Horizontal orientation facilitates pollen transfer and rain damage avoidance in actinomorphic flowers of Platycodon grandiflorus

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ABSTRACT

In zoophilous plants, floral orientation evolves under both biotic and abiotic pressure to enhance pollination success. However, the adaptive significance of horizontal orientation in radially symmetrical (actinomorphic) flowers remains largely unknown, although that of bilaterally symmetrical flowers has been well studied.

We experimentally altered floral angle in a population of insect-pollinated *Platycodon grandiflorus* flowers to examine the effects of floral orientation on pollinator behavior, pollination success and pollen rain damage avoidance. To further investigate the potential pollen damage by rain, we obtained the past precipitation in the study area during the flowering season, and experimentally tested *P. grandiflorus* pollen damage by water.

Horizontally oriented flowers received more pollinator visits and had pollen grains on the stigma in male and/or female phases than downward- and/or upward-oriented flowers, and avoided pollen damage by rainfall better than upward-oriented flowers. A pollen germination experiment showed that approximately 30% of pollen grains burst in distilled water, indicating that pollen damage by rainfall may be significant in *P. grandiflorus*.

Our field experiments revealed that upward-oriented flowers cannot avoid pollen damage by rainfall during the flowering period, and that both upward- and downward-oriented flowers experience pollinator limitation in female success. Therefore, horizontal flower orientation appears to be adaptive in this insect-pollinated actinomorphic species that blooms during the rainy season.

Keywords: actinomorphy, floral orientation, rain drop, generalist pollination

INTRODUCTION

Angiosperm floral diversity is thought to have evolved under selection mediated by both biotic and abiotic pressure (Darwin, 1862; Grant & Grant, 1965; Stebbins, 1970; Fenster et al. 2004; Wilmer, 2011). In animal-pollinated species, most floral traits including size, shape, color, scent, and flowering timing have been adapted to enhance pollen transfer by pollinators; some of these traits simultaneously provide protection against harmful abiotic factors, such as rain and extreme low/high temperature (Kudo, 1995; Huang et al. 2002; Patiño et al. 2002). Sun et al. (2008) reported that the bracts of *Davidia involucrata* function not only for pollinator attraction but also for protecting pollen from rain damage. Vertical direction of flower orientation (including upward, horizontal, oblique and downward orientation), which is regulated by flower stalk angle, evolves under both biotic and abiotic pressure to enhance pollination success in zoophilous plants (Hocking & Sharplin, 1965; Kevan, 1975; Kudo, 1995; Tadey & Aizen, 2001; Huang et al. 2002; Patiño et al. 2002; Galen & Stanton, 2003; Ushimaru et al. 2009; Haverkamp et al. 2019).

Flower orientation influences the attraction and control of landing position of specialized pollinators (Ushimaru & Hyodo, 2005; Fenster et al. 2009). Bilaterally symmetrical (zygomorphic) flowers usually exhibit a horizontal orientation (Neal et al. 1998), which can attract more pollinators and enhance behaviors that lead to higher pollen transfer success compared to upward or downward orientation (Ushimaru & Hyodo, 2005; Ushimaru et al. 2009; Wang et al. 2014a). In contrast, optimal flower orientation varies among radially symmetrical (actinomorphic) flowers, likely depending on their range of pollinators. For example, upward and heliotropic orientation increases floral temperature, which attracts many fly pollinators to alpine, arctic, and early spring blooming plants with dish-shaped actinomorphic flowers (Hocking & Sharplin, 1965; Kevan, 1975; Kudo, 1995). In some actinomorphic flowers, downward orientation likely limits their pollinators to specialized groups such as hummingbirds and large bees in concert with tubular corollas, thereby improving pollination efficiency, whereas upward floral orientation facilitates nocturnal hawkmoth pollination in actinomorphic species with long nectar holders (Fulton & Hodges, 1999; Aizen, 2003; Campbell et al. 2016).

The primary selective agents driving floral angle evolution in some zoophilous plants are abiotic factors, rather than pollinators (Huang et al. 2002; Tadey & Aizen, 2001; Wang et al. 2010; Lin & Forrest, 2019). Downward flower orientation is thought to have evolved to avoid pollen damage and nectar dilution due to rainfall, as well as exposure to solar radiation, in actinomorphic flowers (Huang et al. 2002; Tadey & Aizen, 2001; Wang et al. 2010; Lin & Forrest, 2019). Rainfall effects may be the more important drivers of downward floral orientation than pollinator-mediated selection especially in generalist actinomorphic flowers pollinated by a wide range of pollinator groups (two or more functional groups) whose compositions and visit frequencies are generally not influenced by floral angle (Huang et al. 2002; Wang et al. 2010; Lin & Forrest, 2019).

To date, the adaptive significance of horizontal orientation in actinomorphic flowers under biotic and abiotic selection pressure has not been examined, although that in zygomorphic flowers has been relatively well studied. Generally, specialized zygomorphic flowers exhibit higher pollination success in flowers with a horizontal orientation (Neal et al. 1998; Ushimaru et al. 2009; Wang et al. 2014a; Armbruster & Muchhala, 2020). Only one study has examined the role of horizontal orientation in a generalist zygomorphic species, in which horizontally oriented flowers were found to have higher pollen transfer success than those oriented upward or downward (Yu et al. 2020). In the same species, horizontal flower orientation was discussed to reduce pollen damage by rainwater compared to upward-orientated flowers; however, its anthers were always wet during rainfall, irrespective of flower angle (Yu et al. 2020). Together, these findings suggest that horizontal orientation has evolved mainly to attract and control pollinators in zygomorphic flowers. To our knowledge, the functional roles of horizontal orientation in actinomorphic flowers with generalist systems remain unknown. We predict that pollinator-mediated selection on flower orientation is relatively weak also in generalist actinomorphic flowers.

In this study, we conducted field and laboratory experiments to examine the functional roles of horizontal floral orientation on the attraction and landing control of pollinators, pollen transfer, and avoidance of rainfall damage to pollen in the Japanese bellflower, *Platycodon grandifloras* (Jacq.) A.DC (Campanulaceae), whose actinomorphic flowers are visited by diverse insect groups during the rainy season.

MATERIALS AND METHODS

Study species and site

Platycodon grandiflorus is a perennial herb currently listed as a vulnerable species in the Ministry of the Environment of Japan Red List (2020). This species typically grows on natural and semi-natural grasslands in Japan, the Korean Peninsula, China, and eastern Russia. In Japan, *P. grandiflorus* blooms during the rainy season, from mid-June to mid-September; its blue cup-shaped flowers are self-compatible and protandrous, usually opening for 4–6 days, and require insect pollination for setting fruits and seeds

(Wei et al. 2006). The male and female phases do not overlap and persist for an average of 1.3 days (1–3 days, n = 170) and 2.9 days (2–5 days, n = 132), respectively (Fig. S1b, c). In both male and female phases, flowers are oriented nearly horizontally, with flower angles deviating by an average of 2.4° (−43 to 32°, n = 32; Fig. S2). In male phase flowers, the pistil produces pollen-bearing hairs, on which secondary pollen presentation occurs soon after flower opening, whereas the female phase begins with the curling of the stigmatic lobes, as in other Campanulaceae species (Liu and Huang, 2013; Vranken et al. 2014). Pollen grain viability is > 80% just after flower opening and pollen vigor decreases quickly, to < 30%, within 3 days in cultivated plants (Wei et al. 2006). Individual plants have thick roots and usually do not spread clonally.

We examined a population of *P. grandiflorus* on the Sugadaira plateau, Nagano Prefecture, Japan (36°32’12.9 N, 138°20’53.1 E) in August 2018, 2019, and 2021. *Platycodon grandiflorus* is recorded only on ski slope grasslands that are mowed annually in early September (Yaida et al. 2019). In the study site, we observed several pollinator groups visiting *P. grandiflorus* flowers to forage pollen and nectar; these included large- and medium-sized bees (predominantly *Megachile*, sporadically *Bombus hypocrita* and *Apis* species), small bees (mainly *Andrena* and Halictidae species), syrphid flies (*Episyrphus balteatus*, *Syrphus torvus*, and other syrphid flies), infrequent scolid wasps, butterflies, skippers (e.g., *Ochlodes ochraceus*), and beetles (Fig. S3).

