

1 **Article type** : Research Article

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4 **Title: Horizontal orientation facilitates pollinator attraction and rain avoidance in**
5 **radially symmetrical flowers**

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7 Running head: Horizontal orientation in actinomorphic flowers

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20 **Abstract: 220 words, main text: 4532 words, 38 references, 5 figures, a supplementary**

21 **table, 6 supplementary figures**

22

23 **Abstract**

24 *Premise:* Floral angle, such as upward, horizontal, and downward orientation are known to
25 evolve under both biotic and abiotic agents to enhance pollination success in zoophilous
26 plants. Adaptive significance of horizontal orientation in radially symmetrical
27 (actinomorphic) flowers under biotic and abiotic selection pressures were largely unknown,
28 although those in bilaterally symmetrical flowers have been well studied.

29 *Methods:* Using experimentally angle changed flowers, we examined the effects of flower
30 angle on pollinator behaviors, pollination success and rain avoidance in a population of
31 insect-pollinated *Platycodon grandiflorus*. We further investigated the frequency and
32 amount of precipitation in the flowering season and pollen damage by water in this species.

33 *Main results:* Horizontally oriented flowers received more visitations and pollen grains on
34 the stigma in male and/or female phases than downward and/or upward oriented flowers
35 and avoided pollen damage by rainfall compared to upward oriented flowers. The pollen
36 germination experiment showed that approximately 30% of pollen grains burst in distilled
37 water, thus pollen damage by rainfall was potentially serious in *P. grandiflorus*.

38 *Conclusion:* In this study, our field experiments revealed that upward flowers cannot avoid
39 damage from rainfall during the flowering period whereas both upward and downward
40 flowers suffered from pollinator limitation in female success. Thus, horizontal flower
41 orientation is suggested to be adaptive in this insect-pollinated actinomorphic species which
42 blooms in the rainy season.

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

44 **Introduction**

45 Present floral diversity in angiosperms are considered to have evolved under selections
46 mediated by both biotic and abiotic agents (Darwin, 1862; Grant and Grant, 1965; Stebbins,
47 1970; Fenster et al., 2004; Wilmer, 2011). In animal-pollinated species, most floral traits
48 such as size, shape, color, scent, flowering timing have adapted to enhance pollen transfer
49 by their respective pollinators, while some of the traits simultaneously function as
50 protection against harmful abiotic factors such as rain and very low/high temperature
51 (Kudo, 1995; Huang et al., 2002; Patino et al., 2002). Flower angle (e.g. vertical direction
52 of flower orientation including upward, horizontal, oblique and downward orientation)
53 regulated by flower stalk angle is a trait which evolves under both biotic and abiotic agents
54 to enhance pollination success in zoophilous plants (Hocking and Sharplin, 1965; Kevan,
55 1975; Kudo, 1995; Tadey and Aizen, 2001; Huang et al., 2002; Patino et al., 2002; Galen
56 and Stanton, 2003; Ushimaru et al., 2009; Haverkamp et al., 2019).

57 Flower angle is known to influence attraction to and behavioral control of specialized
58 pollinators (Fenster et al., 2009; Ushimaru and Hyodo, 2005). Bilaterally symmetrical
59 (zygomorphic) flowers usually exhibit horizontal orientation (Neal et al., 1998), which can
60 attract more pollinators and enhance their legitimate behaviors for achieving higher pollen
61 transfer success compared to upward or downward orientation (Ushimaru and Hyodo,
62 2005; Ushimaru et al., 2009; Wang et al., 2014a). In contrast, the optimal flower angle may
63 vary among radially symmetrical (actinomorphic) flowers likely depending on pollinator
64 composition. For example, upward and heliotropic orientation increases floral temperature
65 to attract many fly pollinators in alpine, arctic and early-spring blooming plants with dish-

66 shaped actinomorphic flowers (Hocking and Sharplin, 1965; Kevan, 1975; Kudo, 1995).
67 Downward orientation of tubulous actinomorphic flowers likely limits pollinators to some
68 specialized groups such as humming birds and large bees, improving pollination efficiency
69 whereas upward-orientation facilitate nocturnal hawkmoth pollination in actinomorphic
70 species (Fulton and Hodges, 1999; Aizen, 2003; Campbell et al., 2016).

71 Meanwhile, pollinators are unlikely the primary selective agent driving the evolution
72 of floral angle but abiotic factors put more severe pressures in some zoophilous plants
73 (Haung et al., 2002; Tadey and Aizen, 2001; Wang et al., 2010; Lin and Forrest, 2019).
74 Downward flower orientation is thought to have evolved to avoid pollen damage and nectar
75 dilution by rainfall and exposure to solar radiation in actinomorphic flowers (Huang et al.,
76 2002; Tadey and Aizen, 2001; Wang et al., 2010; Lin and Forrest, 2019). In some generalist
77 actinomorphic flowers which are pollinated by a wider range of pollinator groups (two or
78 more functional groups), flower angle usually less influences pollinator composition and
79 visit frequency, so that the effects of rainfall might be a more important driving force
80 selecting downward orientation (Huang et al., 2002; Wang et al., 2010; Lin and Forrest,
81 2019).

82 The adaptive significances of horizontal orientation in actinomorphic flowers under
83 biotic and abiotic selection pressures have not been examined to date, although those in
84 zygomorphic flowers are relatively well studied. Generally, specialized zygomorphic
85 flowers exhibit higher pollination success when flowers face horizontally (Neal et al., 1998;
86 Ushimaru et al., 2009; Wang et al., 2014a; Armbruster and Muchhala, 2020). So far only a
87 single study examined the role of horizontal orientation in a generalist zygomorphic

88 species, in which horizontal oriented flowers achieved higher pollen transfer success than
89 upward and downward orientated flowers (Yu et al., 2020). In the same species, horizontal
90 flower orientation could reduce pollen damage by rainwater compared to upward orientated
91 flowers, but the anthers were always wet during rainfall irrespective of flower angle (Yu et
92 al., 2020). These together indicate that horizontal orientation have mainly evolved to attract
93 and control pollinators in zygomorphic flowers. Meanwhile, to the best of our knowledge,
94 functional roles of horizontal orientation in actinomorphic flowers with generalist systems
95 were still unknown. We may expect that pollinator-mediated selection on flower angle
96 would be less strong in generalist actinomorphic flowers.

97 Here, we report a case study on functional roles of horizontal orientation in
98 attraction and landing control of pollinators, pollen transfer and rainfall avoidance in
99 actinomorphic *Platycodon grandiflorus* whose flowers are visited by diverse insect groups
100 in the rainy season. We conducted a flower angle-change experiment to examine the effects
101 of flower angle on pollinator behaviors and pollination success. We also examined pollen
102 viability in water and sucrose solutions and precipitation during the flowering season in the
103 study site to investigate possibility of pollen damage by rainfall. Flowers of *P. grandiflorus*
104 are protandrous and male and female phases do not overlap temporally within each flower.
105 We therefore examined and discussed whether there were difference in the effects of flower
106 angle between male and female phases in this study.

