similar pollinating stamens and higher rates of pollen production to ensure higher chances of pollen donor success. Because this is a case study of a single species, more andromonoecious species should be examined to generalize our findings.

ACKNOWLEDGMENTS

We thank A. Nikkeshi and N. Ohmido for their advice on the pollen counting and pollen tube observation methods, respectively. We also thank the members of the Biodiversity, Evolutionary Ecology, and Minamoto Laboratories of Kobe University for their valuable comments on our study. We are grateful to the land owners who allowed our field surveys to be carried out on their properties. This work was supported by Grants-in-Aid for Scientific Research (nos. 16K07517, 17J01902, 19H03303, 19K06855, and 20J01271) from the Japan Society for the Promotion of Science.

AUTHOR CONTRIBUTIONS

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

DATA AVAILABILITY

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

SUPPORTING INFORMATION

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

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Figure legends

Fig. **1**. Perfect (left) and staminate (right) flowers of *Commelina communis*. Both were the second-opening flowers of each inflorescence on the same individual. The photograph was taken in the middle of the flowering season.

Fig. **2**. Scatterplot of a principal component analysis of floral traits (a), and the relationship between principal component 1 (PC1) and the observation date for each flower type (b). PC1 explained 77.9% of the variation, was positively correlated with all measurements, and was taken to represent flower size (Table S3). Red circles, perfect flowers; blue circles, staminate flowers. Red and blue lines in (b) indicate the estimated mean values for perfect and staminate flowers, respectively, based on the results from generalized linear mixed model analysis. Boxplots in (b) indicate PC1 values for perfect (P) and staminate (S) flowers, and asterisks indicate a significant difference (*** $p < 0.001$).

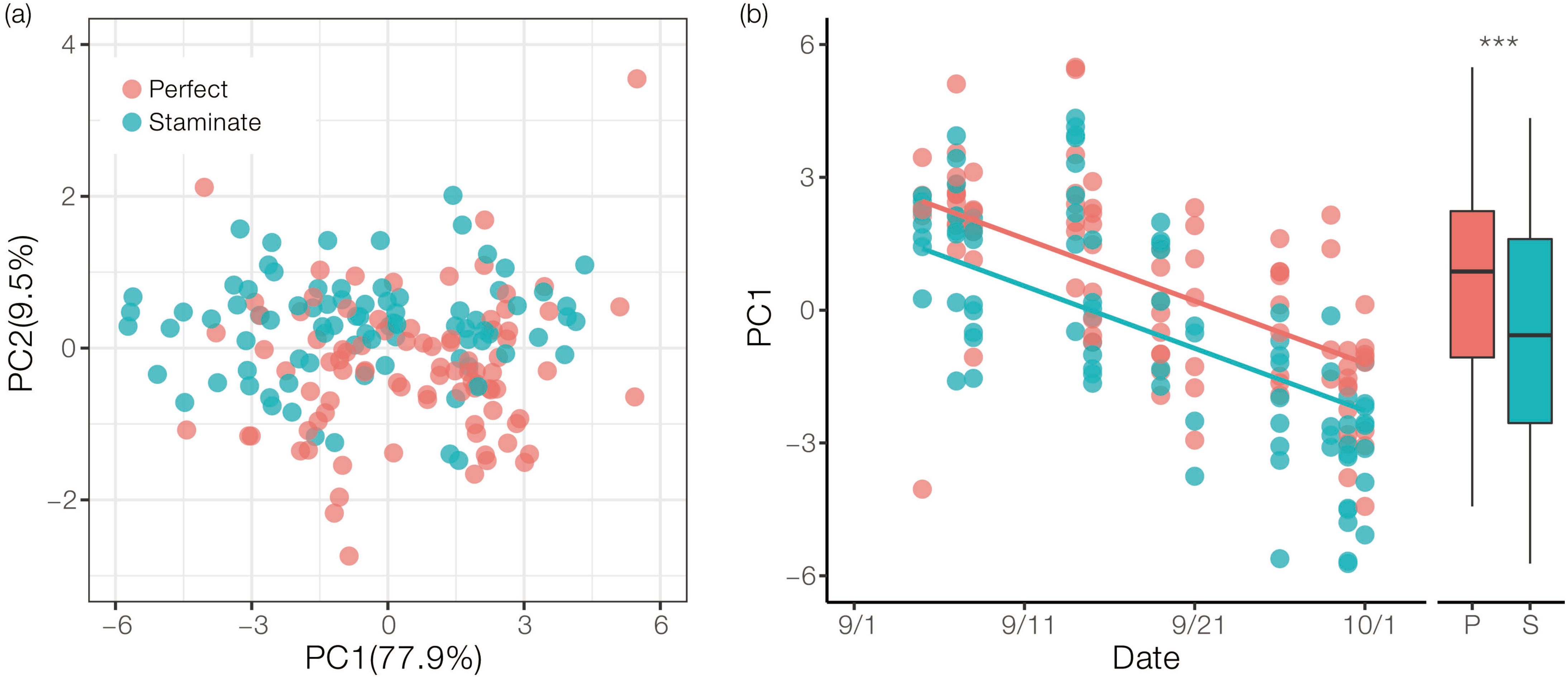
Fig. **3**. Boxplots of floral traits of perfect (P) and staminate (S) flowers: petal length (a) and width (b), S-anther length (c) and width (d), M-stamen (e) and L-stamen (f) lengths, and the number of pollen grains on an M-anther (g) and L-anther (h). The Wald test result for each comparison is indicated as follows: *** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$; ns, not significant.

