

1 **The functional roles of 3D heterostyly and floral visitors in the reproductive**
2 **biology of *Turnera subulata* (Turneroideae: Passifloraceae)**

3

4 Marina Teixeira Achkar^a, André Rodrigo Rech^b, Leonardo Ré Jorge^c, W. Scott
5 Armbruster^d, Odair José Garcia de Almeida^{a,*}

6 ^aPlant morphology lab, Biosciences Institute, Coastal campus, São Paulo State
7 University – UNESP;

8 ^bUniversidade Federal dos Vales do Jequitinhonha e Mucuri (UFVJM) –
9 Licenciatura em Educação do Campo.

10 ^cDepartamento de Biologia Animal, Universidade Estadual de Campinas
11 (UNICAMP)

12 ^dSchool of Biological Sciences, University of Portsmouth, Portsmouth, UK

13 *Corresponding author: Odair José Garcia de Almeida

14 E-mail address: odair.almeida@unesp.br

15 **Abstract**

16 Heterostyly is a floral polymorphism that reduces conflicts between sexual functions
17 (sexual interference), such as self-pollination in self-incompatible flowers and loss of
18 pollen to incompatible stigmas. In many distylous pollination systems, there is
19 remarkable structural fit between the sexual organs of flowers and the parts of the
20 pollinator body parts where compatible pollen is deposited. Nevertheless, inter-morph
21 pollen transfer is often asymmetrical, with short-styled flowers (S-morph) receiving less
22 compatible pollen than long-styled flowers (L-morph). One way to reduce the problem
23 of sexual interference in short-styled flowers is to place stigmas outside of the flower
24 centre, as seen in three-dimensionally (3D) heterostylous flowers. Heterostyly in
25 *Turnera* has been extensively studied; however, 3D heterostyly has not been previously
26 reported in this genus. The aims of this study were: 1) to elucidate the pollination
27 system of *T. subulata*; and 2) to assess whether 3D heterostyly promotes disassortative
28 pollination and reduces self-pollination in short-styled flowers in this taxon. The study
29 population of *Turnera subulata* exhibited an isoplethic (1:1) ratio, with two well-
30 defined morphs, and a high floral accuracy index for both morphs. Results from an
31 experiment in which we manipulated the 3D orientation of the style showed that
32 compatible crosses were favoured by the 3D flower morphology, consistent with a high
33 floral accuracy. Our results also demonstrate the functional importance of 3D
34 heterostyly in reducing intramorph pollination, as it increases the amount of cross pollen
35 reaching the S-morph flowers. Our results provide the first observation of the presence
36 of 3D heterostyly in this well-studied species and demonstrate the importance of this
37 kind of morphological specialization ensuring efficiency in an ecologically generalized
38 pollination system.

39 **Key-words:** Floral accuracy, L-morph flower, S-morph flower, Disassortative
40 pollination, Generalized pollination system

41 **1. Introduction**

42 The reciprocal correspondence of anther and stigma positions in the vertical
43 dimension (heterostyly) was the phenomenon that perhaps most amazed Darwin in his
44 studies of floral morphology and pollination (Darwin, 1905, p. 74). He discussed the
45 evolution and function of heterostyly in great detail and established that only inter-
46 morph pollinations produced viable seeds (Cohen, 2010; Darwin, 1877). Since then,
47 heterostyly has been shown to reduce sexual interference and self-pollination, and
48 promote disassortative (inter-morph) mating (Barrett et al., 2000; Barrett and Shore,
49 2008). Indeed, reciprocal differences in the position of reproductive organs between
50 morphs makes inter-morph cross-pollen deposition more likely than intra-morph
51 deposition, at least in some systems (Barrett et al., 2000; Keller et al., 2014; Wu et al.,
52 2018). Nevertheless, breakdown of intra-morph compatibility, has also been shown,
53 especially in self-compatible monomorphic populations colonizing new areas (Barrett
54 2002; Castro et al., 2013).

55 In many distylous pollination systems, there is remarkable structural fit between
56 the stigmas of each morph and the part of the pollinator body where compatible
57 (disassortative) pollen is deposited (Costa et al., 2017). However, inter-morph pollen
58 transfer may not always be “on target”, with short-styled flowers (S-morph) receiving
59 fewer compatible pollen grains than long-styled flowers (L-morph), the occurrence of
60 which is sometimes related to pollinator behaviour (Keller et al., 2014). Another
61 deviation from perfect disassortative pollen transfer happens when the deposition of
62 self-pollen precedes, and therefore limits or prevents, cross-pollination. In *Lythrum*
63 *salicaria* the S-morph had more interference from self-pollination than the other floral
64 morphs, compromising seed set of this floral morph (Waites and Ågren, 2006).

65 One peculiar variation of the classical distylous systems, happens when gynoecia
66 and androecia vary not only in height but also occur in reciprocal positions laterally
67 creating a three dimensional variation. Previously described 3D heterostyly involve
68 short-styled flowers with stigmas projected through the basal portion of the stamens,
69 and therefore projecting away from the centre, functionally creating a second whorl
70 (Armbruster et al., 2006). One expected advantage of the 3D heterostylous flowers is an
71 increased rate of compatible pollen receipt and a reduction in self-pollination, because
72 stigmas are no longer positioned right below the anthers (Armbruster et al., 2006). Even
73 though 3D heterostyly has been described (see also Turketti et al., 2012), to the best of
74 our knowledge there is no experimental evidence testing the hypothesis that the 3D
75 morphological inversion of the position of fertile organs increases the receipt of
76 compatible pollen or reduces the adherence of self-pollen to the stigmatic surfaces of
77 short-styled flowers.

78 Three-dimensional heterostyly was first described in *Linum suffruticosum* L., a
79 species that has positional reciprocity between L-morph and S-morph flowers in three
80 dimensions (Armbruster et al., 2006). The inter-morph reciprocity is achieved with both
81 anthers of long stamens (S-morph) and stigmas of long styles (L-morph) contacting the
82 dorsal region of the bodies of nectar-seeking pollinators, while the anthers of short
83 stamens (L-morph) and the stigmas of short styles (S-morph) contact their ventral
84 surface. Therefore, the three dimensional position of stigmas should insure pollen
85 deposition with the same or at least similar efficiency for both morphs of *L.*
86 *suffruticosum*. However, Armbruster et al. (2006) did not present experimental evidence
87 that 3D heterostyly effectively increases disassortative mating in *L. suffruticosum*.
88 Turketti et al. (2012) reported a complex case of three-dimensional reciprocity in
89 tristylous populations of *Oxalis* L. section *Sagittatae* in South Africa. This species is

90 self-compatible, making the system more reliant on reciprocity, and requiring consistent
91 and precise floral visitation by pollinators, to achieve out-crossing. No data on
92 pollination efficiency were presented, however.

93 Heterostyly in *Turnera* L. has been extensively studied (Baker and Shore, 1995;
94 Barrett, 1978, 1990; Barrett and Shore, 1987; Belaoussoff and Shore, 1995; Bentley,
95 1979; Medeiros and Schlindwein, 2003; Schlindwein and Medeiros, 2006; Shore, 1991;
96 Shore and Barrett, 1984, 1985, 1987; Swamy and Bahadur, 1984). However, 3D
97 heterostyly has not been reported previously in this genus. Baker and Shore (1995)
98 studied the pollination of *T. ulmifolia* L. and suggested pollination success was due to
99 adaptation to a diverse range of pollinators, i.e. due to it being an ecological generalist
100 in terms of pollination (Ollerton et al., 2007). The flowers of this species were reported
101 to attract a diverse group of insects that feed on pollen and/or nectar (Barrett, 1978). In
102 this study we focus on *Turnera subulata* Sm. [syn = *T. ulmifolia* var. *elegans* (Otto ex
103 Nees) Urb.]. Although this species also has an open floral morphology and wide
104 diversity of floral visitors (Barrett 1978, ARR unpublished observations), consistent
105 with it having an ecologically generalized pollination system, preliminary observations
106 suggested that the flowers were phenotypically specialized (sensu Ollerton et al., 2007),
107 exhibited 3D heterostyly,. We therefore addressed the following questions: 1) How
108 does the pollination system of *T. subulata* work? 2) How accurate are the 3D flowers of
109 *T. subulata* (cf. Armbruster et al., 2009, 2017)?; and, 3) Is 3D heterostyly able to
110 promote disassortative (compatible) pollination and, in the short-styled flowers of *T.*
111 *subulata*, to reduce self-pollen deposition?

