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1 **Changes in floral biology and inbreeding depression in native and invaded regions of**
2 *Datura stramonium*

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23 **ABSTRACT**

- 24 • Plant populations invading new environments might compromise their fitness
25 contribution to the next generation, owing to the lack of native specialist pollinators
26 and/or potential mates. Thus, changes in plant mating system and traits linked to it
27 are expected in populations colonising new environments where selection would
28 favour selfing and floral traits that maximise reproductive output. To test this, we
29 studied native (Mexico) and non-native (Spain) populations of the obligate sex
30 reproducing annual weed *Datura stramonium*.
- 31 • Flower size, herkogamy, total number of seeds per plant, number of visits by and type
32 of pollinators, and inbreeding depression, were assessed in four native and five non-
33 native populations. Finally, we measured phenotypic selection on corolla size and
34 herkogamy in each population.
- 35 • Flower size and herkogamy showed wide and similar variation in both ranges.
36 However, the largest flower size was found in one non-native population whereas the
37 highest herkogamy was detected in one native population. On average, flowers in the
38 native range received more visits. Hawkmoths were the main visitors in the native
39 populations while only bees were observed visiting flowers in Spain. Only in the
40 native range inbreeding depression was detected. Selection to reduce herkogamy was
41 found only in one native population.
- 42 • Absence of inbreeding depression and selection on floral traits suggest a change in
43 mating system of *D. stramonium* in a new range and generalist pollinators may
44 promote high reproductive success in non-native range. Selection against deleterious

45 alleles may explain the reduction of inbreeding depression, promoting the evolution
46 of selfing, which also would facilitate invasion.

47

48 **Keywords** herkogamy; inbreeding depression; mating system; Mediterranean; phenotypic
49 natural selection; pollinator; reproductive assurance; selfing syndrome; invasive weeds.

50

51 INTRODUCTION

52 In flowering plants, long distance dispersal could result in colonisation of novel
53 environments, following the establishment and range expansion (Pannell 2015). This
54 process, which generally involves single or few individuals, largely depends on the genetic
55 constitution of colonisers and on their effective reproduction in the novel environment
56 (Baker 1955; Barrett *et al.* 2008).

57 After dispersal, populations in the new range are subject to severe reductions in
58 population size associated to founder events, bottlenecks and genetic drift. These processes
59 have detrimental effects on introduced plant populations, reducing their genetic diversity
60 and outcrossing rates, increasing homozygosity within population, and limiting the adaptive
61 evolution of traits under new ecological conditions (Charlesworth 2003; Dlugosch & Parker
62 2008; Charlesworth *et al.* 2009; Eckert *et al.* 2009). However, despite negative effects
63 expected by the reduction of genetic diversity, some introduced species perform
64 successfully in the new distribution ranges (*i.e.* “the paradox in invasion biology”; Sax &
65 Brown 2000), although not all species become invasive in a new environment.

66 In order to understand the invasion paradox it is necessary to analyse the changes
67 brought about by the factors of the new environments and the target characters related to
68 the ability to colonise; for instance, increased growth rate, competitive ability or changes in
69 the mating and/or reproductive systems (van Kleunen *et al.* 2015). Particularly, in animal-
70 pollinated flowering plants, a main limiting barrier for successful reproduction during the
71 colonisation process is the scarcity or lack of potential mates as well as the absence of
72 effective pollinators in the new environment (Moodley *et al.* 2016). Baker (1955) noted that
73 compatible species capable to self-fertilize could be more successful colonisers than self-
74 incompatible ones; this is the so-called Baker’s law (Stebbins 1957). He suggested that

75 during dispersal and establishment phases, colonising populations are small and would
76 suffer a reduction in the services of their native pollinators. Contrary to obligated out-
77 crossers (i.e. self-incompatibility systems, SI), self-compatible species can maximize their
78 fecundity through self-fertilization. Hence, uni-parental reproduction can be advantageous,
79 enabling demographic growth to alien populations (Baker 1955; Barrett *et al.* 2008;
80 Richardson & Pyšek 2012; Barrett 2015). Empirical evidence supports Baker's law,
81 pointing that self-compatible species are more prone to be invasive than self-incompatible
82 ones (Pannell 2015).