Flower diameter changes during opening

We measured the front diameter of the corolla opening in the male (ca. 0.5–1 days after opening) and female phases (> 3 days after opening), using a digital caliper to examine the effects of temporal changes in petal lobe opening on pollinator attraction and rainfall avoidance.

Effects of floral angle on pollinator behavior

In 2018, we experimentally prepared three types of flowers by varying the floral angle: control flowers with no change in angle (Con); flowers with faces turned upright (Up); and flowers with faces turned downward (Down). The angles of all experimental flowers were fixed during anthesis using short wires (Fig. 1). The same treatment was administered to all opening flowers within the same individual plant (total of 123 flowers; 44 Con, 42 Up, and 37 Down); in total, there were 84 plants (31 Con, 28 Up, and 25 Down). A maximum of four flowers per plant was subjected to subsequent observations. The number of open flowers per individual varied from 1 to 3, with an average of 1.4 per site. All experimental plants and their flowers were identified.

To test the effects of floral angle on pollinator behavior, we observed pollinator visits to the experimental flowers. For each trial, we arbitrarily selected 3–10 neighboring experimental plants with different flower types and observed their pollinators during 20-min periods. We conducted 41 observation trials for a total of 820 min of observation from 08:00 to 15:00 on sunny days (when pollinators were active). Before each trial, we recorded the sexual phase (male or female) of each experimental flower. For most flowers, we conducted repeat observations at different times of the same day, or during the same sexual phase on different days, and in different sexual phases. The average number of observations per flower was 3.36 (range: 1–8), and 16 flowers were observed only once. We observed 25 and 56 flowers during the male and female phases, respectively, and 42 flowers during both phases. Among all trials, we observed 95 male Con, 87 male Up, 97 male Down, 48 female Con, 52 female Up, and

34 female Down flowers.

During the observation period, we recorded two types of pollinator behavior: approaching and landing. We further divided landing behavior into legitimate landing, in which the pollinator touched any part of pollen-bearing hairs (i.e., secondary presented pollen) on the pistil in a male phase flower or the stigma in a female phase flower during landing, and petal landing, in which the pollinator landed on a petal and collected pollen without touching these organs (Ushimaru & Hyodo, 2005). We counted the occurrences of each behavior on each experimental flower during each observation trial. Because the flowers of *P. grandiflorus* are protandrous, and its male and female phases do not overlap temporally within each flower we explored difference in the effects of flower angle on pollinator behavior between the male and female phases.

Effects of floral angle on pollen transfer success

We estimated pollen removal by counting pollen grains remaining on the pistil hairs of 60 experimental flowers and 10 newly opened, unvisited flowers in 2019 (Harder 1990; Ushimaru et al. 2006; Ushimaru et al. 2014; Katsuhara et al. 2017; Ushimaru et al. 2021). We collected a single pistil from each of 20 naturally pollinated flowers (20 individuals) in each of the Con, Up, and Down treatments, for a total of 60 pistils; these were stored separately. We confirmed that these flowers had nearly reached the end of the male phase by carefully checking the condition of the pistil (e.g., stigmatic lobe status). In addition, a single pistil containing secondary pollen was collected from each of 10 unvisited flowers (10 individuals) after opening; these pistils were stored separately in 1.0 mL 99.9% ethanol. We vortexed each sample and estimated the number of pollen grains per sample by counting pollen grains in three 10.0-μL drops per sample under a microscope. To quantify pollen removal from each experimental flower, we calculated pollen removal by subtracting the estimated number of pollen grains remaining in each experimental flower from the mean estimated number of pollen grains among newly opened flowers (Ushimaru et al. 2014; Ushimaru et al. 2021).

We also examined the effects of floral angle on pollen receipt by collecting one pistil from each of 20 flowers in the Con, Up, and Down treatments at ca. 3 days after the start of the female phase, in early August 2019. These flowers were exposed to natural pollinator visits for ca. 5 days following bud break. We removed the stigma from each pistil, stored it separately, and subsequently counted the pollen grains deposited on the stigma under a microscope (magnification, ×40) in the laboratory.

Effects of precipitation on experimental flowers during the flowering season

In 2019, we conducted qualitative observations of several male- and female-phase control and experimental flowers after rainfall, to determine whether flower parts related to mating, including anthers, pistil hairs, and stigmas were dry, wet, or soaked.

To examine the combined effects of flower angle and precipitation, we prepared 61 experimental flowers (10 male Con, 10 male Up, 10 male Down, 11 female Con, 10 female Up, and 10 female Down) on 61 individuals and determined whether the flower base, sexual organs, and pollen were dry, wet, or soaked after 24 h of rain on August 16, 2021.

We obtained meteorological data for the past decade (2012–2021) from the nearest Automated Meteorological Data Acquisition System (AMEDAS) station (36°31’9 N, 138°19’5 E), to examine the effects of daily precipitation during the *P. grandiflorus* flowering season (July 15 to September 15) at the study site.

Pollen germination in water and sucrose solution

To determine the influence of rainfall on pollen grain viability, we examined pollen germination and burst rates in water or sucrose solution according to established methods (Dafni, 1992; Huang et al. 2002). We collected pollen grains from the dehisced anthers of 10 flowers immediately after flower opening; the grains were preserved at ca. −20°C in a freezer in the laboratory. We placed pollen grains from each flower on a glass slide containing 0, 5, 10, 15, or 20% (g/g × 100) sucrose solution, where 0% indicates distilled water. On each slide, we examined the status of 45–235 pollen grains (average, 145.7) and counted the germinated and burst pollen grains after 24 h under a light microscope. This germination/burst evaluation was repeated 10 times per sucrose concentration.

Statistical analyses

We compared flower diameters between sexual phases using a generalized linear mixed model (GLMM) with a Gaussian error distribution and the identity link function; flower diameter, sexual phase (male/female), and flower ID were the response and explanatory variables and random term, respectively. Next, we examined the effect of floral angle on pollinator behavior using GLMMs with negative binomial error distribution and logarithmic link functions. The numbers of approaches, legitimate landings, and petal landings per 20-min period per flower were the response variables. In all models, we included treatment (Con /Up /Down), sexual phase, and their interaction as explanatory variables, display size (number of flowers per individual) and observation time (start time, minutes from 08:00) as covariates, and nested flower and individual identities as random terms. We further conducted a post-hoc multiple comparison among three treatments by a Tukey method. We applied the same GLMM analyses to the approaching and landing behavior of the three major pollinator groups.

Next, we compared pollen removal and receipt between Con and other (Up or Down) flowers using generalized linear models (GLMs) with negative binomial errors and logarithmic links and a post-hoc multiple comparison by a Tukey method. The model incorporated treatment as the explanatory variable and pollen removal or pollen receipt as the response variable. Then, we examined differences in the ratios of flowers showing different responses to precipitation using Fisher's exact test. Finally, we compared the ratios of germinated and burst pollen grains using GLMMs with negative binomial errors and logarithmic links, in which pollen grain number, sucrose concentration, total number of examined pollen grains, and flower ID were the response and explanatory variables, offset term, and random term, respectively. All statistical analyses were performed using R software (R Development Core Team 2017), largely with the glmmADMB (Fournier et al. 2012) and the multcomp packages (Hothorn et al. 2008).

RESULTS

Flower diameter

We observed a significant increase in flower diameter between the male (mean ± standard error [SE], 5.0 ± 0.24 cm; range: 2.0–5.1 cm) and female phases (5.8 ± 0.49 cm, 2.0–7.3 cm, $z = 0.07$, $P < 0.001$; Table S1), indicating that petal lobes were more open and more likely to be bent backward in the female phase. Note that the entire style was sheltered under the umbrella of the upper petal lobe during the male phase in horizontally orientated flowers (Fig. 1a).

Effects of floral angle on pollinator behavior

The dominant pollinators visiting the experimental flowers were large- and medium-sized bees (226 approaches, 54.4 % of all approaches), followed by small bees (100 approaches, 24.1 %) and syrphid flies (74 approaches, 17.8 %). The ratios of the three major pollinator groups did not differ significantly among flower types or between sexual phases for legitimate landings (Fig. S4b).