112

113 **2. Materials and Methods**

114 *2.1. Study Species*

115 *Turnera subulata* was chosen as an experimental species for this study because of its
116 unusual floral morphology with presumed 3D heterostyly. It is a rhizomatous shrub with
117 heterostylous flowers whose corolla is made of yellow petals with lighter apices and a
118 purplish spot at the base; the androecium is composed of flattened-subulate stamens
119 with yellow filaments, which are tightly merged to form a staminal tube; the anthers are
120 orange with basi-dorsal filament insertion, bearing yellow pollen. The gynoecium
121 comprises an ovoid or conical tricarpellar, trichomatous ovary, three yellow styles
122 (measuring 6.5 - 9 mm in L-morph, and 3-5 mm in S-morph), and yellow stigmas. The
123 fruits are subglobose, and the seeds are claviform with unilateral aryls (Arbo, 2005;
124 Woodson-Jr. et al, 1967). The flowers of *T. subulata* have three separate styles, each
125 with a three-branched stigma oriented in a dimension perpendicular to the floral axis in
126 the S-morph flowers. The species is ruderal, occurring in clearings in woods and
127 disturbed areas, including roadsides and urban centres.

128

129 2.2. Study Population

130 Experiments and observations were performed with a population of more than 300
131 individuals of *T. subulata* in the city of Campinas, at the Ecological Park “Prof.
132 Hermógenes de Freitas Leitão Filho” (22°48’44” / 47°04’26”), in an area of semi-natural
133 vegetation (135,000 m²). The study population flowers year round, but with a flowering
134 peak from July to February. In December 2015, July 2016, and January 2017 we
135 collected data for three weeks, with daily observations from pre-anthesis to the end of
136 insect visitation or until flowers closed. For comparison of flower visitors and results of
137 incompatibility tests, we used data collected in January 2015 from a population in the
138 city of Presidente Figueiredo, Amazonas (02°73’17” / 60°01’29”).

139

140 2.3. *Isoplethy and Morphometric Analysis*

141 Members of both plant morphs were counted in the population to determine if
142 the population was isoplethic (equal morph frequencies). The nature of floral
143 heterostyly was evaluated morphometrically by measuring 40 flowers of each morph,
144 from which two petals had been removed so that the reproductive organs could be
145 photographed from a fixed distance. To avoid damage or change in functional distances,
146 measurements of stamens (height) and style (length) were taken from photographs using
147 a caliper-ruler as a scale to maintain floral functional dimensions. Afterwards,
148 measurements were recorded using the Image J software, and from these parameters the
149 floral reciprocal accuracy was calculated using the following formulas (Armbruster et
150 al., 2009; see also Armbruster et al. 2017):

151

152 Tall Organ Inaccuracy

153
$$TOI = (\overline{LS} - \overline{SP})^2 + V_{LS} + V_{SP}$$

154 Short Organs inaccuracy

155
$$SOI = (\overline{SS} - \overline{LP})^2 + V_{SS} + V_{LP}$$

156

157 Where LS means Mean Anther Height of L-morph; SP means Mean Stigma Height of
158 S-morph; SS means Mean Anther Height of S-morph; and LP means Mean Stigma
159 Height of L-morph. The V's represent the respective variances.

160 To compare the inaccuracy values proportionally to the organ size, the final values were
161 scaled according to the following formula:

162

163 Mean-Squared-Scaled Inaccuracy of Tall Organs

164
$$MSTOI = (TOI * 100 / ((LS + LP)/2)^2) * 100$$

165 Mean-Squared Inaccuracy of Short Organs

166 $MSSOI = (SOI * 100 / ((SS+ SP)/2)^2)*100$

167

168 *2.4. Frequencies and Efficiencies of Floral Visitors*

169 In order to measure the efficiency of each floral visitor as a potential pollinator
170 we used tests of single-visit pollen deposition onto stigmas of virgin flowers, based on
171 Freitas (2013). To ensure all flowers analyzed were virgin, floral buds were bagged,
172 preventing visitor interference. Flowers were individually uncovered, and one of the
173 three stigmas was immediately collected as an unvisited control group. The flower was
174 then observed until it received its first visit; immediately after the visit, another stigma
175 was collected and stored separately for pollen quantification. We counted pollen under a
176 light microscope, using slides coated with a medium made of gelatin-glycerin and basic
177 fuchsin to enhance contrast of the pollen grain relative to stigma tissue (Beattie, 1971).

178 To determine visitation frequency, we observed flowers for periods of 10
179 minutes per morph per day, for a total of 80 minutes for each morph (S-morph and L-
180 morph). Visitors were considered pollinators when the difference between the number
181 of pollen grains on the visited stigma and the virgin stigma was greater than zero (King
182 et al., 2013). To ensure that we correctly inferred the morph of the pollen-donor plant
183 we considered differences in pollen size. We tested and confirmed that pollen grains of
184 the L- and S-morphs differ in size (Mean L: 54.43 versus Mean S: 74.04; t: -8.9873 p<
185 0.0001 - see also Fig. 03 from Schlindwein and Medeiros 2006). Because Barrett (1978)
186 recorded that there may be some overlap in pollen size from S- and L-morph flowers,
187 we ignored pollen grains between 60 and 70 μm in size. Figure S.1 (in the
188 supplementary material), shows the range of sizes of pollen from S- and L-morph
189 flowers of our experimental species.

190

191 *2.5. Incompatibility Tests*

192 We determined the fruit-production rates for each possible cross within the
193 population: inter-morph (L-morph vs. S-morph), intra-morph (L-morph vs L-morph;
194 and S-morph vs. S-morph), and self-pollination. In total, we bagged 120 floral buds
195 from 76 L-morph and 77 S-morph plants. After anthesis started we made all crosses
196 manually, making sure stigmas were well covered with pollen. Pollen used for crosses
197 was a mixture from at least five different anthers (one per flower) from five different
198 individuals of the same morphs. We performed 40 inter-morph crosses (20 with S-
199 morph pollen deposited on L-morph stigmas and 20 with L-morph pollen deposited on
200 S-morph stigmas); 40 intra-morph crossings (20 with L-morph pollen deposited on L-
201 morph stigmas and 20 with S-morph pollen deposited on S-morph stigmas); and 40 self-
202 pollinations (20 S-morph and 20 L-morph flowers). Flowers were marked and identified
203 according to the crossing they were subjected to and then re-bagged until the end of
204 anthesis. After seven days, we evaluated the fruit set.

205 Along with incompatibility tests involving fruit production, we measured pollen-
206 tube growth after inter-morph, intra-morph, and self-pollinations. In order to check for
207 pollen-tube growth we run the same set of pollination tests using ten flowers in each
208 treatment. After the pollination test flowers were kept bagged for at least 12 hours. We
209 then collected carpels, fixed them in 50% FAA (Johansen, 1940), and observed the
210 extent of pollen-tube growth under a fluorescence microscope in the lab. For the pollen-
211 tube growth analysis, we stained the stigmas/styles with aniline-blue solution, as
212 suggested by Martin (1959), allowing the pollen grain and pollen tubes to fluorescence
213 under illumination with a Blue Glass (BG) filter.