83 Following Baker's ideas, under absence of mates or pollen limitation scenarios,
84 selfing can confer "reproductive assurance" to colonising plants, and hence, selected over
85 out-crossing. The "reproductive assurance" (Schoen & Lloyd 1992) and the "automatic
86 advantage of selfing" (Fisher 1941) are two main hypotheses to explain the evolution of
87 selfing in plant populations. Further, since selfing increases homozygosity, it may promote
88 the maintenance of superior homozygotes genotypes to particular combinations of
89 environmental conditions (Pannell 2015). However, the selfing evolution may depend on
90 other factors like behaviour and quality of pollinators (Moeller & Geber 2005; Karron *et al.*
91 2012; Rodger *et al.* 2013; Pannell 2015), and on the magnitude of the inbreeding
92 depression (Lande & Schemske 1985).

93 Inbreeding depression (ID), the reduction of fitness of offspring derived from
94 selfing relative to those derived from out-crossing due to genetic load and/or
95 overdominance in loci linked to fitness, is considered a main selective limit for the
96 evolution of selfing (Lande & Schemske 1985). However, selection against homozygote
97 individuals along generations is expected to reduce genetic load and hence ID (genetic
98 purge) (Lande & Schemske 1985; Yahara 1992; Dart & Eckert 2013). In highly selfing

99 populations, a negative correlation between selfing rate and ID is expected (Lande &
100 Schemske 1985). During the colonisation event, the dynamics of ID may initially
101 exacerbate the negative effects associated to bottlenecks, founder events and genetic drift,
102 reducing the genetic and phenotypic variation, compromising the potential adaptive
103 response to the new environment.

104 The evolution of self-fertilization is generally accompanied by correlated changes in
105 flower features (i.e. “selfing syndrome”) (Lloyd 1979). Flowers traits can be modified by
106 different ways: by selection on characters related to self-fertilization, like herkogamy
107 (Takebayashi *et al.* 2006; Bobdyl-Roels & Kelly 2011), dichogamy (Webb & Lloyd 1986),
108 or by differential selection on resource allocation to female, male, and attractiveness
109 functions (e.g., flower size and display) (Cruden 1977; Lloyd 1987; Charlesworth &
110 Morgan 1991). This may occur in new environments where pollination by generalists could
111 relax prior selection on flower traits exerted by the native pollinators, or where different
112 abiotic conditions impose severe limitations for pollinators (Memmott & Waser 2002;
113 Goulson & Hanley 2004; Morales & Aizen 2006). However, changes in flower traits also
114 can occur by pleiotropic effects of genes associated directly to the size of flower organs
115 when selfing increases, as it has been demonstrated for petal size during the evolution of
116 selfing in *Capsella* (Sicard *et al.* 2016). Besides changes in floral design, floral display can
117 evolve intimately linked to mating system; large floral displays can incur a cost by reducing
118 the opportunities for outcrossing (Barrett and Harder 1996; Harder and Barrett 1995).

119 In this study with *Datura stramonium* L., an annual plant native to North America
120 (Symon & Haegi 1991), we aim to determine whether changes in floral traits and
121 inbreeding depression associated to plant mating system have occurred during the
122 colonisation of a new and different environment (the Mediterranean-climate areas in Spain

123 which the species encountered early in its Old World spread; Sanz Elorza *et al.* 2004).
124 Many Mediterranean plants behave as aggressive weeds in other parts of the world, while
125 the Mediterranean region is less prone to be invaded, especially on mainland (Hulme *et al.*
126 2008; Arianoutsou *et al.* 2013). However, there are some paradigmatic cases of aliens that
127 in some cases became noxious weeds in the Mediterranean, despite they came from native
128 regions of contrasted environmental conditions, as it occurs with *D. stramonium*.
129 Specifically, we assess if populations from the native and non-native ranges differ in (i)
130 flower size and herkogamy; (ii) the number of visits by pollinators to flowers and the type
131 of pollinators; (iii) the direction and intensity of selection on flower size and herkogamy;
132 and (iv) the magnitude of inbreeding depression, related to the amount of genetic variation
133 of populations. We hypothesize that native populations will show higher corolla size,
134 positive or approach herkogamy (stigma protruding anthers), and more phenotypic
135 variation on these characters than non-native populations. On the other hand, in the native
136 range, we expect higher visitation to flowers by specialized pollinator. Further, a reduced
137 herkogamy and flower size due to selection is expected in populations in the new range
138 (Ollerton *et al.* 2012). Finally, we expect higher genetic variation in the native populations
139 than in the new range and thus higher inbreeding depression in the former.