For all pollinators, the approach frequency per flower did not differ significantly among flower types during the male and female phases (Fig. 2a, Table S1). The number of approaches per flower by all pollinators decreased significantly with increasing display size ($z = -3.17$, $P = 0.002$; Table S1). Approach frequency by LM bees significant decreased in the female phase ($z = -0.707$, $P = 0.017$, Table S1), and those by small bees decreased with larger display sizes (z = -3.02, P = 0.003, Table S1). The number of approaches increased significantly with observation time for all pollinators and bee sizes (All pollinators: z = 2.44, $P = 0.002$; LM bees: z = 2.42, $P = 0.016$; S bees: z = 2.55, $P = 0.011$; Table S1).

There were significantly fewer legitimate landings by all pollinators and bees on Down flowers than on controls, but there was no significant difference between Con and Up flowers (Down: z = -3.29, $P = 0.001$; Up: z = -0.030, $P = 0.978$; Fig. 2b, Table S1), and significantly fewer for all pollinators in female- than male-phase flowers in the GLMM ($z = -3.34$, $P < 0.001$; Fig. 2b, Table S1). Based on the post-hoc Tukey–Kramer test results, a significant difference in legitimate landing frequency by all pollinators was only found between Down and Con flowers in the male phase ($P < 0.05$; Fig. 2b).

Legitimate landings per flowers by all pollinators was not differ significantly among the three flower types in the female phase, although Down and Up flowers received relatively fewer legitimate landings than controls (Fig. 2b). Legitimate landings increased with observation time in all pollinators ($z = 3.630$, $P < 0.001$; Table S1), but decreased with increasing display size only in small bees ($z = -2.380$, $P = 0.017$; Table S1).

Petal landings by all pollinators and each pollinator group were not influenced by any of the explanatory variables (Table S1). The interaction between flower angle and sexual phase had no significant effect on any response variable in any pollinator group, except for approach frequency by small bees, which increased significantly in female-phase Up flowers than in male-phase controls (z = -2.460, $P = 0.014$; Table S1).

Effects of floral angle on pollen transfer success

More than 95% of pollen grains were removed from pistil hairs during the male phase in all flower types; a significantly higher pollen removal was found in Up and Down flowers than in controls and higher pollen removal was found in Down than Up (Up: z = 31.330, $P < 0.001$; Down: z = 15.430, $P < 0.001$; Fig. 3b, Table S1). In contrast, the stigmas of Up and Down flowers received significantly fewer pollen grains (ca. 45 and 30 grains on average, respectively) than those of Con flowers, which received > 65 pollen grains on average, and Down flowers received more pollen grains than Up flowers (Up: z = -10.130, $P < 0.001$; Down: z = -16.630, P < 0.001; Fig. 3a, Table S1).

Effects of precipitation on flowers during the flowering season

At daily precipitation rates of > 3.5 mm, Up flowers accumulated water and the anthers and pistil hairs within were soaked in the male phase, whereas parts of the stigma lobes of Up flowers were wet or soaked in the female phase in 2019 (Fig. S5a, b). In Down flowers, mating-related parts remained dry, although the backs of the petals were wet. The rear upper petals and front lower petal lobes were wet in Con and intact flowers, but mating-related organs were rarely wet; the stigma surfaces were also rarely wet in female-phase flowers with widely opened petal lobes.

On August 16, 2021, the flower base was soaked or wet after 6.0 mm of rain in many Up flowers, although little water remained in the flower cups due to stem oscillation in strong winds. We observed a single plant in which the pistil of an Up flower was broken by rain (Fig. S5c). Significantly more Up flowers had wet or soaked pollen, flower bases, and/or mating-related organs than other flower types (Fig. 4a). In Con and Down flowers, the lobes and backs of petals were often wet, but mating-related organs remained dry throughout the experiment ($P < 0.001$, Fisher's exact test; Fig. 4b).

During the past decade, rainfall events with ≥ 3.5 mm precipitation, which was sufficient to soak most parts of the pistil in Up flowers, persisted for 10–21 days (mean, 15.9 days) during the flowering period (Fig. S6). Heavy rainfall events (> 10 mm/h) occurred frequently in the study area. Periods of > 4.2 sequential days (the average longevity of a flower) without rainfall were observed in less than half of the flowering season in 2012–2021, indicating that most flowers in the study area experienced at least some rainfall while they were open (Fig. S6). Periods of > 5 sequential days without rain occurred in the study area only three times in 2018, and once in 2019, during the flowering period (Fig. S6).

Pollen germination in water and sucrose solution

On average, 56.3% of pollen grains germinated in the 5–20% sucrose solution, whereas only 0.04% of grains germinated in distilled water (Fig. 5), with significant differences between distilled water and sucrose solution (Table S1). Approximately 30% of pollen grains burst in distilled water, whereas only < 0.007% of pollen grains burst in 5–20% sucrose solution, which was significantly lower than the rate in distilled water. No pollen burst was observed in 20% sucrose solution.

DISCUSSION

In this study, we examined the effects of horizontal flower orientation on pollinator behavior and rain protection in *P. grandiflorus*. Our field experiments showed that upward-oriented flowers experienced more pollen and pistil damage due to rainfall and greater pollen limitation than control flowers, whereas downward-oriented flowers received fewer pollinator visits in the male phase and had fewer pollen grains on their stigmas than controls unlike our prediction. Thus, horizontal flower orientation may have evolved under both pollinator- and rain-mediated selection in *P. grandiflorus*.

Effects of floral angle on pollinator behavior and pollen transfer

Although all flower types experienced similar numbers of approaches by all pollinator groups, downward flower orientation reduced the frequency of legitimate bee landings compared with horizontal and upward flower orientations during the male and female phases, although the pattern was not significant in the female phase. Thus, floral orientation influenced pollinator landing behavior to a greater extent than pollinator attraction. These results are consistent with those for the zygomorphic flowers of *Commelina communis* (Ushimaru & Hyodo 2005), but not with patterns in another species with zygomorphic flowers, *Corydaris sheareri*, whose upward-oriented flowers showed pollinator limitation (Wang et al. 2014a), or in *Geranium refractum* (Wang et al. 2014b) or *Mertensia* species (Lin et al. 2019), whose downward-oriented actinomorphic flowers did not experience pollinator limitation. These discrepancies between the present and previous studies (Wang et al. 2014a, b; Lin et al. 2019) may be due to differences among pollination systems, i.e., a combination of floral characteristics and pollinators. Bumblebees less discriminate downward-oriented flowers with open or cup shapes than leafcutter bees, small bees, syrphid flies, and lepidopterans do (Huang et al. 2002; Ushimaru & Hyodo 2005; Wang et al. 2014a, b; Lin et al. 2019; Yu et al. 2020). Long-tubed flowers are typically not preferred by diurnal pollinators when experimentally turned upright (Wang et al. 2014a; Yu et al. 2020), whereas upright open or cup-shaped flowers are visited by various groups of pollinators that use petals as landing platforms (Huang et al. 2002; Ushimaru & Hyodo 2005; Wang et al. 2014b; Lin et al. 2019). In this study, we observed that leafcutter bees, small bees, and syrphid flies (the dominant pollinators of *P. grandiflorus*) sometimes took longer to find footholds on downward-oriented flowers than on other flower types (supplemental movie). This longer handling time may have led to discrimination against downward-oriented flowers by these pollinators.

Pollen removal per flower was significantly enhanced by upward and downward flower orientation compared to horizontal orientation. Upright flowers experienced more legitimate landings than controls during the male phase although the difference was not significant, consistent with our pollen removal results. Pollinator-limited downward-oriented flowers also had higher pollen removal rates than controls, perhaps due to accidental pollen loss caused by flower jostling by pollinators or wind. In all flower types, > 95% of pollen grains were removed; therefore, such differences in pollen removal were negligible (Fig. 3a).

In contrast, flower angle treatments greatly reduced pollen receipt on *P. grandiflorus* stigmas, consistent with previous studies on horizontally oriented zygomorphic flowers (Ushimaru et al. 2009; Wang et al. 2014a). Upward and downward flowers received fewer legitimate landings than control flowers during the female phase, although the difference was not significant (Fig. 2a), likely causing lower pollen receipt. However, the cause of lower pollination success in these flowers during the female phase requires further research.