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108

109

110 MATERIALS AND METHODS

111 Study species and site

112 The Japanese bellflower, *Platycodon grandiflorus* (Jacq.) A.DC. (Campanulaceae) is a
113 perennial herb species, which is currently listed as Vulnerable species in the Natural Red
114 list (Ministry of the Environment of Japan, 2020). This species usually grows on natural
115 and semi-natural grasslands in Japan, Korean Peninsula, China and east Russia. In Japan,
116 the species blooms during the rainy season from mid-June to mid-September. Blue cup-
117 shaped insect-pollinated flowers, which are usually open for 4–6 d, are self-compatible and
118 protandrous and require pollination for fruiting and seeding (Wei et al., 2006): male and
119 female phases did not overlap and lasted average 1.3 d (1–3 d, n = 170) and 2.9 d (2–5 d, n
120 =132), respectively (Fig. S1b, c). Both in male and female phases, flowers oriented nearly
121 horizontally: flower angles deviated 2.4° in average (-43–32°, n = 32) from the horizontal
122 direction (Fig. S2). In a male phase flower, the pistil produces pollen-bearing hairs, on
123 which secondary pollen presentation occurs soon after flower opening whereas female
124 phase starts with curling the stigmatic lobes like in other Campanulaceae species (Vranken
125 et al., 2014) More than 80% of pollen grains have viability just after flower opening and
126 pollen vigor quickly decreased to less than 30% three days later in cultivated plants (Wei et
127 al., 2006). An individual of this species has thick roots and usually does not spread clonally.

128 We examined a population of *P. grandiflorus* in the Sugadaira plateau, Nagano
129 Prefecture, Japan (36°32'12.9 N, 138°20'53.1 E) in August 2018–2019, 2021. The species
130 is distributed only on ski slope grasslands which are managed by annual mowing in early
131 September (Yaida et al., 2019). In the study site, we observed several pollinator groups

132 visiting *P. grandiflorus* flowers to forage pollen and nectar: large and medium sized bees
133 (LM bees, mostly *Megachile* bees and more infrequently *Bombus* and *Apis* bees), small-
134 sized bees (S bees, such as *Andrena* and Halictidae species), syrphid flies (*Episyrphus*
135 *balteatus*, *Syrphus torvus* and other syrphid flies), infrequently scolid wasps and butterflies
136 and skippers (e.g., *Ochlodes ochraceus*) and beetles (Fig. S3).

137

138 **Flower diameter change during opening**

139 We measured flower diameters of en face surface at male (ca. 0.5-1d after opening) and
140 female phases (over 3 d after opening) by a digital caliper (in mm) to examine the effects of
141 temporal change in petal lobe opening on pollinator attraction and rainfall avoidance.

142

143 **Effects of floral angle on pollinator behaviors**

144 We experimentally prepared three types of flowers that differed in terms of their floral
145 angle in 2018:

146 (1) Con: control flowers whose angle were not changed,

147 (2) Up: flowers whose faces were turned upright and

148 (3) Down: flowers whose faces were turned downward.

149 Angles of all experimental flowers were fixed by short wires during the anthesis (Fig. 1).

150 The same treatment was done for all opening flowers within the same individual: totally

151 413 (143 Con, 139 Up and 131 Down) flowers on 84 (31 Con, 28 Up and 25 Down)

152 individuals: four flowers on the same plant were examined at the maximum. The number of

153 opening flowers for each individual varied 1 to 3 and the average was 1.4 in the site. All the

154 experimental individuals and their flowers were marked and identified.

155 To test the effects of floral angle on pollinator behaviors, we observed pollinator
156 visits to experimental flowers. For each trial, we arbitrarily selected neighboring 3–10
157 experimental individuals including more than one flower types and observed pollinators to
158 them for 20-min observation. We conducted totally 41 observation trials (820 min
159 observation in total) during the daytime from 08:00 to 15:00 h on sunny days when
160 pollinators were active. Before each trial, we recorded sexual phase (male or female) of
161 each experimental flower. Most flowers were repeatedly observed in different timings
162 during the same sexual phase in the same day and/or on different dates and at different
163 sexual phases: the average number of observation times per flower was 3.36 (1–8) and 16
164 flowers were observed only once. Twenty-five and 56 flowers were observed in either male
165 or female phase, respectively whereas 42 flowers were observed in both male and female
166 phases. In all trials, we observed cumulatively 95 male Con, 87 male Up, 97 male Down,
167 48 female Con, 52 female Up and 34 female Down flowers.

168 During our observation, we recorded two types of pollinator behavior: approach
169 and landing behaviors. Approach occurred when pollinators found a flower and approached
170 it from the front. Landing was defined as a pollinator landing on any part of a flower after
171 approaching it. We further divided landings into the following two types (Ushimaru and
172 Hyodo, 2005): legitimate landing, a pollinator touched any parts of the pistil and stamens
173 during its landing; petal landing, a pollinator landed on any part of petals and foraged or
174 collected pollen without touching secondary presented pollen on the pistil or the stigma
175 lobes. We counted the number of each behavior on each experimental flower for each

176 observation trial.

177

178 **Effects of floral angle on pollen transfer success**

179 We estimated pollen removal by counting the number of pollen grains remaining on the
180 pistil hairs of 60 experimental flowers and that on each of newly opened, unvisited ten
181 flowers in 2019 (c.f.; Harder 1990; Ushimaru et al. 2006; Ushimaru et al. 2014; Katsuhara
182 et al. 2017; Ushimaru et al. 2021). We collected a single pistil from each of naturally-
183 pollinated 20 Con, 20 Up and 20 Down flowers (60 individuals) whose male phases were
184 almost finished by carefully checking the pistil conditions, such as stigmatic lobes status,
185 and stored separately. The pistil with secondary pollen presentation were collected from
186 each of ten unvisited flowers after their opening and was stored in 1.0 mL 99.9 % ethanol
187 as a single sample. We vortexed the sample well and estimated the number of pollen grains
188 per sample by counting the pollen numbers in three 10.0 μ L drops per sample under a
189 microscope. To quantify pollen removal from each experimental flower we calculated
190 pollen removal [(the mean estimated number of pollen grains in the newly opened flowers)
191 - (the estimated number of pollen grains remaining in each experimental flower)] as an
192 integer (Ushimaru et al., 2014; Ushimaru et al., 2021).

193 We also examined the effects of floral angle on pollen receipt by collecting the
194 pistil of a single flower from each of 20 Con, 20 Up and 20 Down individuals ca. 3 d after
195 the start of female phases in early August, 2019. These flowers were exposed to natural
196 pollinator visits from bud break for ca. 5 d. We dissected the stigma from a remaining part
197 of each pistil and stored it separately. We then counted the number of pollen grains

198 deposited on the stigma for each sampled flower under a microscope ($\times 40$) in the
199 laboratory.