140

141 **MATERIALS AND METHODS**

142 **Study species**

143 *Datura stramonium* (Solanaceae) is an annual weed native from North America (Mexico
144 and southern United States) (Weaver & Warwick 1984). In Spain it was introduced after
145 the Conquest of Mexico at least since 1540-1577 (Sanz Elorza *et al.* 2004), probably
146 through the harbours in Spain, which had an intensive trade with America. *Datura*

147 *stramonium* is self-compatible and its flowers are fragrant and produce nectar associated to
148 its native pollinators, hawkmoths (*Manduca sexta*, *M. quinquemaculata* and *Hyles lineata*;
149 Grant 1983), although it is also visited by honeybees (*Apis mellifera*; Sharma 1972). The
150 variation in outcrossing rates in *D. stramonium* could be affected by plant spatial
151 arrangement and, by the stigma position (i.e., herkogamy): positive or approach herkogamy
152 (stigma above anthers) increase outcrossing rates (Motten & Antonovics 1992; Motten &
153 Stone 2000). Also, herkogamy is positively related to inbreeding depression (Stone &
154 Motten 2002). Moreover, Kleunen *et al.* (2007) found that plants' outcrossing rates were
155 not affected by population size. Flowers of *D. stramonium* are tubular, white (recessive) or
156 purplish (dominant) corollas. They open at dusk, last for one night only, are large (*ca.* 10
157 cm) (Stone & Motten 2002), and herkogamy has moderate heritability ($h^2 = 0.3$; Motten &
158 Stone 2000). Flowering time occurs in summer months both in the native and in the non-
159 native (Spain) ranges, despite a different climate regime, wet and dry respectively. Since in
160 *D. stramonium* flowers are produced in successive metamers as plant develops, the number
161 of open flowers per plant is variable and depends on plant size (Camargo *et al.* 2017).
162 Because *D. stramonium* is an annual herb and many generations had passed since its
163 introduction to Spain, it is possible that modifications in flower traits, which may affect
164 mating system, have occurred in invasive populations.

165

166 **Study sites**

167 The study was conducted during two summer seasons (2015-2016). In Spain we sampled
168 five populations in the southern region (Table 1). Five populations were sampled in the
169 region of Andalusia with typical Mediterranean climate, characterized by moist winter and
170 hot and dry summer. Most populations inhabit cultivated and waste areas except in

171 Cardeña, which occurs in a river bank of a protected natural area. In Mexico, four
172 populations we sampled in the central region (Table 1), with summer rains and dry season
173 in winter and spring, and all populations are ruderal.

174

175 **Phenotypic variation in flower traits**

176 In each population, we sampled all reproductive plants available (Table 2). From each plant
177 we collected 2-5 flowers which were longitudinally cut, pressed and dried in an electric
178 stove at 50°C and stored for further measurements. We measured the length of corolla (*i.e.*,
179 flower size) and the minimum distance between anthers and stigma. When stigma was
180 above anthers, herkogamy was considered positive (approach), negative (reverse) when the
181 stigma is below the anthers, or zero when contacting each other. In order to explore the
182 phenotypic variation in herkogamy (*i.e.* positive or negative), we estimated the percentage
183 of plants and flowers of each type in each population.

184 To assess the differences in corolla and herkogamy between ranges, we conducted a
185 mixed-model nested ANOVA with range as a fixed effect and populations nested within
186 range and plants nested within population and range as a random effects. Additionally, in
187 order to account for the variance of flower size (corolla) and herkogamy due to populations,
188 hierarchical analyses of variance were performed separately, where plants were nested in
189 populations and among flowers variance thus corresponds to residual variance.

190 Accordingly, nested ANOVAs were carried out for both the native and non-native ranges
191 for corolla size and herkogamy. Populations and plants within populations were declared as
192 random effects. Analyses were conducted using the JMP statistical package (SAS Institute,
193 v. 7.0.1, 2007).