Sexual phase, display size, and time of day influenced pollinator visitation to *P. grandiflorus* flowers. Female-phase flowers received significantly fewer approaches and landings by large bees than male-phase flowers, suggesting that these bees mainly foraged pollen on flowers. Larger display size tended to decrease pollinator attraction per flower, indicating that the presence of large corollas attracts pollinators, and that the simultaneous opening of multiple flowers per plant may have a negative impact on the reproductive success of individual flowers. This finding may explain the small average floral display size (1.3 flowers per plant) of this species. Bees increased their visitation rates to flowers before and after noon, but no such pattern was observed in syrphid flies. Daily activity patterns are known to vary between bees and flies (Herrera, 1990; Rader et al. 2013; Ushimaru et al. 2021). However, further study is required to elucidate the factors responsible for these differences, including thermoregulatory ability, energy requirements, and their interactions with the habitat environment of each pollinator group or species (Herrera, 1990; Rader et al. 2013)

Roles of horizontal flower orientation in rain avoidance

Pendant and downward flowers function to protect pollen grains from rainfall and sun radiation (Tadey & Aizen 2001; Huang et al. 2002; Haverkamp et al. 2019). In this study, many *P. grandiflorus* pollen grains burst and lost germination ability in water, indicating a significant negative effect of rainfall on pollen viability, as observed in other species (Tadey & Aizen 2001; Huang et al. 2002; Mao & Huang 2009). The anthers, pistil hairs, and stigmas of control as well as downward-oriented flowers remained dry, unlike upward-oriented flowers, which were wet or soaked (Figs. 4, S5d–g). Interestingly, petal lobes did not open as fully in the male compared to female phase, suggesting that they function as an umbrella for pollen grains during rainfall. We frequently observed intact flowers facing downslope, irrespective of slope direction, as has been documented in forest floor flowers on slopes (Ushimaru et al. 2006). We also observed that flowers frequently opened toward the sun, indicating a lack of sun avoidance in the species (A. Ushimaru, personal observation). The flowering phenology of *P. grandiflorus* barely overlaps the rainy and typhoon seasons in Japan. Thus, horizontal orientation was expected to have a rainfall avoidance function in the flowers of this species.

CONCLUSION

In this study, our field experiments using *P. grandiflorus* flowers revealed that upward-facing flowers experienced rainfall damage during the flowering period, whereas downward-facing flowers were pollinator-limited. Thus, horizontal flower orientation likely evolved under both biotic and abiotic pressure in this species. A recent study suggested a similar adaptation in horizontally orientated zygomorphic *Abelia* ×

grandiflora flowers (Yu et al. 2020). However, in that species, a role of horizontal orientation in rain protection was dubious because the anthers and stigmas protrude from the petals and are always wet under rainy conditions (Yu et al. 2020). Thus, this study is the first to demonstrate that horizontal orientation enhances legitimate pollinator landings and female pollination success, and also protects mating-related organs, especially the anthers and secondary-presented pollen grains in the male phase, from rainfall damage in actinomorphic *P. grandiflorus* flowers. Because the adaptive significance of horizontal flower orientation in actinomorphic flowers in generalist pollination systems has been examined only in this species, a wider range of species should be investigated to generalize our findings.

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AUTHOR CONTRIBUTIONS

AU and RI firstly conceived the study and designed the methodology; TN, RI, YAY and AU collected the data; TN and AU analyzed the data and were involved in the writing of the manuscript. All authors contributed critically to the drafts and provided final approval for publication.

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FIGURE LEGEND

Fig. 1 Experimentally manipulated flowers of *Platycodon grandiflorus*. (a) Control (Con); (b) upward-facing (Up); (c) downward-facing (Down).

Fig. 2 Mean numbers of (a) approaches and (b) legitimate landings by pollinators, per 20-min period per flower, for each experimental treatment (Con, Up, or Down) in *Platycodon grandiflorus*. M and F indicate the sexual phase (M, male phase; F, female phase). Different letters indicate significant ($P < 0.05$) differences among treatments based on the Tukey method.

Fig. 3 Mean number of remaining pollen grains (a) on pistil hairs and (b) deposited on the stigma (b) in flowers of each experimental treatment (Con, Up, and Down). Bars are standard errors. Different letters indicate significant ($P < 0.05$) differences among treatments based on the Tukey method.

Fig. 4 Effects of flower angle on rain damage in experimental *Platycodon grandifloras* flowers. The ratios of flowers with (a) mating-related organs or pollen or (b) a flower base that were soaked or wet after ca. 6.0 mm of rainfall differed significantly among flower types ($P < 0.001$; Fisher's exact test).

Fig. 5 Ratios of germinated and burst pollen grains of *Platycodon grandifloras* in various concentrations of sucrose solution.

TABLE S1 Results of generalized linear mixed model (GLMM) and generalized linear model (GLM) analyses. Control and male phase flowers, and distilled water (i.e., 0% sucrose solution) were used as baselines for the treatment, sex, and sucrose concentration variables in the models. Significant effects are indicated in bold ($P <$ 0.05)

Fig. S1 *Platycodon grandifloras* flower (a) soon after opening and during the (b) male and (c) female phases.

Fig. S2 Circular histograms of flower angle in both male- (blue bars) and female-phase (red bars) flowers in 2021. Flower angle did not significantly differ between male- and female-phase flowers ($\chi^2 = 7.771$, $P = 0.169$).

Fig. S3 Diverse pollinators on *Platycodon grandiflorus*: large- and medium-sized bees including (a) *Megachile* sp., (b) *Bombus hypocrita*, (c) *Apis mellifera*; small bees including (d) *Andrena* sp. and (e) *Halictidae* sp.; (f) the syrphid fly *Episyrphus balteatus*; (g) the skipper, *Ochlodes ochraceus*.

Fig. S4 The ratios of (a) approaches and (b) legitimate landings by large- and medium-sized (LM) bees (blue), small (S) bees (light pink), syrphid flies (medium pink), and other pollinators (dark pink) in the experimental flower treatments (Con, control; Up, upward-oriented; Down, downward-oriented). The ratios of the three dominant pollinator groups differed significantly among flower types and between sexual phases in terms of approaches ($\chi2 = 22.534$, $P = 0.012$), but not legitimate landings ($\chi2 = 17.63$, $P = 0.06$).

Fig. S5 Experimental and control *Platycodon grandifloras* flowers after rain. At a rainfall rate of 3.5 mm/day, (a) pistil hairs and anthers of male-phase flowers and (b) stigma of female-phase flower were soaked. (c) At 6.0 mm/day, the stigma of a female-phase upward-oriented flower was broken. In horizontal-oriented control flowers at (d)

10 (e) 6.0, and (f) 2.0 mm/day and in (g) downward-oriented flowers at 6.0 mm/day, the anthers, stigma, and pollen were protected from rain damage.

Fig. S6 Precipitation during the flowering season. Data were obtained from the Automated Meteorological Data Acquisition System (AMDAS) station in Sugadaira, Nagano Prefecture (36°31’9 N, 138°19’5 E).