200

201 **Experimental flowers in the rain and precipitation during the flowering season**

202 In 2019, though it was not quantitatively, we observed several male-phase and female-
203 phase experimental and intact flowers after rainfall to examine whether mating related parts
204 (anthers, pistil hairs and stigma) were wetted, soaked or not.

205 Additionally, to examine the effect of flower angle on rain susceptibility, 61
206 experimental flowers (10 male Con, 10 male Up, 10 male Down, 11 female Con, 10 female
207 Up, 10 female Down) were prepared and observed whether flower base, mating related
208 organs and pollen of these flowers were wet or soaked 24-h after rain in 16 August, 2021.

209 We obtained meteorological data during the last decade (2010–2019) from the
210 nearest AMEDAS (governmental weather observation system in Japan; 36°31'9 N,
211 138°19'5 E) station to examine precipitation (mm per day) during the flowering season (15
212 July–15 September) of *P. grandiflorus* in the study site.

213

214 **Pollen germination in water and sucrose solution**

215 To test how rainfall influence viability of pollen grains, we examined pollen germination
216 and burst in water or sucrose solutions according to the existing method (Dafni, 1992;
217 Huang et al., 2002). We collected pollen grains from 10 flowers just after opening and
218 preserved at ca. $-20\text{ }^{\circ}\text{C}$ in a freezer in our laboratory. We then placed pollen grains from
219 dehisced anthers of a single flower on slide glasses with 0, 5, 10, 15, and 20 % (g/g $\times 100$)

220 sucrose solutions: the 0% solution is just distilled water. For each glass, we examined the
221 fates of 45–235 (average 145.7) pollen grains and counted the number of germinated and
222 burst pollen grains after 24 h under a light microscope. This germination/burst test was
223 repeated ten times for each sucrose concentration.

224

225 **Analyses**

226 We first compared en face flower diameter between sexual phases using a generalized
227 linear mixed model (GLMM, gaussian errors and identity link function) in which the
228 diameter of each flower, sexual phase (male/female) and individual identities were the
229 response and explanatory variables and the random terms, respectively. We then examined
230 the effect of angle changes on pollinator behaviors using GLMMs with Poisson errors and
231 logarithmic link function. The number of approaches, legitimate landings or petal landings
232 per 20 min. per flower was the response variable. In all the models, we included treatment
233 (Con/Up/Down), sexual phase and their interaction as the explanatory variables, display
234 size (the number of flowers per individual) and observation time (start time, minutes from
235 08:00) as covariates and the individual identity as a random term. We also conducted the
236 same GLMM analyses for the two behavior of three major pollinator groups, separately.
237 Secondly, we compared pollen removal and receipt between Con and other (Up and Down)
238 flowers using generalized linear models (GLMs with negative binomial errors and
239 logarithmic link). The model incorporated treatment as the explanatory variable and pollen
240 removal or pollen receipt was the response variable. Thirdly, we examined difference in the
241 ratio of flowers whose flower base or mating related organs among experimental flowers

242 using a Fisher's exact test. We finally compared the ratios of germinated and burst pollen
243 grains (burst or germination) on using GLMMs (negative binomial error and logarithmic
244 link), in which each pollen number, the sucrose concentration category (0/5/10/15/20), and
245 total number of examined pollen grains were the response and explanatory variables, an
246 offset term, and individual identity as a random term respectively. All statistical analyses
247 were done using the software R (R Development Core Team 2015).

248

249 **RESULTS**

250 **Flower diameter change**

251 We found a significant increase in en face flower diameter from male (mean \pm SE, range;
252 5.0 ± 0.24 cm, 2.0–5.1 cm) to female (5.8 ± 0.49 cm, 2.0–7.3 cm) phases (Table S1),
253 indicating that petal lobes opened more and often bent backward in the female phase of
254 each flower. Note that the whole style was under the umbrella of upper petal lobe during
255 the male phase in the horizontally orientated flowers (Fig. 1a).

256

257 **Effects of floral angle on pollinator behaviors**

258 Most dominant pollinators to experimental flowers were LM bees (227 approaches, 56.7 %
259 of total approaches) and S bees (100 approaches, 24.9 %) and syrphid flies (74 approaches,
260 18.5 %) followed. It should be noted that the ratios of the three major pollinator groups did
261 not differ significantly among flower types and between sexual phases for legitimate
262 landings (Fig. S4b).

263 Approach frequency per flower by all pollinators did not differ significantly

264 between Con and other flower types whereas approaches to male-phase flowers were
265 significantly higher than those to female-phase flowers in all the flower types (Fig. 2a,
266 Table S1). Approaches per flower by all pollinators significantly decreased with increasing
267 display size (Table S1). Significant decreases in approach at female phase and with larger
268 display size were found in LM and S bees, respectively. Approaches significantly increased
269 with observation time in all pollinators and bee groups (Table S1).

270 Legitimate landings by all pollinators and bees were significantly fewer on Down
271 flowers than on controls whereas the variable did not differ between Con and Up flowers
272 (Fig. 2b, Table S1). The response variable for all pollinators and LM bees was significantly
273 lower in female phase flowers than in male-phase flowers (Fig. 2b, Table S1). Legitimate
274 landings increased with observation time in all pollinators and bees whereas the variable
275 decreased with increasing display size only in S bees (Table S1).

276 Petal landings by all pollinators and each pollinator group was not influenced by
277 any explanatory variables at all (Table S1). The interaction between flower angle and
278 sexual phase had no significant effects on any response variables in any pollinator groups
279 except for approaches by S bees, such that the variable significantly increased in female-
280 phase Up flowers than male-phase controls (Table S1).

281

282 **Effects of floral angle on pollen transfer success**

283 More than 95 percent of pollen grain were removed from pistil hairs in all the flower types
284 and significant higher pollen removal was found in Up and Down flowers than in controls
285 (Fig. 3a, Table S1). By contrast, the stigmas of Up and Down flowers received significantly

286 fewer pollen grains (ca. 45 and 30 grains in average, respectively) than those of Con
287 flowers which received more than 65 pollen grains in average (Fig. 3b, Table S1).

288

289 **Flowers in rainy conditions and precipitation during the flowering season**

290 When over 3.5 mm precipitation per day was observed, Up flowers accumulated rainfall
291 and the anthers and pistil hairs therein were soaked in the male phase whereas some parts of
292 the stigma lobes of Up flowers were wet or soaked in the female phase in 2019 (Fig. S5a,
293 b). In Down flowers, mating related parts were never wet or soaked, although the back of
294 petals were wet. The back of upper petals and the front of lower petal lobes were wet in
295 Con and intact flowers, but the mating-related organs were rarely wet: stigma surfaces were
296 very infrequently wet in female-phase flowers with very opened petal lobes.

297 The flower base was soaked or wet after 6.0 mm rain in many Up flowers in 16
298 August, 2021, although not much water remained in flower cups owing to stem oscillation
299 by the strong wind. We observed a single case in which the pistil of Up flower was broken
300 by the rain (Fig. S5c). The number of flowers whose pollen, flower base and/or mating
301 related organs were soaked or wet was significantly greater in Up flowers than in other
302 flower types (Fig.4a). In Con and Down flowers, lobes and backs of the petals were often
303 wet but mating related organs were never wet in the experiment (Fig. 4b).