214

215 2.6. *Three-Dimensional (3D) Heterostyly Experiment*

216 Thirty S-morph floral buds in upright positions were bagged until anthesis. When virgin
217 flowers were uncovered, we collected one of the stigmas and prepared it on a slide as
218 the control for pollen counts (hereafter called virgin). We manipulated a second stigma
219 (“experimental stigma”) to move its position to the centre of the flower (simulating
220 standard unidimensional reciprocity, with the gynoeceium in the centre), while the third
221 stigma (called “natural”) was kept in the original/natural position (3D) (Fig. 1). In order
222 to move one stigma to the flower centre (inside stamens ring) we used forceps to gently
223 press the style in between the filaments (Fig. 1 B). Since filaments are positioned very
224 close to each other at the base of the flower, the manipulated style remained in the
225 manipulated position by itself during the course of the experiment.

226 Flowers of *T. subulata* lasts for a single day, therefore after the visitation period
227 when flowers started to close, we collected the remaining two stigmas (the manipulated
228 and the unmanipulated 3D one) for pollen counts. Slides were prepared and pollen was
229 counted using the same method described in the previous section

230

231 2.7. *Data Analysis*

232 We used linear mixed models to evaluate the effect of pollen source (intra- vs. inter-
233 morph pollen transfer) on pollen deposition rates. To include the blocked design of the
234 experiments, in which stigmas from the same flower were used for different treatments,
235 individual flower was treated as a random variable in all models. Starting with a null
236 model using only the random factor and an intercept, we compared models with
237 different sets of fixed effects to test for a treatment effect (virgin flower vs. visited
238 flower; included in all models except the null), such as morph of the pollen donor,
239 morph of the pollen recipient, and the taxonomic group of the floral visitor. To compare

240 the models and to identify which best explains pollen deposition on stigmas, we used
241 the Akaike information criterion (AIC - Akaike, 1974; Burnham and Anderson, 2002),
242 treating models with AIC differences (delta-AICs) less than 2 as equally well-supported
243 (Burnham and Anderson, 2002).

244 We used linear and generalized mixed models to test if there were differences in
245 the total number and proportion of pollen grains from L-morph flowers deposited on the
246 stigma surface in S-morph flowers subjected to different treatments. The response
247 variables were 1) the total number of pollen grains from L-morph flowers deposited on
248 the stigma (legitimate pollen) and 2) the proportion of legitimate pollen relative to the
249 total. Total number was modelled as a linear variable with gaussian error distribution,
250 while the proportion as a logistic variable with a binomial error distribution. Predictor
251 variables were single fixed factors representing the three treatments used: virgin stigma,
252 3D stigma and manipulated stigma (positioned centrally). We used individual flower as
253 a random factor to account for the blocked design of our experiment. We again used the
254 AIC (Akaike, 1974; Burnham and Anderson, 2002) to determine whether this model fit
255 the data better than a null model with only the random factor. Subsequently, we used
256 multiple comparisons through one-way ANOVA, followed by a Tukey test to verify
257 whether the differences were significant between every pair of treatments. The analyses
258 were conducted in the R environment (R Development Core Team, 2006), using the
259 lme4 package for mixed models (Bates et al., 2015). TGgraphics were produced from
260 data analysis with R software (R Development Core Team, 2006) and GraphPad Prism
261 7.00, GraphPad Software, La Jolla California USA (www.graphpad.com).

262

263 **3. Results**

264 *3.1. Isopleth and Morphometric Analysis*

265

266 The sample population of *Turnera subulata* in Campinas comprised 76 L-morph
267 and 77 S-morph plants and was considered isoplethic (1:1 ratio). The morphometric
268 characterization of heterostyly exhibited two-well defined morphotypes ($p = 2.5024E-$
269 48 ; Fig. 3). The inaccuracy of reciprocal organs was 0.04 for tall organs, and 0.05 for
270 short organs, indicating a relatively high levels of reciprocal accuracy in both. Short
271 organs were more accurate when not scaled by mean-squaring, but tall organs were
272 proportionately more accurate (MSTOI 4.03% versus MSSOI 2.28%).

273

274 3.2. Frequencies and Efficiencies of Floral Visitors

275 Flowers were visited most frequently by the non-native bee species *Apis*
276 *mellifera*, followed by the native bees *Trigona* spp. in both locations where pollinator
277 frequencies were recorded. In Campinas, we recorded bees and, very infrequently, a bee
278 fly species (Phthiriinae) visiting the flowers (Fig. 2), while in Presidente Figueiredo
279 (Amazonas), Hesperidae butterflies were also recorded as visitors of the flowers. Bees
280 mostly visited a single flower per individual and collected nectar, although pollen
281 collection was also common. Bees frequently landed onto the stigma as they
282 approaching the L-morph flowers. To collect nectar, bees needed to crawl down towards
283 the flower base touching reproductive organs on the way. There was no difference in the
284 position (dorsal/ventral) of the pollen deposition onto the bee body. S-morph flowers
285 deposited pollen predominantly on the visitor abdomen while L-morph pollen were
286 concentrated on the bee head. Butterflies always collected nectar and mostly touched
287 only the tall organs.

288 Experiments evaluating the efficiency of floral visitors showed that the model
289 with donor morph only, and the model with the donor morph associated with the pollen

290 receiver morph were the most highly ranked and equally likely (Table 1, dAICc 0.2).
291 Since the donor-morph model is the simpler model, it was considered the more
292 appropriate to explain what influenced pollen deposition. Adding pollinators to the
293 model, either as an additive factor or as an interaction factor, did not improve the
294 explanatory power, suggesting that different pollinator species did not substantially
295 influence the amount of pollen transferred. S-morph flowers received more self-pollen
296 on their stigmas and more total pollen after a single visit than L-morph flowers (Fig. 4).
297 Additionally, pollen from S-morph flowers was more frequently found on the stigma
298 surfaces of both S and L-morph flowers (Fig. 4).

299

300 *3.3. Incompatibility Tests*

301 The cross pollination between different morphs (L-morph vs. S-morph) showed
302 high rates of fruit production (94,50%), the cross pollination within the same morphs,
303 but different flowers, (L-morph vs. L-morph and S-morph vs. S-morphs) did not set
304 fruit. Curiously the self-pollinated flowers had a non-zero (but very low – 22%) rate of
305 fruit production.

306

307 *3.4. Three-Dimensional (3D) Heterostyly Experiment*

308 The experiment involving stigma repositioning in the S-morph flowers (Fig. 5)
309 revealed an effect of the three treatment classes (virgin, natural and experiment). The
310 natural (control) treatment (original 3D arrangement) received the most pollen grains
311 ($P < 0.0001$). Additionally, the stigmas in 3D arrangement (natural in S-morph) received
312 more pollen grains from L-morph flowers, both in absolute terms and as a proportion
313 relative to S-morph pollen, compared to the manipulated stigmas (with the 3D
314 arrangement removed, Fig. 6).

315

316 **4. Discussion**

317 This study reports the third plant family with a case of 3D heterostyly in nature
318 and for the first time an important functional role of this mechanism in improving
319 disassortative mating (compatible pollination) and reducing self-pollination in S-morph
320 flowers of the isoplethic populations of *Turnera subulata*. The almost three-fold
321 increase in total number of compatible pollen grains deposited in the 3D arrangement
322 compared to the experimental flowers (116.4 vs. 43.5) is a good index of the gain in
323 reproductive fitness by this type of heterostyly.