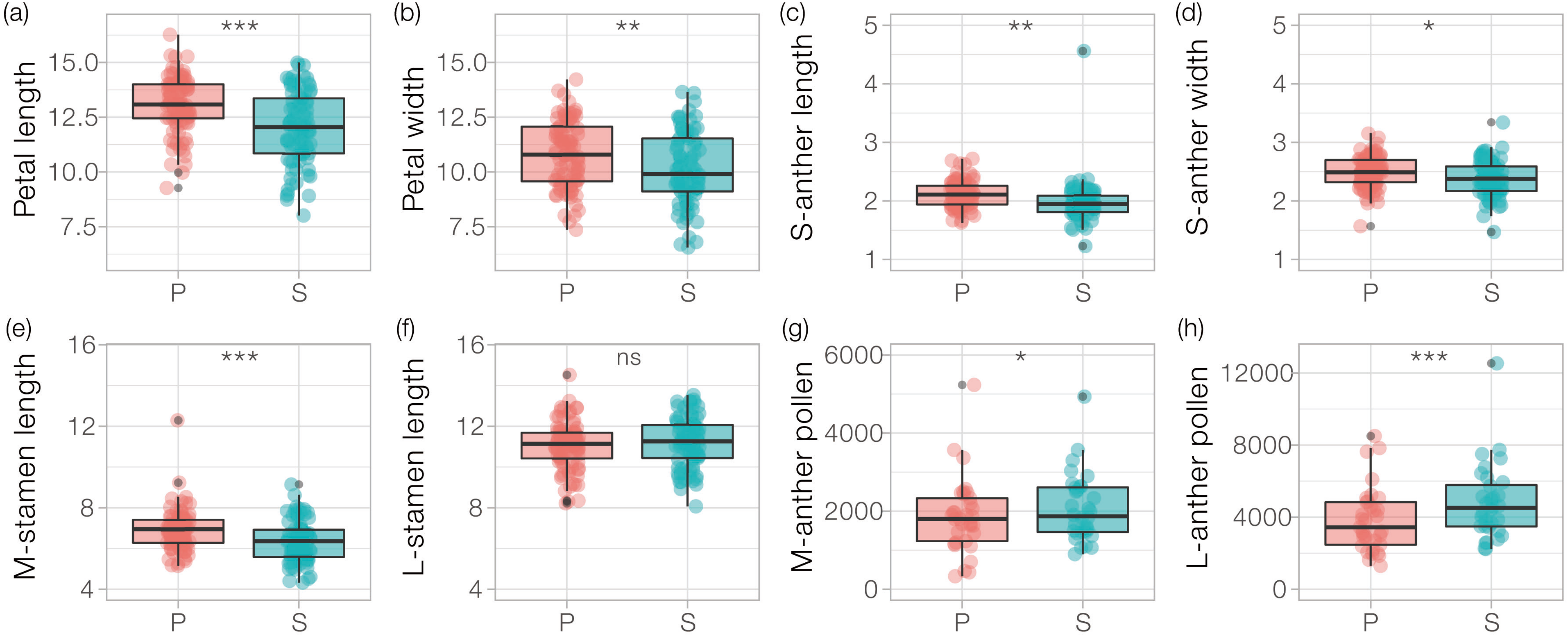
Fig. **4**. Pollinator visitation frequency (a) and pollen removal from M-anthers (b) and L-anthers (c) in perfect (P) and staminate (S) flowers. The Wald test result for each comparison is indicated as follows: *** $p < 0.001$; ** $p < 0.01$; ns, not significant. Pollinator visitation data are presented as the means and standard errors (bars) in (a), and the medians are indicated with bold lines in the boxplots in (b) and (c).