304 In the last decade, ≥ 3.5 mm precipitations, which was enough to soak most part of
305 the pistil in Up flowers, were found for 10–21 (mean, 16.6) d during the flowering period
306 (Fig. S6). Moreover, heavy rain over 10 mm per hour were often observed in the study area.
307 More than 4.2 sequential days (the average longevity of a flower) without rainfall were

308 observed less than half of the flowering season in 2010–2019, indicating that the majority
309 of flowers experienced rainfall to some extent during their opening in the study area (Fig.
310 S6). The number of periods when more than 5 sequential days without rain were only 3
311 times and once in 2018 and 2019, respectively in the study areas during the flowering
312 period (Fig. S6).

313

314 **Pollen germination in water and sucrose solution**

315 On average, 56.3 % of pollen grains germinated in the 5–20 % sucrose solution whereas
316 only 0.04 % of grains germinated in distilled water (Fig. 5). The differences between
317 distilled water and sucrose solutions were significant (Table S1). Approximately 30 % of
318 pollen grains burst in distilled water. By contrast, only less than 0.007 % of pollen grains
319 burst in 5–20 % sucrose solutions and the percentages were significantly lower than that in
320 distilled water: especially no pollen burst was observed in 20 % sucrose solutions.

321

322 **DISCUSSION**

323 In this study, we examined functional roles of horizontal flower orientation with respect to
324 pollinator behavior control and rain protection in actinomorphic *P. grandiflorus*. Our field
325 experiments showed that upward oriented flowers likely suffered more from pollen and
326 pistil damages by rainfall and pollen limitation than controls whereas downward oriented
327 flowers received less visitations and pollen grains on the stigmas compared to control
328 flowers. Thus, horizontal flower orientation might have evolved under both pollinator- and
329 rain-mediated selections in actinomorphic *P. grandiflorus* flowers. We discussed our

330 findings more in detail below.

331

332 **Effects of floral angle on pollinator behaviors and pollen transfer**

333 Although all flower types experienced a similar number of approaches by all pollinator
334 groups, downward flower orientation reduced legitimate landings of bees when comparing
335 to horizontal and upward orientation in both male and female phases unlike our
336 expectation. Thus, floral orientation influenced pollinator landing behavior rather than
337 pollinator attraction. The results are concordant with the findings in zygomorphic
338 *Commelina communis* (Ushimaru and Hyodo 2005), but not with patterns in another
339 zygomorphic species, *Corydalis sheareri* whose upward-oriented flowers also had
340 pollinator limitation (Wang et al., 2014a) as well as in actinomorphic flowers of *Pulsatilla*
341 *cernua* (Haung et al., 2002), *Geranium refractum* (Wang et al. 2014b) and *Mertensia*
342 species (Lin et al., 2019) whose downward-oriented flowers did not suffer from pollinator
343 limitation. The discrepancies between the present and previous studies (Wang et al., 2014a,
344 b; Lin et al., 2019) might be owing to differences in pollination system, i.e., a set of floral
345 characteristics and pollinators. Bumble bees usually less discriminate downward flowers
346 with open or cup shapes but leafcutter and small-sized bees, syrphid flies and lepidopterans
347 do when approaching and/or landing (Haung et al., 2002; Ushimaru and Hyodo 2005; Wang
348 et al., 2014a, b; Lin et al., 2019; Yu et al., 2020). Moreover, long-tubed flowers were often
349 not preferred by diurnal pollinators when facing upright experimentally (Wang et al.,
350 2014a; Yu et al., 2020) whereas upright open or cup-shaped flowers were visited by various
351 groups of pollinators who usually used petals as landing platforms (Huang et al., 2002;

352 Ushimaru and Hyodo 2005; Wang et al. 2014b; Lin et al., 2019). Leafcutter bees, small-
353 sized bees and syrphid flies (the dominant pollinators in *P. grandiflorus*) were sometimes
354 observed to take longer time to find footholds on Down flowers than on other flower types,
355 although we did not quantify the time. The longer handling time might cause discrimination
356 of Down flowers by these pollinators.

357 Pollen removal per flower was significantly enhanced by upward and downward
358 flower orientations compared to horizontal one. Though the difference was not significant,
359 upright flowers experienced more legitimate landings than controls in the male phase, being
360 consistent with the pollen removal result. Meanwhile, pollinator-limited downward flowers
361 had higher pollen removal than controls did as well. We cannot explain this with our data
362 set, but accidental pollen loss owing to shaking flowers by pollinators and wind could be
363 responsible. Anyway, over 95 % of pollen grains were removed from all flower types, so
364 the difference in pollen removal was small (Fig. 3).

365 By contrast, flower angle change treatments largely reduced pollen receipt on the
366 stigma in *P. grandiflorus* like in the previous studies on horizontally-oriented zygomorphic
367 flowers (Ushimaru et al., 2009; Wang et al., 2014a). Pollinator limitation might cause lower
368 pollen receipt in downward flowers. Upward flowers received lower numbers of visits than
369 control flowers in the female phase though not significant (Fig. 2a), likely causing lower
370 pollen receipt. However, further clarification why upward flowers had lower female
371 pollination success would be required in future.

372 Sexual phase, display size and time in the day influenced pollinator visitation to *P.*
373 *grandiflorus* flowers. Female-phase flowers received significantly less approaches and

374 landings by larger-sized bees than male phase flowers, suggesting that these bees foraged
375 mainly pollen on flowers. Larger display sizes tended to decrease pollinator attraction per
376 flower, indicating that relatively large corolla of each flower enough attract pollinators and
377 that simultaneous opening of multiple flowers within an individual might be rather negative
378 for reproductive success of individual flowers. This may explain relatively small average
379 display size (1.3 flowers per plant) of this species. Bees increased their visitations to
380 flowers toward noon and afternoon but no such a pattern was observed for syrphid flies.
381 Daily active patterns are known to vary between bees and flies (Herrera, 1990; Rader et al.,
382 2013; Ushimaru et al., 2021). However, to elucidate factors explaining the difference, more
383 information on thermoregulatory ability, energy requirements and their interactions with
384 habitat environments for each group (or species) were needed (Herrera, 1990; Rader et al.,
385 2013)

386

387 **Role of horizontal flower orientation in rain avoidance**

388 It is well known that pendant and downward flowers have a function to protect pollen
389 grains from rainfall and sun radiation (Tadey and Aizen, 2001; Haung et al., 2002;
390 Haverkamp et al., 2019). In *P. grandiflorus*, many pollen grains burst and lost the
391 germination ability in water, indicating a significant negative effect of rainfall on the pollen
392 viability like in the other species (Tadey and Aizen, 2001; Huang et al., 2002; Mao and
393 Haung, 2009). The anthers, pistil hairs and stigma of control flowers were observed not to
394 be wet and soaked as in experimental downward flowers (Fig. 4, S5d, e, f, g). Interestingly,
395 in the male phase, petal lobes did not fully open compared to those in the female phase,

396 suggesting their function as an umbrella for pollen grains during the rain events. We
397 frequently observed intact flowers facing toward down-slope irrespective of slope direction
398 like in forest-floor flowers on the slopes (Ushimaru et al. 2006) and often opened toward
399 the sun direction in the study site, indicating no sun radiation preference and avoidance in
400 the species (A. Ushimaru, personal observation). Flowering phenology of *P. grandiflorus*
401 just overlaps rainy and typhoon seasons in Japan. Thus, horizontal orientation should have
402 rainfall avoidance function in their flowers.