324 Both tall and short organs presented a high level of reciprocal accuracy, and
325 two well-defined morphs exist (also recorded by Barrett, 1978 and Schlindwein and
326 Medeiros, 2006). The tall organs are proportionally more accurate (i.e. lower inaccuracy
327 in the L-morph stigma relative to the reciprocal (s-morph) anthers, and vice versa),
328 which is a common phenomenon (Armbruster et al. 2009). High reciprocal accuracy is
329 consistent with the results from the 3D cancellation experiment, which shows that
330 compatible crossings (disassortative) were favoured by natural 3D flower morphology.
331 High floral accuracy is not a rule for heterostyly, and some genera such as *Pulmonaria*
332 show extensive variation (up to 20%) in the scaled Inaccuracy Index (Jacquemyn et al.
333 2018). In fact, the existence of intra-morph pollination in non-3D-heterostylous species
334 has raised recurrent questions about the functional and evolutionary significance of
335 heterostyly (discussed in Wu et al. 2018).

336 Although 3D heterostylous flowers may be considered to be phenotypically
337 specialized, due to the elaborate system of reciprocal herkogamy, they also show
338 features which indicate ecologically generalised pollination (Ollerton et al., 2007).
339 Generalized pollination was indicated by the fact that several kinds of floral visitors

340 deposited similar amounts of pollen grains after a single visit. Although the population
341 at Campinas was visited almost entirely by bees, these bees had diverse morphologies
342 and behaviours, arguably belonging to several functional groups (Fenster et al., 2004).
343 In other 3D heterostylous species, *Linum suffruticosum*, pollen was consistently
344 deposited onto either the ventral or dorsal regions of visitors' bodies, therefore flower
345 phenotypic specialization resulted in a specialized use of pollinator body and behaviour
346 (Armbruster et al. 2006). Instead, *T. subulata* seems to rather resemble species of *Oxalis*
347 section *Sagittatae*, where the 3D phenotypic specialization result in a more evenly
348 distribution of pollen on the animal's body, taking advantage of a larger set of animal
349 sizes and behaviour for pollination (Turketti et al. 2012).

350 In line with a relatively generalist pollination system, butterflies of the
351 HesperIIDae family were recorded visiting flowers in the pilot study conducted in
352 Presidente Figueiredo (Amazonas). Furthermore, Barrett (1978) reported butterflies
353 visiting five varieties of *T. ulmifolia* [including *T. ulmifolia* var. *elegans*, synonym of *T.*
354 *subulata*] in Central America (Costa Rica, Nicaragua, Panamá) and South America
355 (Venezuela and Northern Brazil - Amazon region). Schlindwein and Medeiros, (2006)
356 also recorded visits by three species of HesperIIDae, besides one beetle and over 20
357 species of Hymenoptera (mostly bees) in a semi-natural vegetation of "Tabuleiro
358 Nordeste" in João Pessoa city (Northeastern Brazil). Therefore, it seems possible that
359 lepidopterans may also be effective pollinators, despite their absence in the Campinas
360 population. More research is required to fully understand the reproductive biology of *T.*
361 *subulata*, for instance in relation to the report by Medeiros and Schlindwein (2003) of
362 an intimate relationship between *T. subulata* and the oligolectic bee species
363 *Protomeliturga turnerae*, which appears both to mate in the flowers and to collect its
364 pollen.

365 The well recorded asymmetry in pollen transfer of heterostylous flowers was
366 corroborated in our study, with S-morph flowers exporting more pollen (see Keller et
367 al., 2014; Piper and Charlesworth, 1986; Schlindwein and Medeiros, 2006; Waites and
368 Ågren, 2006). The number of pollen grains of S-morph origin deposited on the stigmatic
369 surfaces was higher in both S-morph and L-morph flowers. This pattern makes sense
370 because S-morph flowers have longer and more exposed anthers, thus pollen is more
371 accessible to visitors, and pollen export is therefore likely more efficient (Piper and
372 Charlesworth, 1986). Our results are consistent with those of Swamy and Bahadur
373 (1984), who also recorded S-morph flowers as more efficient pollen donors in the
374 reproductive system of *T. subulata*.

375 Since we could differentiate morph origin by their pollen grain size, we were
376 able to evaluate whether the 3D arrangement enhances disassortative pollen deposition.
377 Indeed, we observed that legitimate pollen flow between morphs was favored by 3D
378 arrangement, as expected for distylous populations (Barrett et al., 2000; Sánchez et al.,
379 2013). Our experiment also revealed that stigmas with a 3D arrangement (S-morph)
380 received significantly higher amounts of pollen grains, and proportionally higher
381 amounts of compatible L-morph morph pollen grains when compared to stigmas with a
382 cancelled 3D arrangement. Therefore, the 3D arrangement increases the amount of
383 legitimate pollen grains received in relation to illegitimate pollen grains (disassortative
384 mating). Thus, 3D heterostyly in *T. subulata* may have evolved for the promotion of
385 legitimate pollen receipt on S-morph stigmas, a matter for future comparative studies
386 separating, for example, intrafloral and intramorph pollen deposition.

387

388 **5. Conclusions**

389 The export of pollen grains from S-morph flowers was significantly higher than export
390 from L-morph flowers, and the 3D arrangement of stigmas in S-morph flowers
391 increased the proportion of legitimate pollen-grains deposited. In addition, we conclude
392 that the identity of floral visitor species was not a significant factor in explaining
393 differences in pollen deposition. Furthermore, experiments revealed that 3D heterostyly
394 improves pollination by reducing reproductive interference from illegitimate crossings
395 and self-pollination, even in a pollination system that can be considered ecologically
396 generalized.

397

398 **Author Contributions**

399 MTA and ARR conceived the ideas and designed methodology;

400 MTA collected the data;

401 MTA, WSA, LRJ, and OJGA analyzed the data;

402 MTA, LRJ, WSA, ARR, and OJGA wrote the manuscript.

403 **Competing interests**

404 The authors declare no competing financial interests

405 **Acknowledgements**

406 We are extremely thankful for the Lab of Biosystematics and Pollination at the
407 University of Campinas (UNICAMP) for laboratory facilities support. We also thank
408 the “Fundação de Amparo à Pesquisa do Estado de São Paulo – FAPESP” for the
409 scholarship for MTA (Process 2015/26134-9).

410 **References**

- 411 Akaike, H., 1974. A new look at the statistical model identification. *IEEE Trans.*
412 *Autom. Control* 19, 716–723. <https://doi.org/10.1109/TAC.1974.1100705>
413 Arbo, M.M., 2005. *Estudios sistemáticos en Turnera* (Turneraceae). III. Series

414 *Anomalae y Turnera*. *Bonplandia*. 14, 115-318.