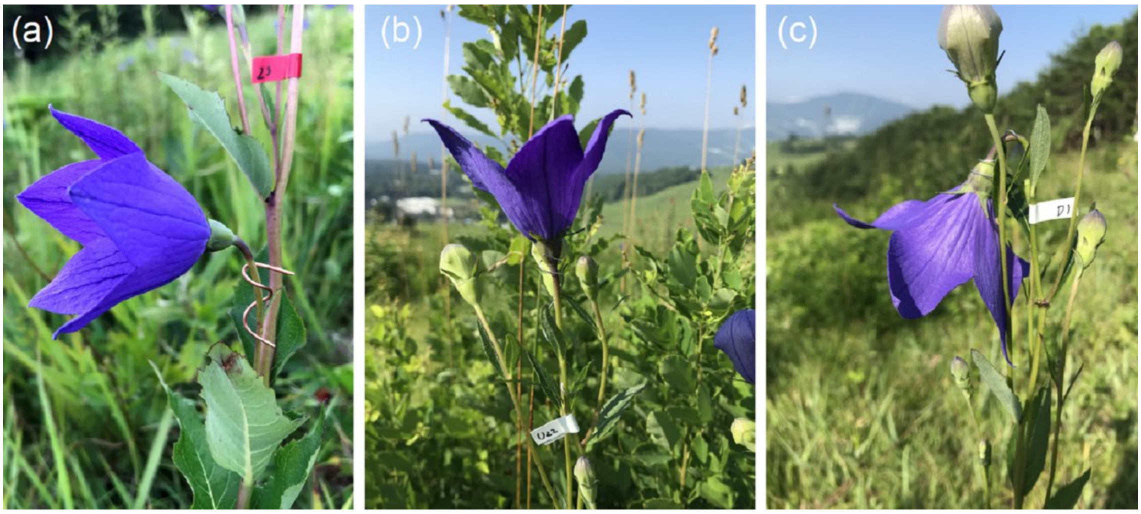


Fig.1

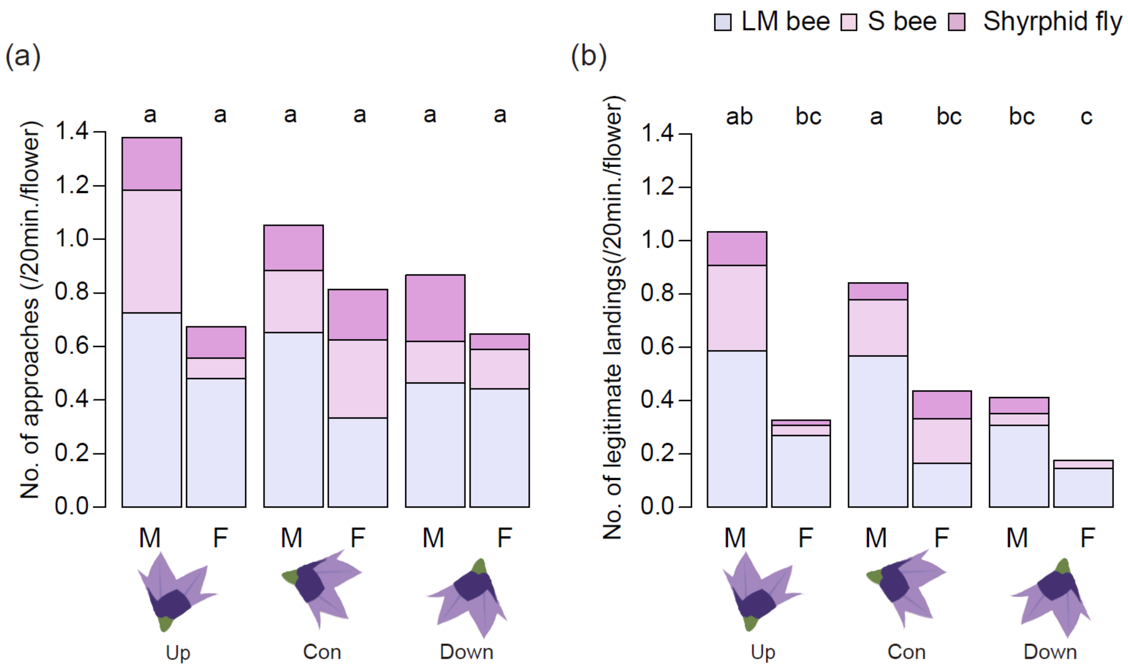

LM bee
S bee
Shyrphid fly
(a)
No. of approaches (/20min./flower)
0.0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
a
a
a
a
a
a
M
F
M
F
M
F
Up
Con
Down
(b)
No. of legitimate landings(/20min./flower)
0.0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
ab
bc
a
bc
bc
c
M
F
M
F
M
F
Up
Con
Down

Fig.2

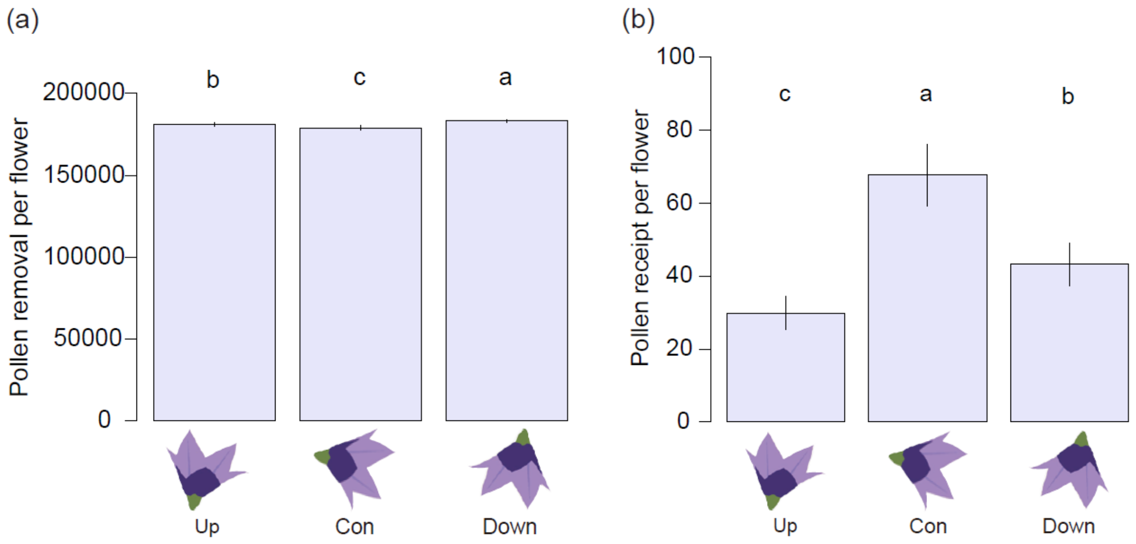

(a)
Pollen removal per flower
200000
150000
100000
50000
0
b
c
a
Up
Con
Down
(b)
Pollen receipt per flower
100
80
60
40
20
0
c
a
b
Up
Con
Down

Fig.3

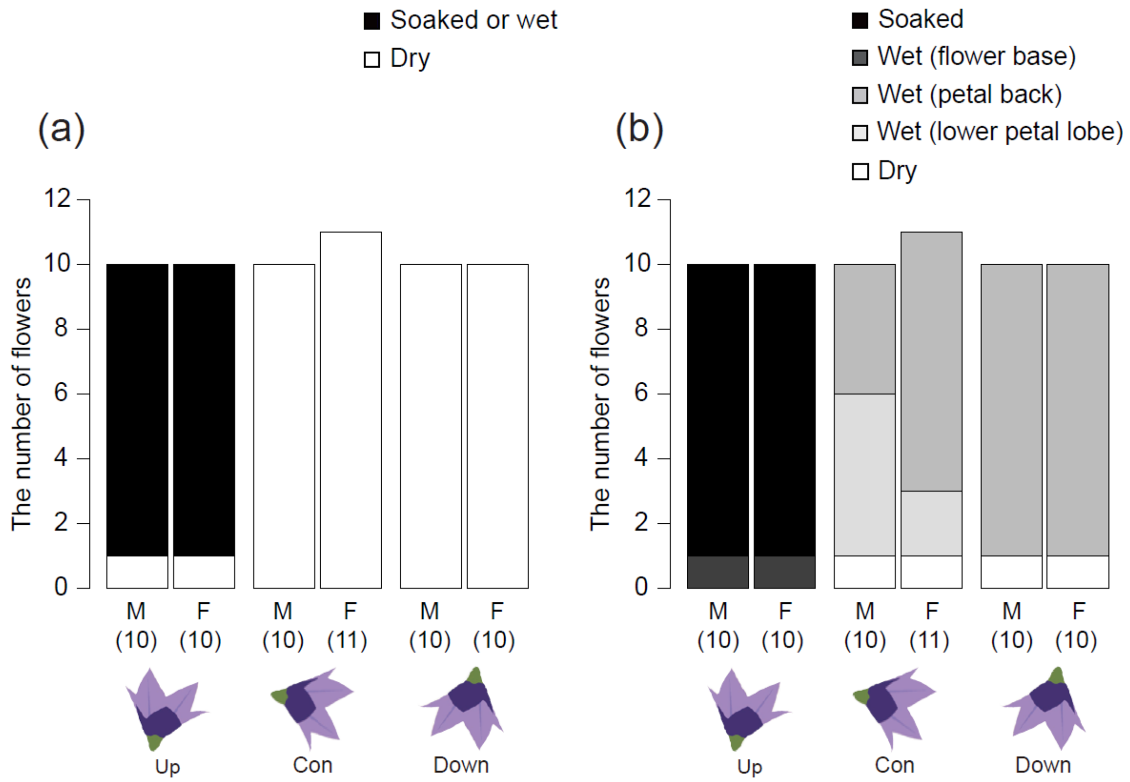

■ Soaked or wet
□ Dry
(a)
The number of flowers
12
10
8
6
4
2
0
M (10)
F (10)
M (10)
F (11)
M (10)
F (10)
Up
Con
Down
■ Soaked
■ Wet (flower base)
■ Wet (petal back)
□ Wet (lower petal lobe)
□ Dry
(b)
The number of flowers
12
10
8
6
4
2
0
M (10)
F (10)
M (10)
F (11)
M (10)
F (10)
Up
Con
Down