194

195 **Selection on flower traits**

196 To estimate the magnitude and direction of selection gradients on herkogamy and flower
197 size, in each populations, in each range, we performed a multiple regression analysis of the
198 total number of seeds per individual as a fitness measure (w) (Lande & Arnold 1983;
199 Brodie *et al.* 1995). First, we obtained the average values of each floral trait per each
200 individual plant. Because absolute maternal fitness (total seed number per individual) varies
201 with plant size (Núñez-Farfán & Dirzo 1994), we included basal stem diameter (as a proxy
202 for plant size; Valverde *et al.* 2015) of each individual plant in the selection analyses.
203 Directional selection gradients (β_i) were estimated as the standardized partial linear
204 regression coefficients of relative fitness (w_r) as a function of flower size, herkogamy and
205 stem size. In each population, predictor variables were standardized ($\bar{x} = 0$ and $s = 1$) and
206 maternal plant fitness was relativized as the total number of seeds per individual, divided
207 by the corresponding mean fitness of each population (Lande & Arnold 1983). Due to small
208 sample size per population ($n \leq 35$ plants), only directional selection gradients were
209 estimated.

210 To obtain the absolute maternal fitness of each individual, we collected all fruits of
211 marked plants at the end of the reproductive season. Fruits were kept in individual paper
212 bags before they open, labelled and dried in an electric stove at 50°C, and stored. Because
213 *D. stramonium* can produce tens of fruits with hundreds of seeds each, we calculated the
214 number of seeds of each fruits, using the relationship between the total numbers of seeds
215 and fruit volume, from a sample of fruits per population. Fruit volume was calculated with
216 the formula of a prolate spheroid as $4/3\pi(a^2b)$, where a is the equatorial radio (width) and b
217 the polar radio (length) of a fruit.

218

219 **Inbreeding depression**

220 To assess the magnitude of inbreeding depression (δ) in native and non-native populations,
221 we conducted controlled pollination experiments in three natural populations, two in Spain
222 (Hinojos and La Zubia) and one in Mexico (Pedregal; data from Núñez-Farfán *et al.* 1996).
223 In each population we selected another sample of individual plants (20-35) with open
224 flowers to perform crosses. In each individual plant, we randomly allocated flowers to: 1)
225 hand-self pollination or 2) hand cross-pollination, emasculating the receptor flowers prior
226 to anthesis. Pollen was applied onto the stigma *ad libitum*. Flowers were bagged using fine-
227 mesh nylon bags. Given that *D. stramonium* flowers last is only one night, both treatments
228 were applied the same night to all individuals of each site. At maturity, all fruits were
229 harvested before opening and releasing the seeds (1.5 months later). We counted the total
230 number of seeds produced per fruit with ImageJ v.1.50d software (Schneider *et al.* 2012).
231 To test differences in seed production between hand cross-pollination vs. hand-self
232 pollination treatments, we used a *t*-test for each population separately.

233 Outbreeding/inbreeding depression (δ) was calculated per plant for each population
234 as $\delta = [1 - (w_s/w_x)]$ if $w_s < w_x$; and $\delta = [(w_x/w_s) - 1]$ when $w_s > w_x$; where w_s corresponds to
235 seed production by self-pollination, and w_x to seed production by outcrossing (Agren &
236 Schemske 1993). The differences between populations in δ were evaluated by means of an
237 ANOVA.

238

239 **Floral visitors**

240 Pollinator visitation rates were quantified in two populations in Spain and three in Mexico
241 (data from 2003; Table 1), as the number of visits per flower every 15 minutes during three
242 hours, with an interval of 5 minutes between observation times. Observations were done
243 from 19:00 h, before flowers open, up to 22:30 h when the activity of pollinators ceased.
244 The type of pollinator was also registered for each visit.

245

246 **RESULTS**

247 **Phenotypic variation in flower traits**

248 Mixed-model nested anova revealed that corolla length and herkogamy did not differ
249 between ranges (corolla: $F_{(1, 7.01)} = 2.40$, $P = 0.1651$; herkogamy: $F_{(1, 7.02)} = 0.08$, $P =$
250 0.7856 , respectively) but populations within range (corolla: $F_{(7, 257.7)} = 61.73$, $P < 0.0001$;
251 herkogamy: $F_{(7, 269.3)} = 35.36$, $P < 0.0001$) and plants within populations (corolla: $F_{(247, 410)}$
252 $= 3.09$, $P < 0.0001$; herkogamy: $F_{(247, 410)} = 1.49$, $P = 0.0002$) were significant. The non-
253 native population Hinojos had the longest corollas and the native population Teotihuacán
254 had the shortest (Table 2). On the other hand, most populations had negative (reverse)
255 average herkogamy (Table 2). The highest average value of positive (approach) herkogamy
256 was found in the Ticumán population, which is also the one with the largest corolla length
257 in the native range (Table 2). An analysis of herkogamy revealed that in two populations,
258 one native and one non-native, all individual plants and flowers had negative herkogamy
259 (Table 3). The Ticumán population had the largest percentage of plants with positive
260 herkogamy (Table 3) and it also has the largest range of corolla size and the second largest
261 range of herkogamy. Interestingly, we detected that in five populations from 29% up to-
262 56% of plants bear flowers with positive and negative herkogamy, revealing intra-plant
263 variation (Table 3). Likewise, at the flower level, 80% of the flowers of the Ticumán