415 Armbruster, W.S., Hansen, T.F., Pélabon, C., Pérez-Barrales, R., Maad, J., 2009. The
416 adaptive accuracy of flowers: Measurement and microevolutionary patterns. *Ann.*
417 *Bot.* <https://doi.org/10.1093/aob/mcp095>

418 Armbruster, W.S., Pérez-Barrales, R., Arroyo, J., Edwards, M.E., Vargas, P., 2006.
419 Three-dimensional reciprocity of floral morphs in wild flax (*Linum suffruticosum*):
420 A new twist on heterostyly. *New Phytol.* 171, 581–590.
421 <https://doi.org/10.1111/j.1469-8137.2006.01749.x>

422 Armbruster, W.S., Bolstad, G.H., Hansen, T.F., Keller, B., Conti, H., Pélabon, C., 2017.
423 The measure and mismeasure of reciprocity in heterostylous flowers. *New Phytol.*
424 215, 906-917. doi: 10.1111/nph.14604

425 Bentley, L.B., 1979. Heterostily in *Turnera trioniflora*, a roadside weed of the Amazon
426 Basin. *Biotropica*. 11, 11-17. Doi: 10.2307/2388164

427 Baker, A.M., Shore, J.S., 1995. Pollen competition in *Turnera ulmifolia* (Turneraceae).
428 *Am. J. Bot.* 82, 717–725. <https://doi.org/10.2307/2445610>

429 Barrett, S.C.H., 1990. The evolution and adaptive significance of heterostyly. *Trends*
430 *Ecol. Evol.* 5, 144–148. [https://doi.org/10.1016/0169-5347\(90\)90220-8](https://doi.org/10.1016/0169-5347(90)90220-8)

431 Barrett, S.C.H., 1978. Heterostyly in a tropical weed: the reproductive biology of the
432 *Turnera ulmifolia* complex (Turneraceae). *Can. J. Bot.* 56, 1713–1725.
433 <https://doi.org/10.1139/b78-203>

434 Barrett, S.C.H., Jesson, L.K., Baker, A.M., 2000. The Evolution and Function of Styler
435 Polymorphisms in Flowering Plants. *Ann. Bot.* 85, 253–265.
436 <https://doi.org/10.1006/anbo.1999.1067>

437 Barrett, S.C.H., Shore, J.S., 2008. New Insights on Heterostyly: Comparative Biology,
438 Ecology and Genetics, in: Franklin-Tong, V. (Ed.), *Self-Incompatibility in*

439 Flowering Plants. Springer Berlin Heidelberg, Berlin, Heidelberg, pp. 3–32.
440 https://doi.org/10.1007/978-3-540-68486-2_1

441 Barrett, S.C.H., Shore, J.S., 1987. Variation and evolution of breeding systems in the
442 *Turnera ulmifolia* L. complex (Turneraceae). *Evolution* (N. Y). 41, 340–354.
443 <https://doi.org/10.2307/2409143>

444 Bates, D., Maechler, M., Bolker, B., Walker, S., 2015. Fitting Linear Mixed-Effects
445 Models using lme4. *J. Stat. Softw.* 67, 1–48. <https://doi.org/10.18637/jss.v067.i01>

446 Beattie, A.J., 1971. A technique for the study of insect-borne pollen. *Pan Pacific*
447 *Entomol.* 47, 82. <https://doi.org/10.1017/S0003598X00016689>

448 Belaoussoff, S., Shore, J.S., 1995. Floral correlates and fitness consequences of mating-
449 system variation in *Turnera ulmifolia*. *Evolution* (N. Y). 49, 545–556.
450 <https://doi.org/10.2307/2410278>

451 Burnham, K.P., Anderson, D.R., 2002. Model Selection and Multimodel Inference: A
452 Practical Information-Theoretic Approach, 2nd ed. Springer New York, New York,
453 NY. <https://doi.org/10.1007/b97636>

454 Castro, S., Ferrero, V., Costa, J., Sousa, A.J., Castro, M., Navarro, L., Loureiro, J.,
455 2013. Reproductive strategy of the invasive *Oxalis pes-caprae*: Distribution
456 patterns of floral morphs, ploidy levels and sexual reproduction. *Biol. Invasions*
457 15, 1863–1875. <https://doi.org/10.1007/s10530-013-0414-2>

458 Cohen, J.I., 2010. “A case to which no parallel exists”: The influence of Darwin’s
459 Different Forms of Flowers. *Am. J. Bot.* 97, 701–716.
460 <https://doi.org/10.3732/ajb.0900395>

461 Costa, J., Castro, S., Loureiro, J., Barrett, S.C.H., 2017. Experimental insights on
462 Darwin’s cross-promotion hypothesis in tristylous purple loosestrife (*Lythrum*
463 *salicaria*). *Am. J. Bot.* 104, 616–626. <https://doi.org/10.3732/ajb.1600408>

464 Darwin, C., 1877. The different forms of flowers on plants of the same species. Murray
465 J, London.

466 Darwin, F., 1905. The life and letters of Charles Darwin. Appleton, D., New York, NY.

467 Fenster, C.B., Armbruster, W.S., Wilson, P., Dudash, M.R., Thomson, J.D., 2004.
468 Pollination syndromes and floral specialization. *Annu. Rev. Ecol. Evol. Syst.*
469 <https://doi.org/10.1146/annurev.ecolsys.34.011802.132347>

470 Freitas, L., 2013. Concepts of pollinator performance: Is a simple approach necessary to
471 achieve a standardized terminology? *Rev. Bras. Bot.* 36, 3–8.
472 <https://doi.org/10.1007/s40415-013-0005-6>

473 Jacquemyn, H., Gielen, M., Brys, R. 2018. Is sexual organ reciprocity related to
474 legitimate pollen deposition in distylous *Pulmonaria* (Boraginaceae)? *Oikos*
475 127(8): 1216-1224. <https://doi.org/10.1111/oik.05122>

476 Johansen, D.A., 1940. Plant microtechnique. McGraw-Hill Book Company, inc., New
477 York.

478 Keller, B., Thomson, J.D., Conti, E., 2014. Heterostyly promotes disassortative
479 pollination and reduces sexual interference in Darwin's primroses: Evidence from
480 experimental studies. *Funct. Ecol.* 28, 1413–1425. [https://doi.org/10.1111/1365-](https://doi.org/10.1111/1365-2435.12274)
481 [2435.12274](https://doi.org/10.1111/1365-2435.12274)

482 King, C., Ballantyne, G., Willmer, P.G., 2013. Why flower visitation is a poor proxy for
483 pollination: Measuring single-visit pollen deposition, with implications for
484 pollination networks and conservation. *Methods Ecol. Evol.* 4, 811–818.
485 <https://doi.org/10.1111/2041-210X.12074>

486 Martin, F.W., 1959. Staining and observing pollen tubes in the style by means of
487 fluorescence. *Biotech. Histochem.* 34, 125–128.
488 <https://doi.org/10.3109/10520295909114663>

489 Medeiros, P.C.R., Schlindwein, C., 2003. Territórios de machos, acasalamento,
490 distribuição e relação com plantas em *Protomelitura trunerae* (Ducke, 1907)
491 (Hymenoptera, Andrenidae). Rev. Bras. Entomol. 47, 589-596.

492 Ollerton, J., Killick, A., Lamborn, E., Watts, S., Whiston, M., 2007. Multiple meanings
493 and modes: On the many ways to be a generalist flower, in: Taxon. pp. 717–728.
494 <https://doi.org/10.2307/25065856>

495 Piper, J., Charlesworth, B., 1986. The evolution of distyly in *Primula vulgaris*. Biol. J.
496 Linn. Soc. <https://doi.org/10.1111/j.1095-8312.1986.tb01827.x>

497 Sánchez, J.M., Ferrero, V., Navarro, L., 2013. Quantifying reciprocity in distylous and
498 tristylous plant populations. Plant Biol. 15, 616–620.
499 <https://doi.org/10.1111/j.1438-8677.2012.00720.x>

500 Schlindwen, C., Medeiros, P.C.R., 2006. Pollination in *Turnera subulata* (Turneraceae):
501 Unilateral reproductive dependence of the narrowly oligolectic bee *Protomelitura*
502 *turnerae* (Hymenoptera, Andrenidae). Flora. 201, 178-188.
503 [doi:10.1016/j.flora.2005.07.002](https://doi.org/10.1016/j.flora.2005.07.002)