403

404 **Conclusion**

405 In this study, our field experiment revealed that upward flowers cannot avoid damage from
406 rainfall during the flowering period whereas downward flowers suffered from pollinator
407 limitation in *P. grandiflorus*. Thus, horizontal flower orientation is suggested to evolve
408 under both biotic and abiotic agents in this species. The very recent study suggested a
409 similar adaptation in horizontally-orientated zygomorphic *Abelia* × *grandiflora* flowers (Yu
410 et al., 2020). However, in the species, the role of horizontal orientation in rain protection is
411 dubious because the anthers and stigmas are protuberant from the petals and always wet
412 under rainy conditions (Yu et al., 2020). Thus, our results firstly demonstrated that
413 horizontal orientation enhanced pollinator legitimate landings and female pollination
414 success as well as protect mating-related organs, especially the anthers and secondary
415 presented pollen grains in the male phase from rainfall damage in actinomorphic *P.*
416 *grandiflorus* flowers. Because the adaptive significances of horizontal flower orientation in
417 actinomorphic flowers with generalist pollination systems were examined only in this

418 species, more other species should be investigated to generalize our findings.

419

420 **ACKNOWLEDGEMENTS**

421 We thank Gaku Hirayama, Fuma Kawakami, Akinari Sato, and Airi Asada for their help in
422 field works and Koki R. Katsuhara for his suggestions on statistical analyses. We are
423 grateful to the Sugadaira-kogen Hare snow resort for allowing our fieldworks. This
424 research results were aided by a research fund (no. R1-3-17) to TN from Nagano Society
425 for the promotion of science and a Grant-in-Aid for Scientific Research Programs
426 (KAKENHI no. 19H03303) to AU from the Japan Society for the Promotion of Science.

427

428 **AUTHOR CONTRIBUTIONS**

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

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434 **References**

- 435 Aizen, M. A. 2003. Influences of animal pollination and seed dispersal on winter flowering
436 in a temperate mistletoe. *Ecology* 84: 2613–2627.
- 437 Armbruster, W. S., and N. Muchhala. 2020. Floral reorientation: the restoration of
438 pollination accuracy after accidents. *New Phytologist* 227: 232–243.
- 439 Campbell, D.R., A. Jürgens, and S. D. Johnson. 2016. Reproductive isolation between

- 440 *Zaluzianskya* species: the influence of volatiles and flower orientation on hawkmoth
441 foraging choices. *New Phytologist*, 210, 333–342.
- 442 Dafni, A. 1992. *Pollination ecology: a practical approach*. Oxford University Press, Oxford,
443 UK.
- 444 Darwin, C. 1862. On the Two Forms, or Dimorphic Condition, in the Species of *Primula*,
445 and on their remarkable Sexual Relations. *Botanical Journal of the Linnean Society*
446 6: 77–96.
- 447 Fenster, C. B., W. S. Armbruster, P. Wilson, M. R. Dudash, and J. D. Thomson. 2004.
448 Pollination Syndromes and Floral Specialization. *Annual Review of Ecology,*
449 *Evolution, and Systematics* 35: 375–403.
- 450 Fenster, C. B., W. S. Armbruster, and M. R. Dudash. 2009. Specialization of Flowers: Is
451 Floral Orientation an Overlooked First Step? *The New Phytologist* 183: 502–506.
- 452 Fulton, M., and S. A. Hodges. 1999. Floral isolation between *Aquilegia formosa* and
453 *Aquilegia pubescens*. *Proceedings of the Royal Society of London. Series B:*
454 *Biological Sciences* 266: 2247–2252.
- 455 Galen, C., and M. L. Stanton. 2003. Sunny-side up: flower heliotropism as a source of
456 parental environmental effects on pollen quality and performance in the snow
457 buttercup, *Ranunculus adoneus* (Ranunculaceae). *American Journal of Botany* 90:
458 724–729.
- 459 Grant, V., and K. A. Grant. 1965. *Flower pollination in the phlox family*.
460 Columbia University Press, New York, New York, USA.
- 461 Harder, L. D. 1990. *Pollen Removal by Bumble Bees and Its Implications for Pollen*

- 462 Dispersal. *Ecology* 71: 1110–1125.
- 463 Haverkamp, A., X. Li, B. S. Hansson, I. T. Baldwin, M. Knaden, and F. Yon. 2019. Flower
464 movement balances pollinator needs and pollen protection. *Ecology* 100: e02553.
- 465 Hocking, B., and C. D. Sharplin. 1965. Flower Basking by Arctic Insects. *Nature* 206: 215.
- 466 Herrera, C. M. 1990. Daily Patterns of Pollinator Activity, Differential Pollinating
467 Effectiveness, and Floral Resource Availability, in a Summer-Flowering
468 Mediterranean Shrub. *Oikos* 58: 277–288.
- 469 Huang, S.-Q., Y. Takahashi, and A. Dafni. 2002. Why does the flower stalk of *Pulsatilla*
470 *cernua* (Ranunculaceae) bend during anthesis? *American Journal of Botany* 89:
471 1599–1603.
- 472 Katsuhara, K. R., S. Kitamura, and A. Ushimaru. 2017. Functional significance of petals as
473 landing sites in fungus-gnat pollinated flowers of *Mitella pauciflora* (Saxifragaceae).
474 *Functional Ecology* 31: 1193–1200.
- 475 Kevan, P. G. 1975. Sun-Tracking Solar Furnaces in High Arctic Flowers: Significance for
476 Pollination and Insects. *Science* 189: 723–726.
- 477 Kudo, G. 1995. Ecological Significance of Flower Heliotropism in the Spring Ephemeral
478 *Adonis ramosa* (Ranunculaceae). *Oikos* 72: 14–20.
- 479 Lin, S.-Y., and J. R. K. Forrest. 2019. The function of floral orientation in bluebells:
480 interactions with pollinators and rain in two species of *Mertensia* (Boraginaceae).
481 *Journal of Plant Ecology* 12: 113–123.
- 482 Mao, Y.-Y., and S.-Q. Huang. 2009. Pollen resistance to water in 80 angiosperm species:
483 flower structures protect rain-susceptible pollen. *New Phytologist* 183: 892–899.