Fig.4

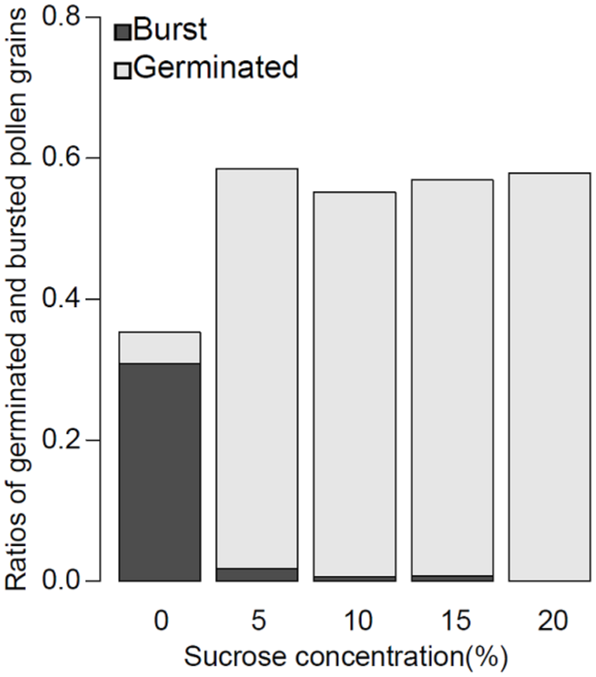

Ratios of germinated and bursted pollen grains
0.8
0.6
0.4
0.2
0.0
Burst
Germinated
0
5
10
15
20
Sucrose concentration(%)

Fig.5

TABLE S1

Response variable/ explanatory variable	Estimated coefficient	S.E.	*z* value	*P*
Flower diameter				
Intercept	4.699	0.110	42.480	**<0.001**
Sexual phase (female)	0.279	0.070	3.980	**<0.001**
Pollinator Observation				
All approaches per 20min. per flower/				
Intercept	0.419	0.251	1.670	0.095
Treatment (Down)	-0.230	0.182	-1.260	0.206
Treatment (Up)	0.161	0.172	0.940	0.349
Sexual phase (female)	-0.343	0.213	-1.610	0.107
Display size	-0.288	0.091	-3.170	**0.002**
Observation time	0.001	0.000	2.440	**0.015**
Angle (Down)×Sex (female)	-0.076	0.344	-0.220	0.826
Angle (Up)×Sex (female)	-0.001	0.301	-0.820	0.415
All legitimate landings per 20min. per flower /				
Intercept	-0.064	0.264	-0.240	0.807
Treatment (Down)	-0.787	0.239	-3.290	**0.001**

Treatment (Up)	-0.006	0.207	-0.030	0.978
Sexual phase (female)	-0.925	0.277	-3.340	**<0.001**
Display size	0.222	0.119	-1.870	0.061
Observation time	0.002	0.000	3.630	**<0.001**
Angle (Down)×Sex (female)	0.111	0.512	0.220	0.828
Angle (Up)×Sex(female)	0.049	0.383	0.130	0.898
All petal landings per 20min. per flower/				
Intercept	-0.379	0.366	-1.030	0.301
Treatment (Down)	-0.432	0.314	-1.370	0.170
Treatment (Up)	-0.097	0.297	-0.330	0.743
Sexual phase (female)	-0.510	0.378	-1.350	0.177
Display size	-0.322	0.160	-2.010	0.044
Observation time	0.000	0.001	-0.660	0.509
Treatment (Down)×Sex (female)	0.027	0.616	0.040	0.965
Treatment (Up)×Sex (female)	-0.590	0.593	-0.990	0.320
LM bee approaches per 20min. per flower/				
Intercept	0.319	0.255	-1.250	0.211
Treatment (Down)	-3.492	0.209	-1.670	0.095
Treatment (Up)	0.034	0.197	0.170	0.863
Sexual phase (female)	-0.707	0.297	-2.380	**0.017**
Display size	-0.181	0.101	-1.790	0.073

Observation time	0.001	0.000	2.300	**0.021**
Treatment (Down)×Sex (female)	0.640	0.431	1.490	0.137
Treatment (Up)×Sex (female)	0.396	0.393	1.010	0.313
LM bee legitimate landings per 20min. per flower/				
Intercept	-0.487	0.298	-1.630	0.103
Treatment (Down)	-0.632	0.252	-2.510	**0.012**
Treatment (Up)	-0.049	0.221	-0.220	0.823
Sexual phase (female)	-1.292	0.402	3.220	**0.001**
Display size	-0.198	0.125	-1.590	0.114
Observation time	0.001	0.001	2.420	**0.016**
Treatment (Down)×Sex (female)	0.536	0.636	0.840	0.400
Treatment (Up)×Sex (female)	0.615	0.509	1.210	0.227
LM bee petal landings per 20min. per flower/				
Intercept	-1.120	0.679	-1.640	0.100
Treatment (Down)	-0.030	0.576	-0.050	0.958
Treatment (Up)	-0.666	0.615	-1.080	0.279
Sexual phase (female)	-24.000	46400.000	0.000	1.000
Display size	-0.472	0.357	-1.320	0.186
Observation time	-0.004	0.001	-2.680	**0.007**
Treatment (Down)×Sex (female)	23.100	46400.000	0.000	1.000

Treatment (Up)×Sex (female)	1.510	62600.000	0.000	1.000
S bee approaches per 20min. per flower/				
Intercept	-0.870	0.454	-1.920	0.055
Treatment (Down)	-0.395	0.406	-0.970	0.331
Treatment (Up)	0.420	0.352	1.190	0.233
Sexual phase (female)	0.025	0.389	0.070	0.948
Display size	-0.673	0.223	-3.020	**0.003**
Observation time	0.002	0.001	2.550	**0.011**
Treatment (Down)×Sex (female)	-0.602	0.738	-0.820	0.415
Treatment (Up)×Sex (female)	-1.663	0.676	-2.460	**0.014**
S bee legitimate landings/ per 20min. per flower/				
Intercept	-1.422	0.569	-2.500	**0.012**
Treatment (Down)	-1.680	0.620	-2.710	**0.007**
Treatment (Up)	0.120	0.410	0.290	0.770
Sexual phase (female)	-0.388	0.474	-0.820	0.413
Display size	-0.697	0.293	-2.380	**0.017**
Observation time	0.003	0.001	3.650	**<0.001**
Treatment (Down)×Sex (female)	-0.117	1.273	-0.090	0.927
Treatment (Up)×Sex (female)	-1.542	0.900	-1.710	0.087
S bee petal landings/ per 20min. per flower/				