504 Shore, J.S., 1991. Tetrasomie inheritance and isozyme variation in *Turnera ulmifolia*
505 vars. *elegans* Urb. and *intermedia* Urb. (Turneraceae). Heredity (Edinb). 66, 305–
506 312. <https://doi.org/10.1038/hdy.1991.39>

507 Shore, J.S., Barrett, S.C.H., 1987. Inheritance of floral and isozyme polymorphisms in
508 *Turnera ulmifolia* L. J. Hered. 78, 44–48.
509 <https://doi.org/10.1093/oxfordjournals.jhered.a110306>

510 Shore, J.S., Barrett, S.C.H., 1985. The genetics of distyly and homostyly in *Turnera*
511 *ulmifolia* l. (turneraceae). Heredity (Edinb). 55, 167–174.
512 <https://doi.org/10.1038/hdy.1985.88>

513 Shore, J.S., Barrett, S.C.H., 1984. The effect of pollination intensity and incompatible

514 pollen on seed set in *Turnera ulmifolia* (Turneraceae). *Can. J. Bot.* 62, 1298–1303.
515 <https://doi.org/10.1139/b84-175>

516 Swamy, N.R., Bahadur, B., 1984. Pollen flow in dimorphic *Turnera subulata*
517 (Turneraceae). *New Phytol.* 98,205-209. <https://www.jstor.org/stable/2433990>

518 Team, R.D.C., 2006. R: A language and environment for statistical computing.

519 Turketti, S.S., Esler, K.J., Dreyer, L.L., 2012. Three-dimensional reciprocity: A new
520 form of tristylly in South African *Oxalis* (Oxalidaceae) species and its implications
521 for reproduction. *South African J. Bot.* 78, 195–202.
522 <https://doi.org/10.1016/j.sajb.2011.06.014>

523 Waites, A.R., Ågren, J., 2006. Stigma receptivity and effects of prior self-pollination on
524 seed set in tristylous *Lythrum salicaria* (Lythraceae). *Am. J. Bot.* 93, 142–147.
525 <https://doi.org/10.3732/ajb.93.1.142>

526 Woodson-Jr., R.E., Scherv, R.W., Robyns, A., 1967. Flora of Panama. Part VI Family
527 Turneraceae. *Ann. Missouri Bot. Gard.* 54, 85–94.

528 Wu, L.Y., Chang, F.F., Liu, S.J., Armbruster, W.S., Huang, S.Q., 2018. Heterostyly
529 promotes compatible pollination in buckwheats: Comparisons of intraflower,
530 intraplant, and interplant pollen flow in distylous and homostylous *Fagopyrum*.
531 *Am. J. Bot.* <https://doi.org/10.1002/ajb2.1013>

532

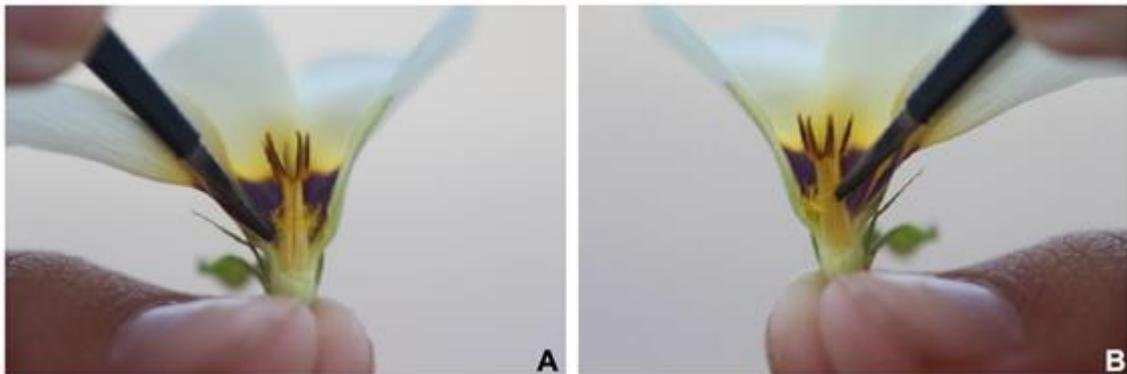
533

534 **Table 1.** Model selection evaluating the factors explaining pollen deposition in *T.*
 535 *subulata*. dAIC represents the delta AICs (difference between Akaike information
 536 criteria; Akaike, 1974) for sequential models. df represents the degrees of freedom for
 537 each model. “Donor” refers to donor morph, and “receiver” refers to recipient morph.

Model	dAIC	df
Pollen donor	0.0	5
Pollen donor + receiver	0.2	6
Pollen Receiver + flower visitor	6.4	17
All factors added	5.2	12
Pollen donor + flower visitor	16.7	18
Pollen receiver	15.2	5
Flower visitor	22.3	10
Null	45.1	3

538

539 **Figures**



540

541 **Fig. 1.** Cancellation of the 3D conformation, moving one of the stigmas to the central
542 position of the flower (among the stamens) of *Turnera subulata*. A. Natural 3D stigmas.
543 B. One stigma in natural 3D position and another one moved to the central part of the
544 flower (cancellation of the three-dimensionality of the stigma).



545

546 **Fig. 2.** Flowers visitors of *Turnera subulata* in the Campinas – São Paulo population.

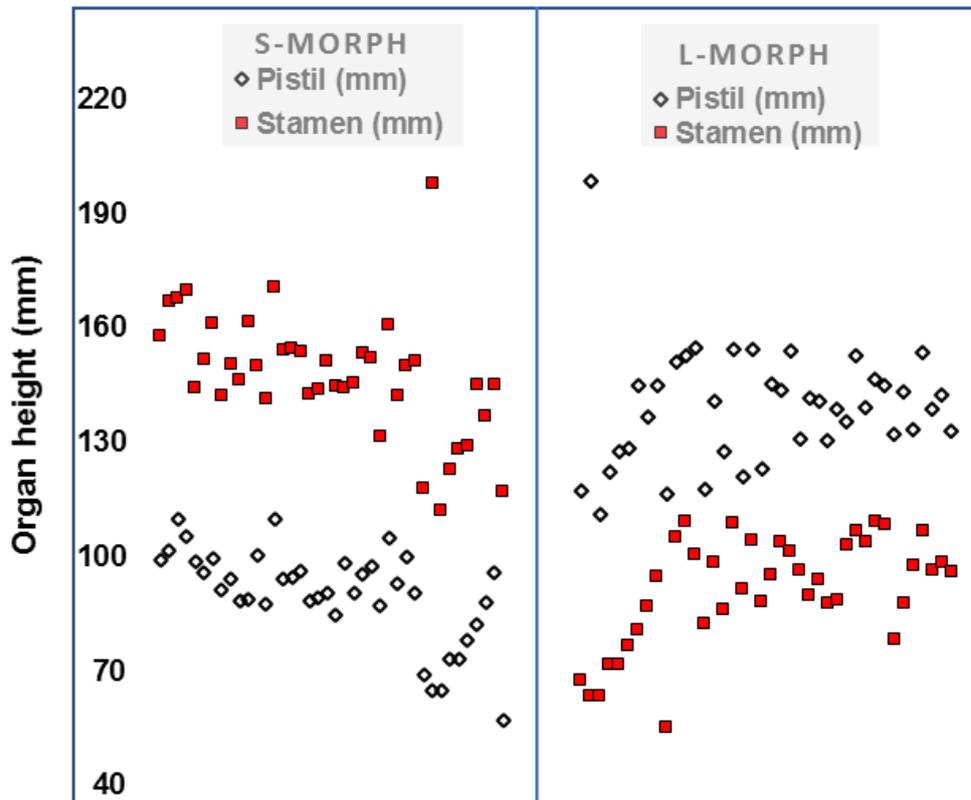
547 A) *Exomalopsis* sp. B) Phthiriinae sp. C) Halictidae sp. D) *Trigona spinipes*. E) *Apis*

548 *mellifera*. F) *Tetragonisca angustula*.