- 484 Neal, P. R., A. Dafni, and M. Giurfa. 1998. Floral symmetry and its role in plant-pollinator
485 systems: Terminology, Distribution, and Hypotheses. *Annual Review of Ecology and*
486 *Systematics* 29: 345–373.
- 487 Patiño, S., C. Jeffree, and J. Grace. 2002. The ecological role of orientation in tropical
488 convolvulaceous flowers. *Oecologia* 130: 373–379.
- 489 Rader, R., W. Edwards, D. A. Westcott, S. A. Cunningham, and B. G. Howlett. 2013.
490 Diurnal effectiveness of pollination by bees and flies in agricultural *Brassica rapa*:
491 Implications for ecosystem resilience. *Basic and Applied Ecology* 14: 20–27.
- 492 R Development Core Team. 2015. R: A Language and Environment for Statistical
493 Computing. R Foundation for Statistical Computing, Vienna.
- 494 Stebbins, G. L. 1970. Adaptive Radiation of Reproductive Characteristics in Angiosperms,
495 I: Pollination Mechanisms. *Annual Review of Ecology and Systematics* 1: 307–326.
- 496 Tadey, M., and M. A. Aizen. 2001. Why do flowers of a hummingbird-pollinated mistletoe
497 face down? *Functional Ecology* 15: 782–790.
- 498 Ushimaru, A., and F. Hyodo. 2005. Why do bilaterally symmetrical flowers orient
499 vertically? Flower orientation influences pollinator landing behaviour. *Evolutionary*
500 *Ecology Research* 7: 151–160.
- 501 Ushimaru, A., D. Kawase, and A. Imamura. 2006. Flowers adaptively face down-slope in
502 10 forest-floor herbs. *Functional Ecology* 20: 585–591.
- 503 Ushimaru, A., I. Dohzono, Y. Takami, and F. Hyodo. 2009. Flower orientation enhances
504 pollen transfer in bilaterally symmetrical flowers. *Oecologia* 160: 667–674.
- 505 Ushimaru, A., A. Kobayashi, and I. Dohzono. 2014. Does Urbanization Promote Floral

- 506 Diversification? Implications from Changes in Herkogamy with Pollinator
507 Availability in an Urban-Rural Area. *The American Naturalist* 184: 258–267.
- 508 Ushimaru, A., I. Rin, and K. R. Katsuhara. 2021. Covering and shading by neighbouring
509 plants diminish pollinator visits to and reproductive success of a forest edge-
510 specialist dwarf species. *Plant Biology* 23: 711–718.
- 511 Vranken, S., R. Brys, M. Hoffmann, and H. Jacquemyn. 2014. Secondary pollen
512 presentation and the temporal dynamics of stylar hair retraction and style elongation
513 in *Campanula trachelium* (Campanulaceae). *Plant Biology* 16: 669–676.
- 514 Wang, H., S. Tie, D. Yu, Y.-H. Guo, and C.-F. Yang. 2014a. Change of Floral Orientation
515 within an Inflorescence Affects Pollinator Behavior and Pollination Efficiency in a
516 Bee-Pollinated Plant, *Corydalis shearerii*. *PLOS ONE* 9: e95381.
- 517 Wang, H., C.-L. Xiao, R. W. Gituru, Z. Xiong, D. Yu, Y.-H. Guo, and C.-F. Yang. 2014b.
518 Change of floral orientation affects pollinator diversity and their relative importance
519 in an alpine plant with generalized pollination system, *Geranium refractum*
520 (Geraniaceae). *Plant Ecology* 215: 1211–1219.
- 521 Wang, Y., L.-H. Meng, Y.-P. Yang, and Y.-W. Duan. 2010. Change in floral orientation in
522 *Anisodus luridus* (Solanaceae) protects pollen grains and facilitates development of
523 fertilized ovules. *American Journal of Botany* 97: 1618–1624.
- 524 Wei J, Huang L, Chen S, Cheng H, Yang C, Chu Q. 2006 Study on the stigma/pollen vigor
525 and self-compatibility of *Platycodon grandiflorum*. *China Journal of Chinese Materia*
526 *Medica* 31, 366–8.
- 527 Willmer, P. 2011. *Pollination and Floral Ecology*. Princeton University Press.

- 528 Yaida, A. Y., T. Nagai, K. Oguro, K. Katsuhara, K. Uchida, T. Kenta, and A. Ushimaru.
529 2019. Ski runs as an alternative habitat for threatened grassland plant species in
530 Japan. *Palaearctic Grasslands*. 42: 16–22.
- 531 Yu, Y.-M., X.-X. Li, D. Xie, and H. Wang. 2020. Horizontal orientation of zygomorphic
532 flowers: significance for rain protection and pollen transfer. *Plant Biology*. 23: 156-
533 161
- 534