Intercept	-1.211	0.687	-1.760	0.078
Treatment (Down)	-1.540	0.728	-2.110	**0.035**
Treatment (Up)	0.053	0.503	0.110	0.916
Sexual phase (female)	-0.298	0.576	-0.520	0.605
Display size	-0.894	0.346	-2.590	**0.010**
Observation time	0.001	0.001	1.170	0.242
Treatment (Down)×Sex (female)	0.428	1.188	0.360	0.719
Treatment (Up)×Sex (female)	-1.968	1.197	-1.640	0.100
Syrphid fly approaches/ per 20min. per flower/				
Intercept	-0.908	0.456	-1.090	**0.046**
Treatment (Down)	0.380	0.352	1.080	0.280
Treatment (Up)	0.030	0.381	0.080	0.936
Sexual phase	-0.040	0.456	-0.090	0.929
Display size	-0.336	0.182	-1.840	0.065
Observation time	-0.002	0.001	-1.700	0.089
Treatment (Down)×Sex (female)	-1.590	0.884	-1.800	0.072
Treatment (Up)×Sex (female)	0.440	0.685	-0.640	0.520
Syrphid fly legitimate landings/ per 20min. per flower/				
Intercept	-2.960	0.865	-3.420	**<0.001**
Treatment (Down)	-0.163	0.705	-0.230	0.817

Treatment (Up)	0.643	0.634	1.010	0.310
Sexual phase (female)	-0.198	0.710	0.280	0.781
Display size	-0.037	0.320	-0.110	0.909
Observation time	-0.002	0.002	-1.090	0.276
Treatment (Down)×Sex (female)	-20.100	14600.000	0.000	0.999
Treatment (Up)×Sex (female)	-2.130	1.300	-1.640	0.102
Syrphid fly petal landings/ per 20min. per flower/				
Intercept	-2.280	0.831	-2.740	**0.006**
Treatment (Down)	0.468	0.713	0.660	0.511
Treatment (Up)	0.217	0.748	0.290	0.772
Sexual phase (female)	-0.213	0.919	-0.230	0.817
Display size	-0.161	0.324	-0.500	0.616
Observation time	-0.004	0.002	-2.030	**0.043**
Treatment (Down)×Sex (female)	-14.492	915.550	-0.020	0.987
Treatment (Up)×Sex (female)	-0.316	1.286	-0.250	0.806
Pollen transfer success				
Number of pollen grains (female)/				
Intercept	4.214	0.027	155.020	**<0.001**
Treatment (Down)	-0.816	0.049	-16.630	**<0.001**
Treatment (Up)	-0.448	0.044	-10.130	**<0.001**

Number of pollen removal (male)/				
Intercept	12.100	0.001	22883.900	**<0.001**
Treatment (Down)	0.023	0.001	31.330	**<0.001**
Treatment (Up)	0.011	0.001	15.430	**<0.001**
Pollen germination experiments				
Ratio of burst pollen grains (gaussian) /				
Intercept	-1.053	0.248	-4.250	**<0.001**
Sucrose concentration (5 %)	-3.333	0.368	-9.060	**<0.001**
Sucrose concentration (10%)	-4.483	0.472	-9.500	**<0.001**
Sucrose concentration (15%)	-4.050	0.416	-9.750	**<0.001**
Sucrose concentration (20%)	-25.004	9080.300	0.000	1.000
Ratio of germination (gaussian)/				
Intercept	-3.909	0.277	-14.100	**<0.001**
Sucrose concentration (5%)	3.365	0.297	11.300	**<0.001**
Sucrose concentration (10%)	3.317	0.296	11.200	**<0.001**
Sucrose concentration (15%)	3.367	0.297	11.300	**<0.001**
Sucrose concentration (20%)	3.426	0.298	11.500	**<0.001**