549

550

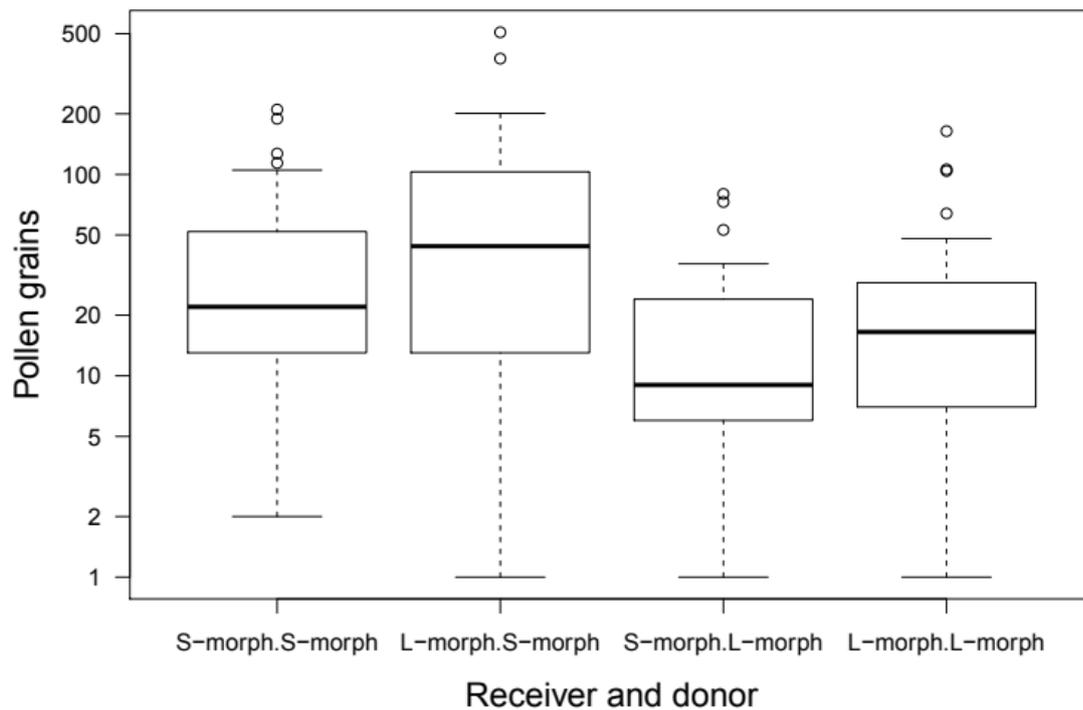
551



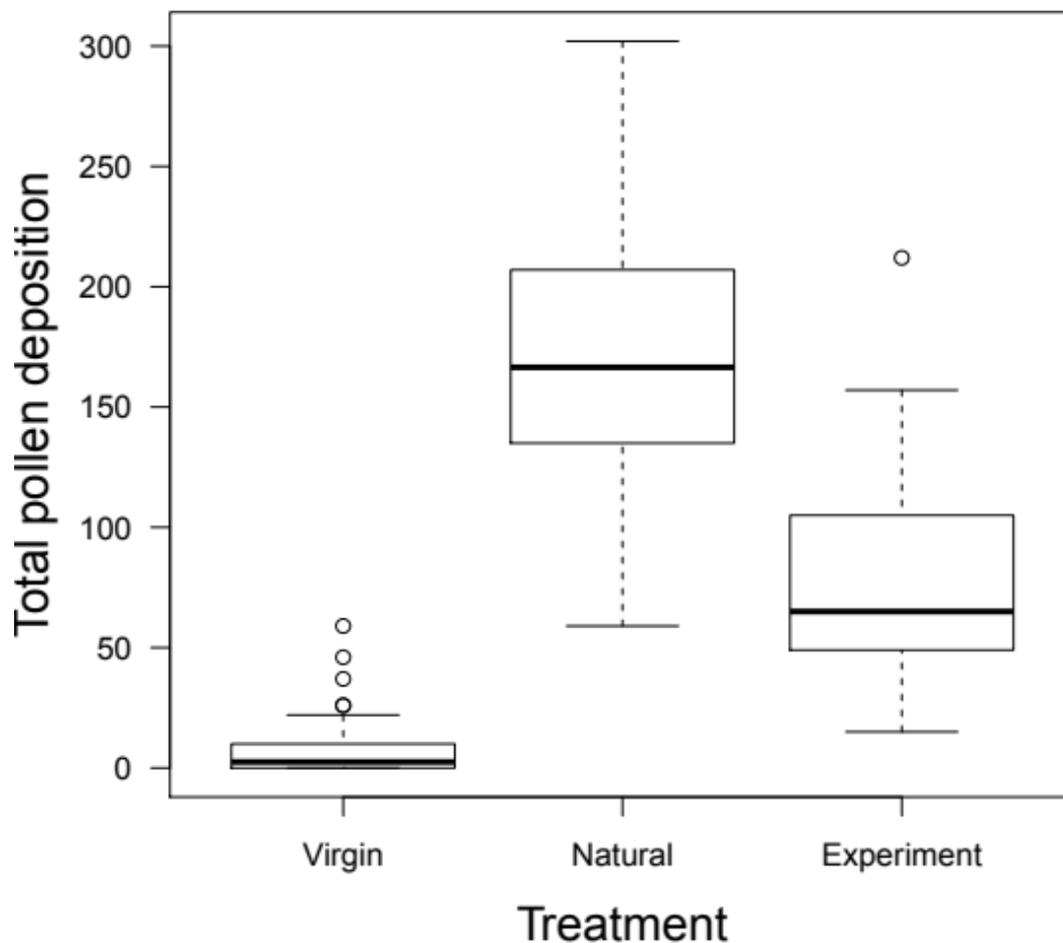
552

553 **Fig. 3.** Distyly in *Turnera subulata*. Range of variation in the pistil and stamen heights,
 554 in a total of 80 flowers (40 S-Morpho, and 40 L-Morpho). For each flower the length of
 555 the style (from the ovary base to the tip of the stigma), and the length of the stamen
 556 (from the ovary base to the tip of the anthers) was measured.