264 population had positive herkogamy, whereas most flowers showed negative herkogamy
265 (from 50 up to 100%) in eight populations (Table 3).

266 Hierarchical analyses of variation in corolla indicate that the largest fraction of
267 variation is significantly explained by the populations effect (Table 4) revealing phenotypic
268 population differentiation in both ranges. A lower but significant fraction of the variance
269 was explained by variation among plants within population suggesting genetic variance
270 given that environmental conditions within populations are apparently very homogeneous
271 (Table 4). In contrast although the fraction of variance in herkogamy explained by
272 population and plants within population effects were significant, we found that the largest
273 fraction of variance is accounted by flowers within individual plants level (Table 4).

274

275

276 **Floral visitors**

277 The likelihood of a flower to be visited by pollinators varied between native and non-native
278 populations of *D. stramonium* (Table 5). An ANOVA showed significant differences
279 among populations (Table 5A). On average, flowers received *ca.* three-fold more visits in
280 Mexico than in Spain (Table 5A). However, population variation was observed as one
281 native population had an average value similar to that of plants in Spain (Tula population;
282 Table 5A). Moreover, bees of different sizes are the main flower visitors in Spain whereas
283 sphingid moths are the main flower visitors in the native populations studied (Table 5B).
284 Notwithstanding, pollinators are almost absent or very scarce in some native populations
285 (A. López-Velázquez and J. Núñez-Farfán, unpubl. data). It is remarkable the fact that in
286 non-native populations the average of open flowers per night per individual was 2.08

287 (range: 2-5 flowers) in Hinojos and, 7.24 (range: 1-15 flowers) in La Zubia; while in the
288 native one, Santo Domingo, an average of 2.77 (range: 1-5) open flowers was recorded.

289

290 **Inbreeding depression**

291 Controlled self- and out-cross pollinations carried out in native and non-native populations,
292 show that both types of crosses differed in the total number of sound seeds produced only
293 in the native population (Table 6A). Estimation of outbreeding/inbreeding depression per
294 plant in each population revealed no differences in average seed production between selfing
295 and outcrossing in the two Spanish populations studied (Table 6B), but significant
296 inbreeding depression was detected in the native population (Table 6B). The distribution of
297 δ at plant level shows outbreeding as well inbreeding depression in each population (Fig.
298 1). However, the highest frequency of plants with positive δ 's was found in the native
299 population (Fig. 1)

300

301 **Selection on flower traits**

302 In most Spanish populations of *D. stramonium* phenotypic selection analyses showed that
303 stem diameter was positive and significantly related to plant fitness (Table 7). Plant size
304 explained between 29 - 53% of variance in plant fitness in each population (Table 7). In
305 contrast, only in one out four native populations, this relationship was significant (Table 7).
306 Selection on corolla size and herkogamy was not significant for most populations (Table 7).
307 Only in the native population of Santo Domingo herkogamy was selected against (Table 7).
308 Selection against herkogamy was also detected in the native Ticumán populations, however
309 this selection gradient was marginally significant (Table 7). Within-population variation in

310 herkogamy was the highest in these two later native populations, which facilitated these
311 results.