Table 1. Coefficients of variation (CVs) and variation test (F-test) results for each floral trait. P, perfect flowers; S, staminate flowers; S-, M-, L-anther, anther on a short, medium, or long stamen, respectively. Boldface indicates significant effects ($p < 0.05$).

	CV (%)		Variation test	
Trait	P	S	F value	*p*
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


Perfect
Staminate


(a)
Perfect
Staminate
PC2(9.5%)
4
2
0
−2
−6
−3
0
3
6
PC1(77.9%)
(b)
PC1
6
3
0
−3
−6
***
9/1
9/11
9/21
10/1
Date
P
S


(a)
Petal length
***
15.0
12.5
10.0
7.5
P
S
(b)
Petal width
**
15.0
12.5
10.0
7.5
P
S
(c)
S-anther length
**
5
4
3
2
1
P
S
(d)
S-anther width
*
5
4
3
2
1
P
S
(e)
M-stamen length
***
16
12
8
4
P
S
(f)
L-stamen length
ns
16
12
8
4
P
S
(g)
M-anther pollen
*
6000
4000
2000
0
P
S
(h)
L-anther pollen
***
12000
8000
4000
0
P
S

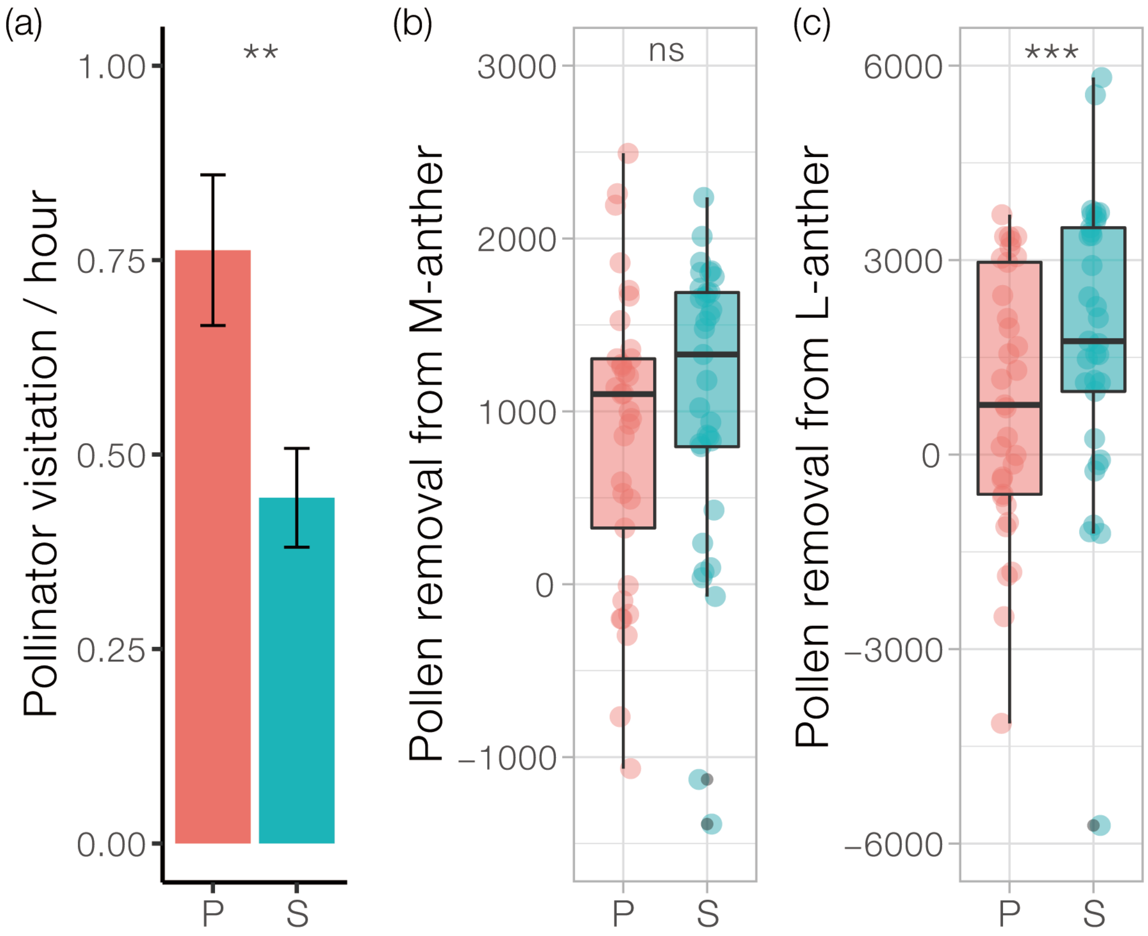

(a)
Pollinator visitation / hour
1.00
0.75
0.50
0.25
0.00
**
P
S
(b)
Pollen removal from M-anther
3000
2000
1000
0
−1000
ns
P
S
(c)
Pollen removal from L-anther
6000
3000
0
−3000
−6000
***
P
S

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

		P	S
Pollen fertility			
	Proportion of mature fruit	88% (21/24)	70% (19/27)
Pollen tube growth			
	Proportion of styles with pollen growth to the ovary (3 h after pollination)	39% (7/18)	58% (11/19)
	Proportion of styles with pollen growth to 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 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 perfect flowers with pollen grains collected from perfect and staminate flowers and examined the fruit set after anthesis in a population near Ogo01 (see Table S2). The petals of experimentally pollinated perfect flowers were removed to eliminate pollinator visitation. We conducted the experiment over 3 days and obtained fruit set data from 24 and 27 flowers pollinated using pollen from perfect and staminate flowers, respectively.

To examine pollen tube growth, we hand-pollinated petal-removed perfect flowers with pollen from each flower type in a similar way and collected and stored their pistils in 99% ethanol for 3 or 6 h after pollen application. Then, we rehydrated the 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 slide, crushed them with a glass cover, and observed pollen tube growth to the ovary within the style via fluorescence microscopy. These experiments were conducted over

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

For pollen fertility, we constructed a generalized linear mixed model (GLMM; binomial error and logit link), including fruit set, pollen source (perfect/staminate), and observation date as the response variable, explanatory variable, and random term, respectively. For pollen tube growth, we constructed a GLMM (binomial error and logit link) in which the proportion of styles with pollen growth to the ovary was the response variable, the source of pollen (perfect/staminate) and time after pollen application were the explanatory variables, and observation date and population identity were random terms. We examined the significance of each explanatory variable in the models using the Wald test. We performed all analyses using R, with the glmmADMB package.

Results: According to the GLMM analyses, pollen source had no significant effect on 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 proportion increased significantly with time after pollen application ($p = 0.006$).

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

Prefecture				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)
	Population	lat	long								
Hyogo											
	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

na, not applicable

Table S3. Results of principal component analysis (PCA). The values show the PC axis loadings obtained for each floral trait.