557



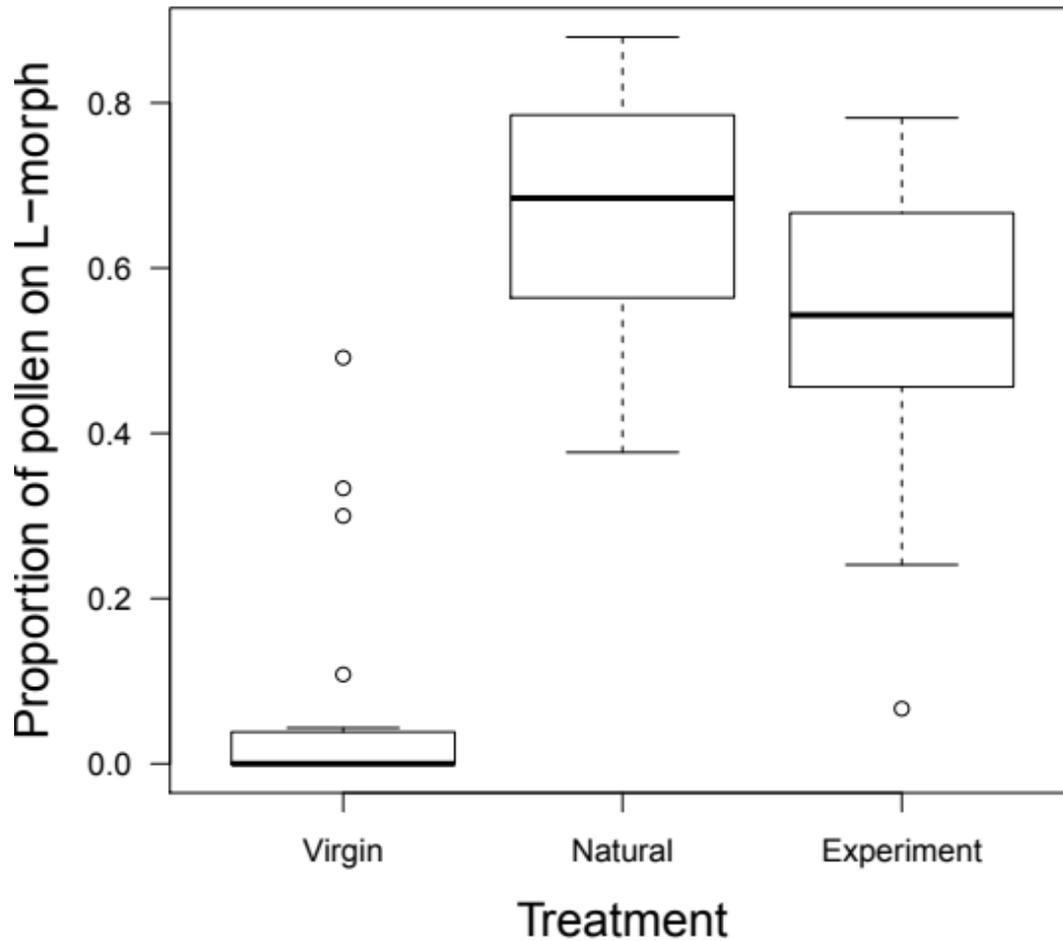
558 **Fig. 4.** Pollen-deposition on stigmas of *Turnera subulata* after a single visit by a
 559 pollinator, grouped according to the floral morph pollen donor and the floral morph
 560 pollen-receiver. The boxes represent median and 1st and 3rd quartiles, and the whiskers
 561 the extreme values up to 1.5 times the interquartile range. Values beyond this limit are
 562 represented as dots above whiskers.
 563



564

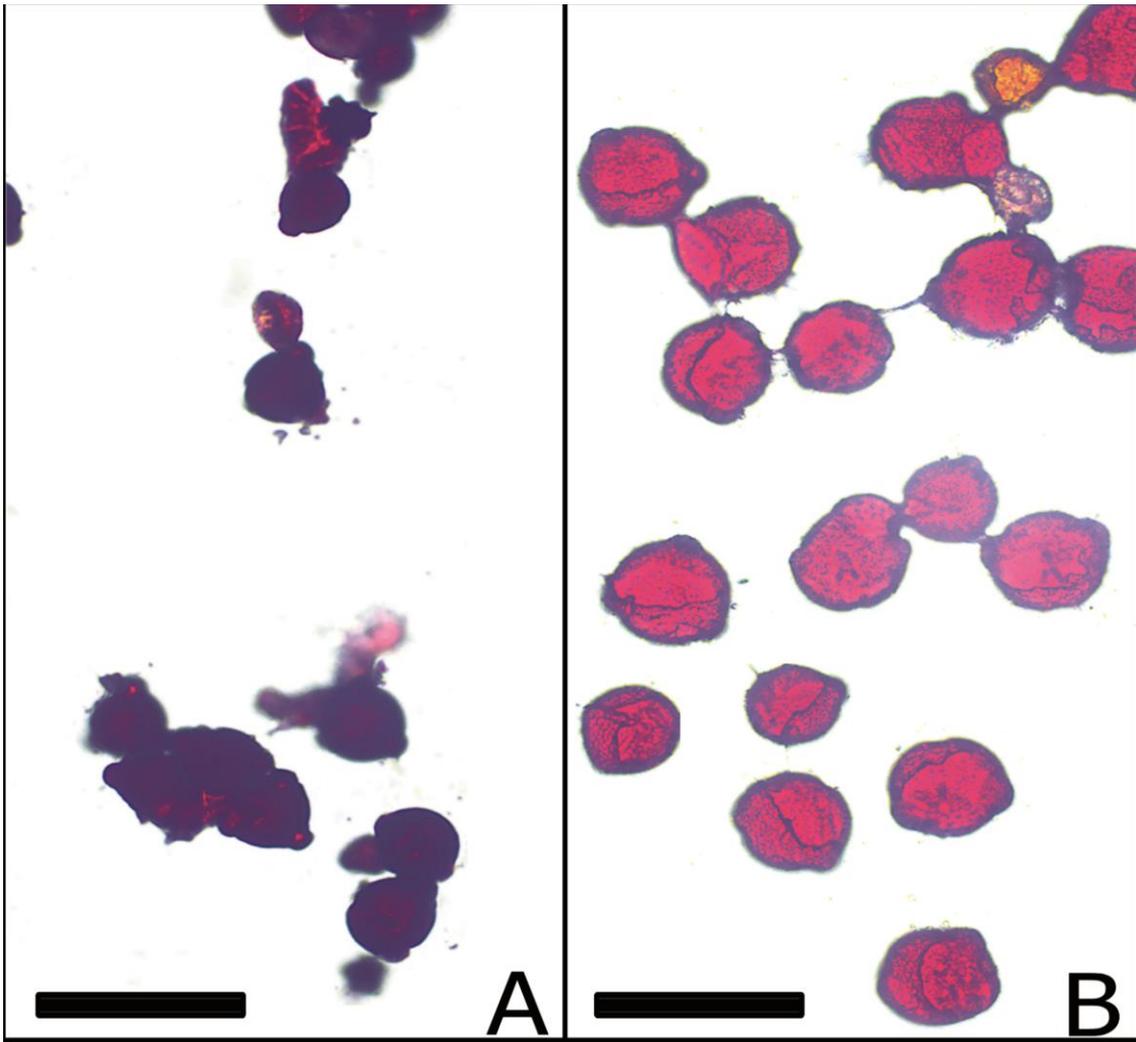
565 **Fig. 5.** Total pollen-deposition on the S-morph stigmas of *T. subulata*. Treatments
 566 tested were as follows: Virgin refers to stigmas collected before any visit of pollinators;
 567 Natural refers to stigmas in its original arrangement; Experiment refers to manipulated
 568 stigmas for which the 3D position was cancelled. Natural and experimental stigmas
 569 were collected at the end of anthesis. The boxes represent median and 1st and 3rd
 570 quartiles, and the whiskers the extreme values up to 1.5 times the interquartile range.
 571 Values above this limit are represented as dots above whiskers (post-hoc comparisons,
 572 all $p < 0.001$).

573



575

576 **Fig. 6.** Proportion of pollen-grains received from L-morph flowers in relation to all the
 577 pollen-grains deposited on the stigma of the S-morph flowers during the 3D
 578 cancellation experiment. The boxes represent median and 1st and 3rd quartiles, and the
 579 whiskers the extreme values up to 1.5 times the interquartile range. Values above this
 580 limit are represented as dots above whiskers (post-hoc comparisons, all $p < 0.001$).



582

583 **Fig. S1.** Range of sizes of pollen grains from S- and L-morph flowers of *T. subulata*. A.

584 Pollen grains from a L-morph flower. B. Pollen grains from a S-morph flower. Scale

585 bars = 100 μ m.

586