Fig.S1

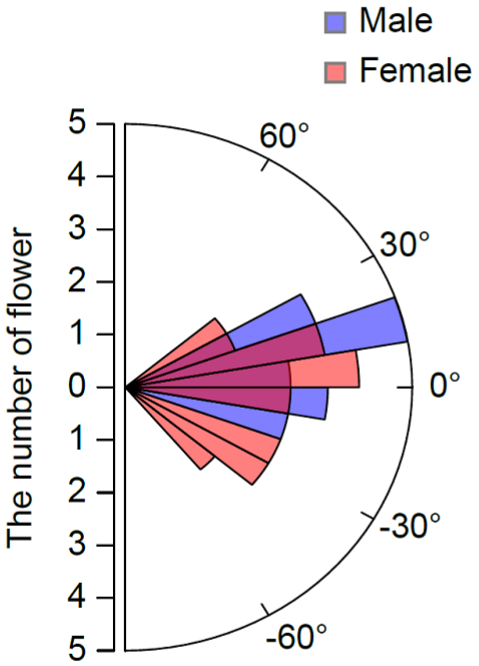


Fig.S2

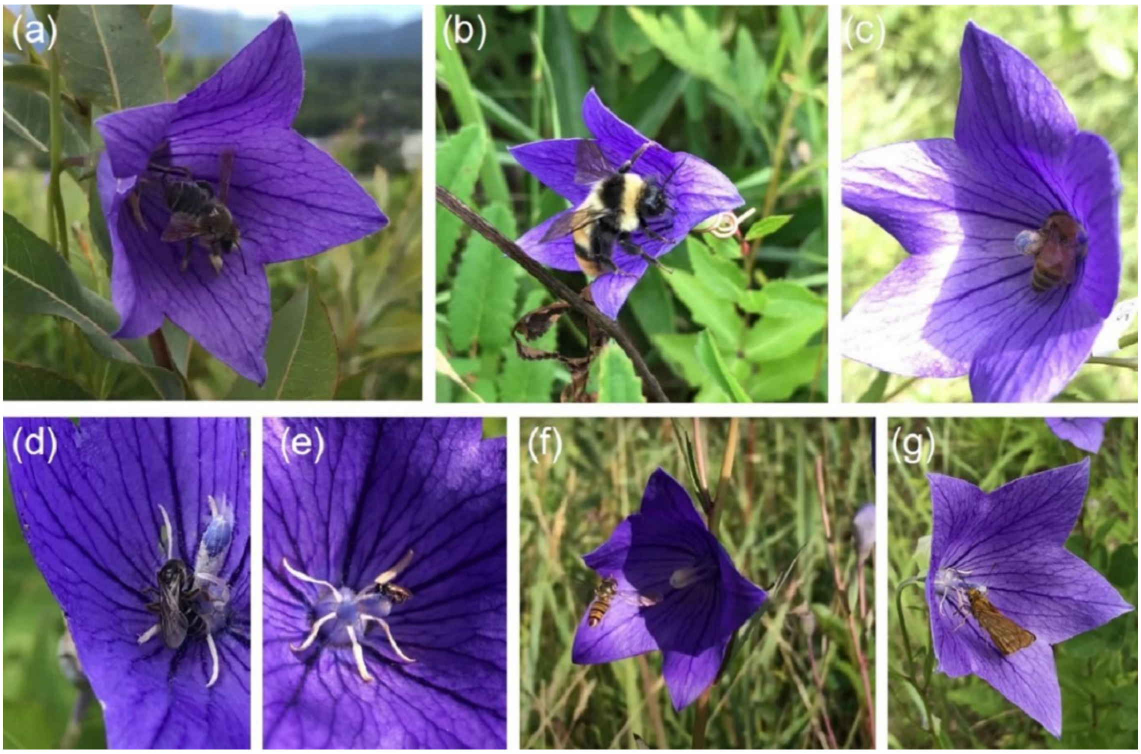


Fig.S3

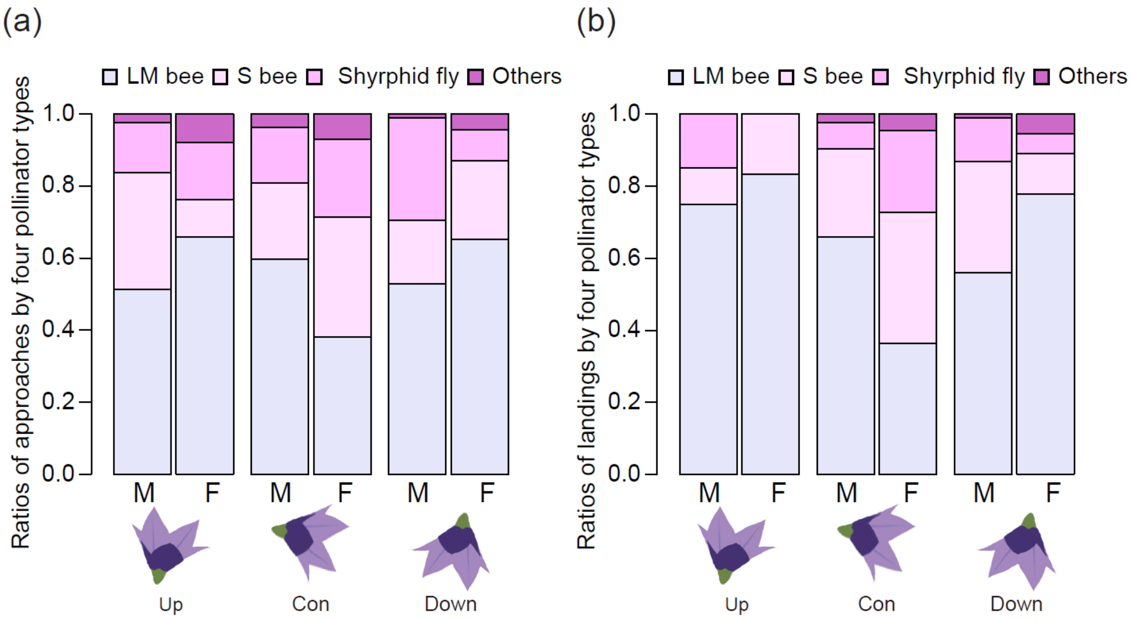
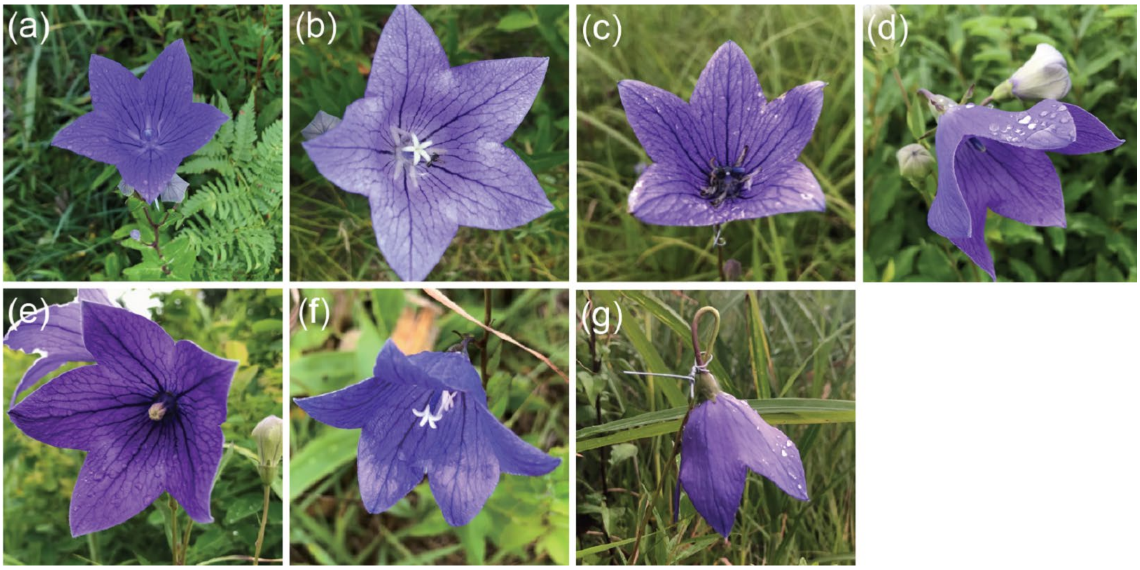

(a)
LM bee
S bee
Shyrphid fly
Others
Ratios of approaches by four pollinator types
1.0
0.8
0.6
0.4
0.2
0.0
M
F
M
F
M
F
Up
Con
Down
(b)
LM bee
S bee
Shyrphid fly
Others
Ratios of landings by four pollinator types
1.0
0.8
0.6
0.4
0.2
0.0
M
F
M
F
M
F
Up
Con
Down

Fig.S4

790



791 Fig.S5

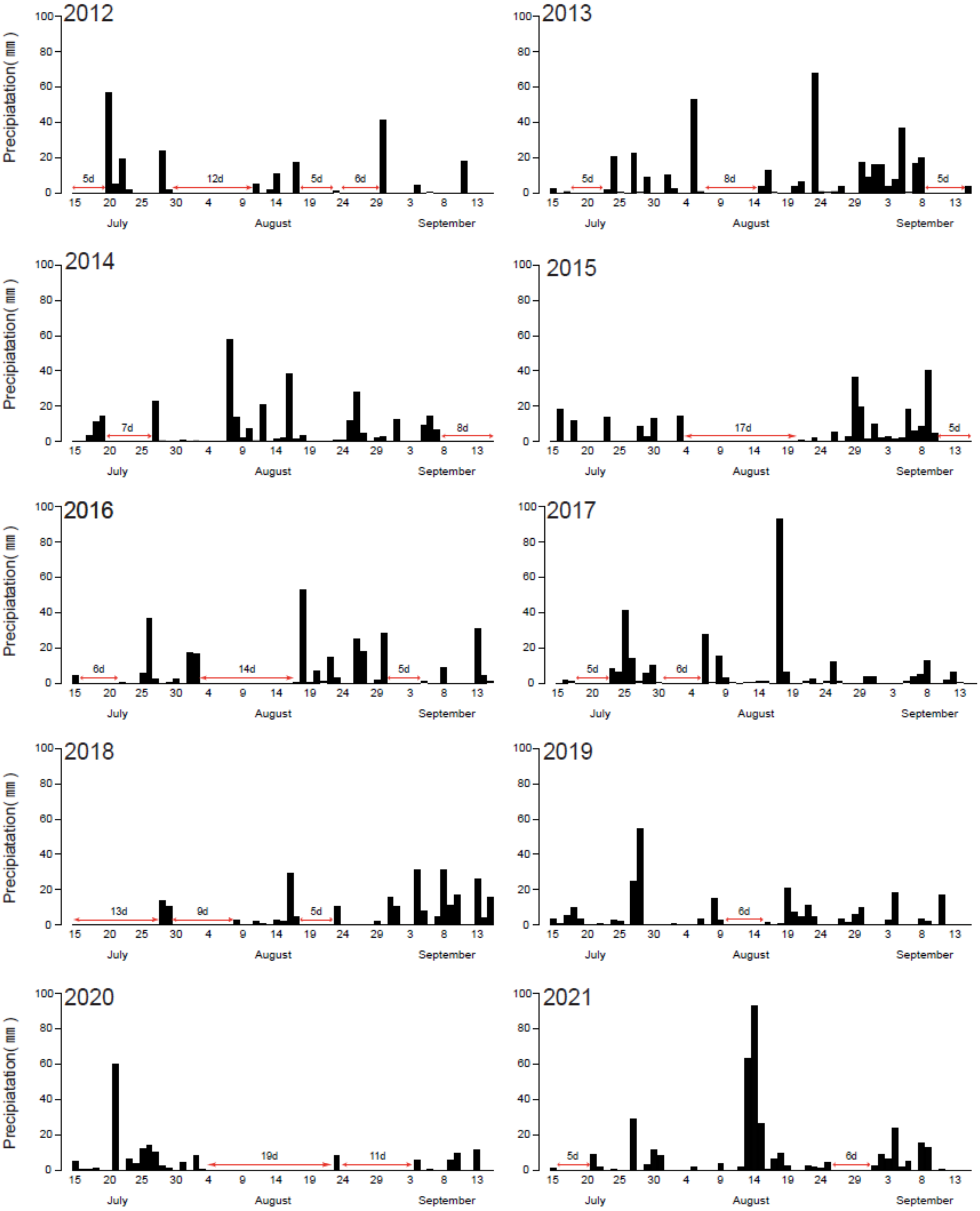

2012
5d
12d
5d
6d
2013
5d
8d
5d
2014
7d
8d
2015
17d
5d
2016
6d
14d
5d
2017
5d
6d
2018
13d
9d
5d
2019
6d
2020
19d
11d
2021
5d
6d
Precipiatation(mm)
0
20
40
60
80
100
15
20
25
30
4
9
14
19
24
29
3
8
13
July
August
September

Fig.S6