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

Table S4. Results of generalized linear mixed model analyses. The Wald test results are indicated as follows: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

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	***

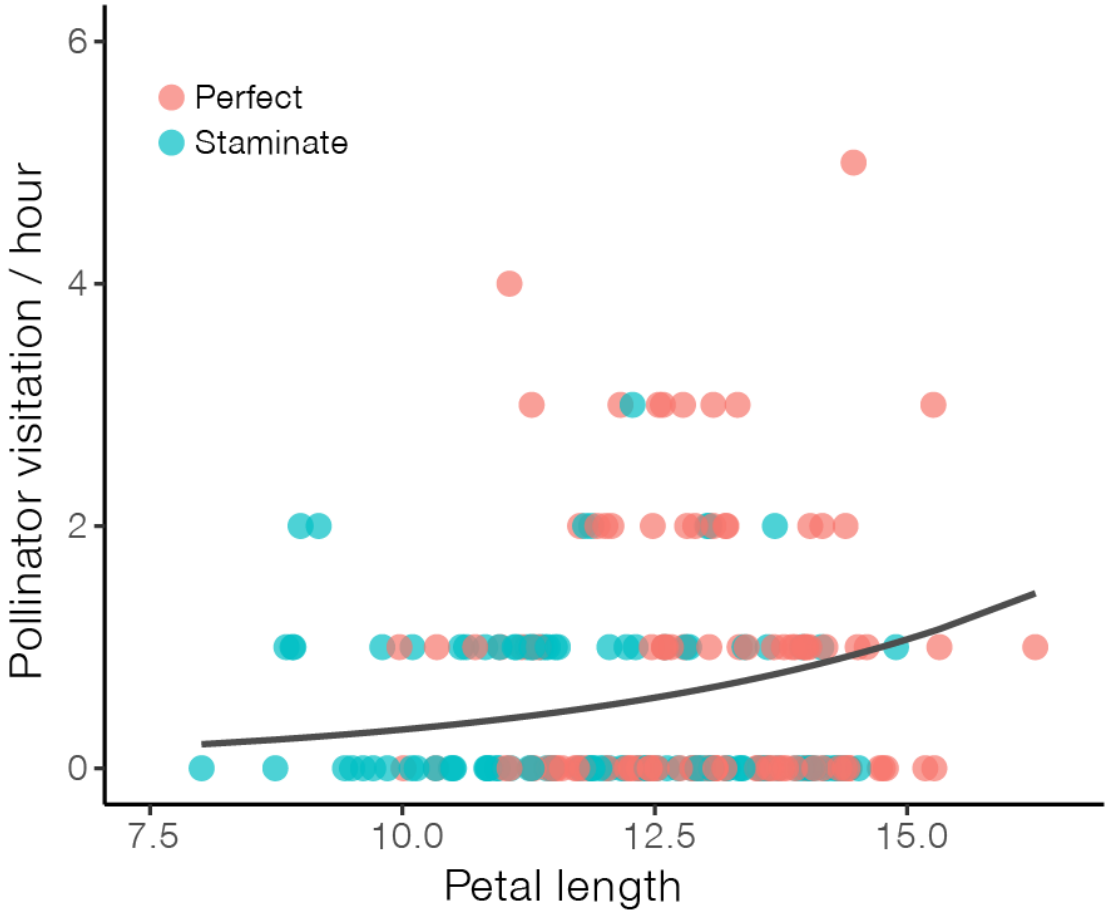


Fig. S1. Relationships between pollinator visits and petal length in perfect and staminate flowers. Red circles, perfect flowers; blue circles, staminate flowers; black line, mean value based on GLMM result.

Method: To test whether the smaller petals of staminate versus perfect flowers explain the reduced rate of pollinator visitation to staminate flowers, we performed a GLMM analysis (Poisson error and log link) that incorporated the total visits per hour per flower as the response variable, flower type (perfect/staminate), petal length, and their interaction as explanatory variables, and population identity and observation date as independent random terms. We used 113/118 perfect and 104/108 staminate flowers for which floral traits were measured and pollinator visits were observed, respectively. We examined the significance of each explanatory variable in the models using the Wald test. We performed all analyses using R, with the glmmADMB package.

Results: From the GLMM analysis, pollinator visitation increased significantly with petal length ($p = 0.02$), whereas flower type and the interaction between flower type and petal length were not significant ($p = 0.14$ and 0.08, respectively). These results suggest that staminate flowers receive fewer pollinator visits because staminate flowers have smaller blue petals compared to perfect flowers.