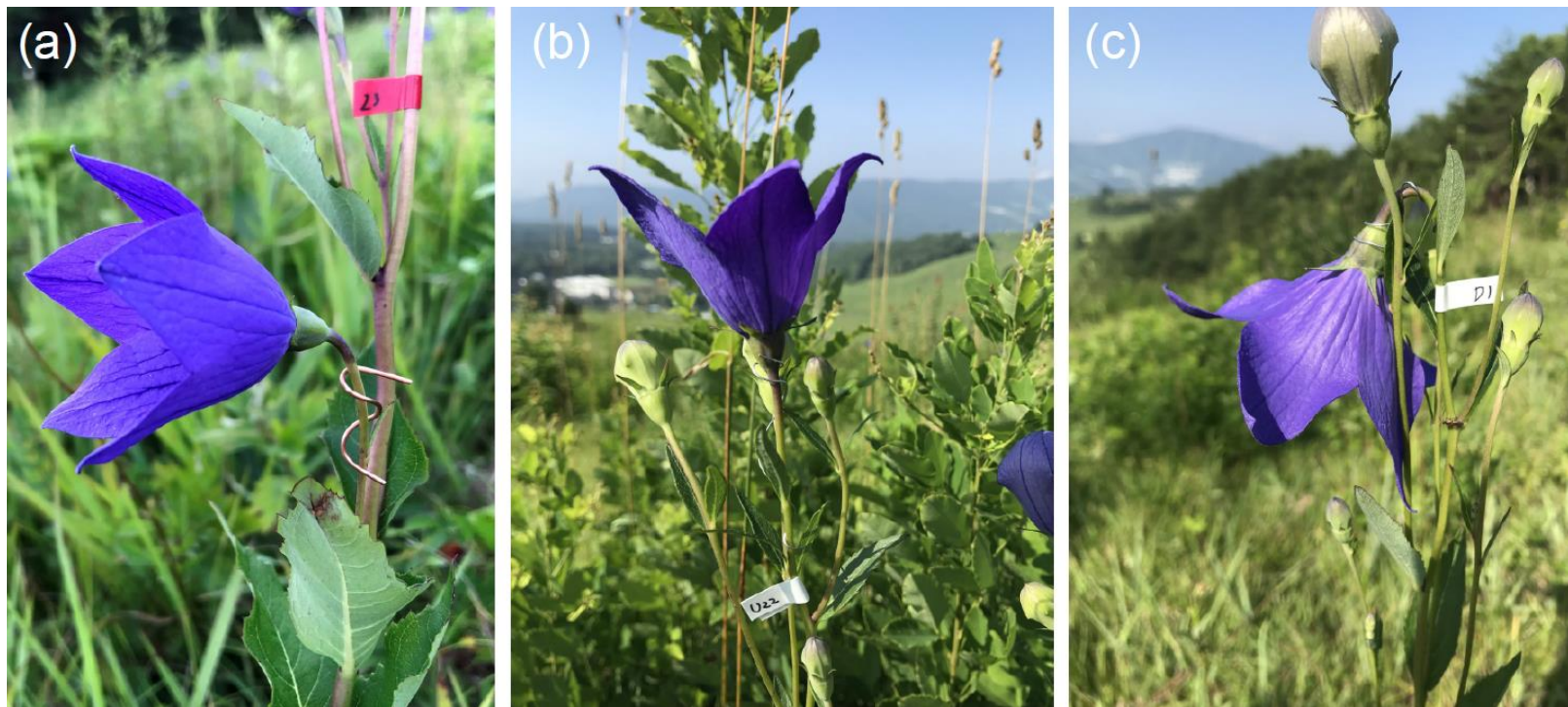


FIGURE 1 Photos of experimental flowers of *Platycodon grandiflorus*: control (Con, a), upward-facing (Up, b), and downward-facing (Down, c) flowers. Main floral axes of Con and manipulated (Up and Down) flowers were prepared nearly horizontal and vertical, respectively.

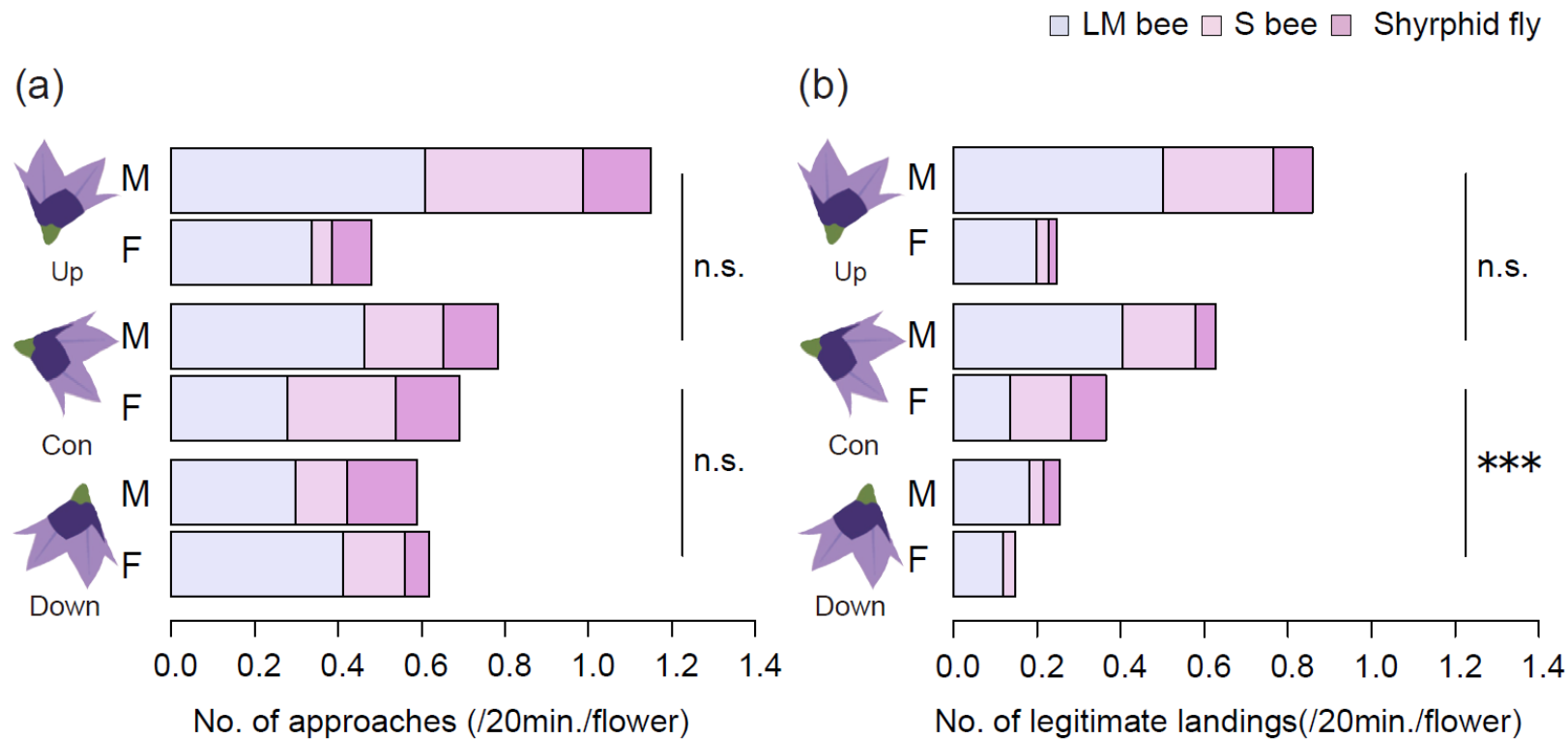


FIGURE 2 Mean numbers of approaches (a) and legitimate landings (b) by pollinators per 20 min per flower for the experimental (Con, Up, and Down) flowers of *Platycodon grandiflorus*. M and F show each sexual phase (M: male phase; F: female phase). ***, $P < 0.001$; n.s., $P > 0.1$, by GLMM.

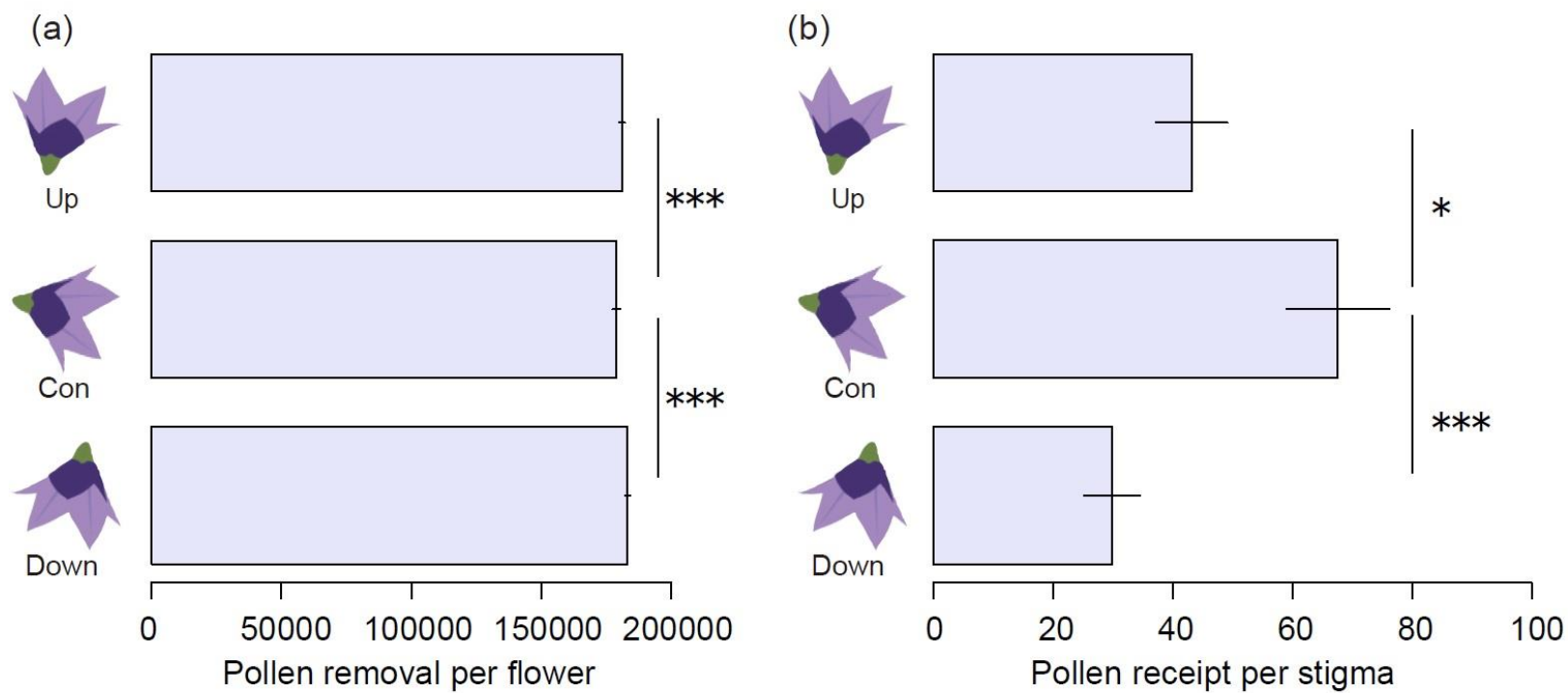


FIGURE 3 Mean number of remaining pollen grains on the pistil hairs (a) and deposited on the stigma (b) for the experimental (Con, Up, and Down) flowers. Bars show standard errors. ***, $P < 0.001$; *, $P < 0.05$ by GLM.

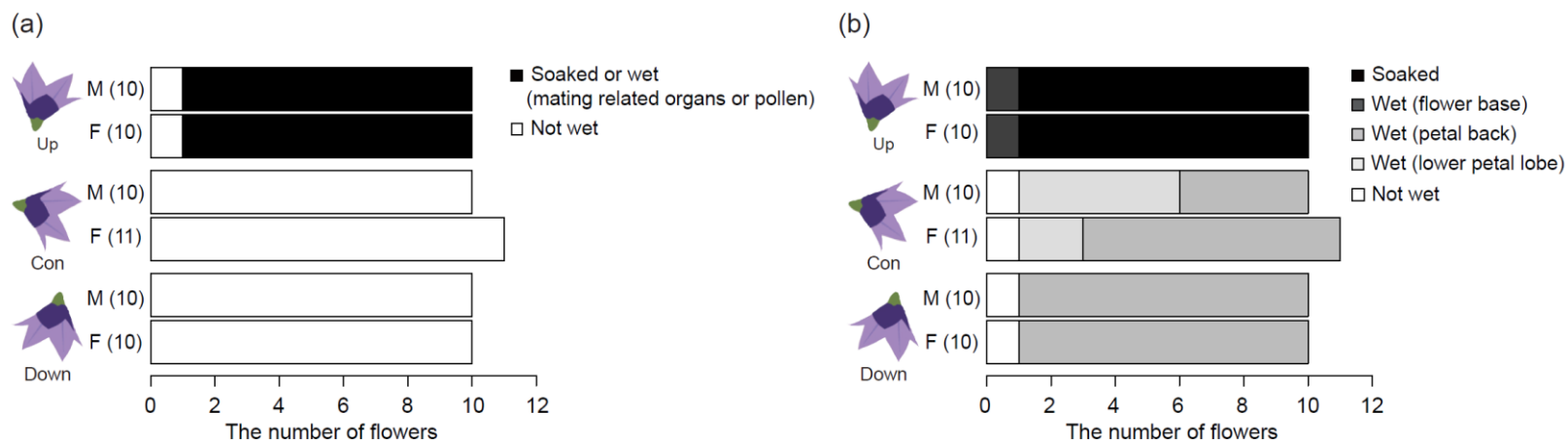


FIGURE 4. The effect of flower angle on rain susceptibility in experimental *Platycodon grandifloras* flowers. The ratios of flowers whose flower base (a) and mating related organs or pollen (b) were soaked or wet after ca. 6.0 mm rainfall differed significantly among flower types (a, b; Fisher's exact test, $p < 0.001$).

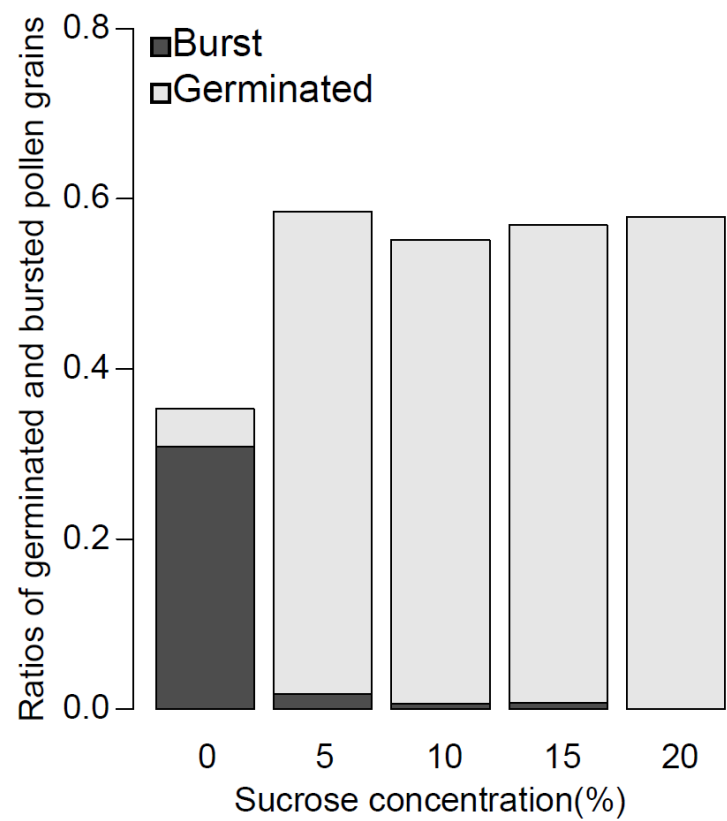


FIGURE 5 Ratios of germinated and burst pollen grains of *Platycodon grandifloras* in sucrose solution with various concentrations.