312

313 **DISCUSSION**

314 During the invasion of novel environments, plant populations may face new physical and
315 biotic factors in relation to their native environments (Eckert *et al.* 2009). In the case of
316 animal-pollinated plants, two plausible scenarios are expected in the new environment: an
317 absence of specialized pollinators or a different set of generalist, opportunistic pollinators
318 (Aigner 2006). This, coupled with the reduced availability of potential mates, predicts
319 changes in plant mating system and flower morphology, among others (Baker 1955;
320 Stebbins 1957; Sicard & Lenhard 2011; Ollerton *et al.* 2012; Pannell 2015). These changes
321 may result from selection for selfing and/or relaxed selection exerted by pollinators on
322 flower traits (e.g., size, herkogamy). Our results indicate partial agreement with
323 expectations. First, populations of both ranges of *D. stramonium* did not differ in the
324 average size of flowers and herkogamy. However, there is broad phenotypic variation in
325 these two traits within the native and non-native ranges. Second, while Spanish populations
326 of *D. stramonium* were visited mainly by bees (mostly honeybees), Mexican populations
327 were visited by legitimate pollinators as expected from the flower syndrome (e.g.,
328 hawkmoths); visitation rates were higher for Mexican populations. Third, inbreeding
329 depression was detected in a Mexican population whereas it was nil for all the non-native
330 populations examined. Finally, selection on herkogamy was detected in only one Mexican
331 population. Thus, results suggest that an evolutionary change in mating system has
332 occurred in populations of *D. stramonium* in Spain. We should admit that we have not
333 currently got estimates of selfing/outcrossing rates in natural conditions, but it is reasonable

334 to infer that differences in inbreeding depression should be due to differences in mating
335 systems.

336 In general, the higher visit rate by pollinators in the native range does not
337 correspond with a higher seed set in comparison with the non-native range. Thus, a first
338 account would determine a lower pollination efficiency of legitimate pollinators in terms of
339 seeds set. However, low visit rate and very high fruit and seed set in the non-native range
340 probably mean that most seeds are produced by selfing, as in fact it has been determined for
341 honeybees visiting Mediterranean native flowers (González-Varo *et al.* 2009, 2010).
342 Additionally, we cannot exclude some maternal effects if non-native populations grow in
343 richer or more humid soils. Furthermore, in the case of one Spanish population (La Zubia),
344 the high number of open flowers per night and per individual could promote higher levels
345 of geitonogamy which may enhance the selfing rate and therefore low levels of inbreeding
346 depression, as observed. This scenario has probably being very common during the
347 colonisation of the Mediterranean in agricultural and waste places, where honeybees are
348 most frequent and behave in such opportunistic manner. In native areas, however, there
349 seems to be a pollen-limited seed set, with pollinators provoking a highly variable selfed-
350 outcrossed seed output, and correlated variation in seed quality. In these circumstances,
351 outcrossing would be still advantageous through heterozygote advantage, and hence
352 inbreeding is not purged. Hawkmoth visits flowers necessarily to obtain nectar and have a
353 flight pattern of visiting many flowers in long flights between plants, in contrast we
354 observed that all bees were collecting both nectar and pollen and spent long time in each
355 flower and then visited a nearby flower, usually in the same plant. As a result, pollen
356 transfer will be very different in promoting outcrossing or selfing after visits by these
357 contrasted types of pollinators (Herrera 1987; Barluenga *et al.* 2011).

358 During the transition to selfing, morphological and physiological changes in
359 reproductive traits are expected, such as reduced flower size and level of herkogamy
360 (Bobblyl-Roels & Kelly 2011; Sicard & Lenhard 2011). Sicard & Lenhard (2011) have
361 synthesized four possible, non-mutually exclusive, scenarios for the changes in flower size
362 and herkogamy. These include resource allocation to individual flowers, selection for more
363 efficient pollination, selection for rapid plant and flower maturation at marginal habitats,
364 and florivory. In this study, native and non-native populations did not differ in average
365 flower size; in fact, the Spanish population Hinojos had the longest flowers of *D.*
366 *stramonium*. That is to say, data do not agree with first and third scenarios. Regarding the
367 fourth scenario, previous study showed that herbivory levels are very low (Valverde *et al.*
368 2015) as to affect flower size. In theory, there is a possible fifth scenario which considers
369 that the changes of flower traits, specifically those related to flower size, are a consequence
370 of the genetic modulation that occurs in selfing populations, as observed in *Capsella*
371 *rubella* (Sicard *et al.* 2016). However, in the case of *D. stramonium*, it seems that flower
372 size is very variable even within populations, which makes difficult to be associated with
373 selfing rates. In spite of differences among individuals within population in flower size
374 explains a significant amount of the variance, suggesting genetic variation of this character,
375 a similar amount is explained by flowers within individuals (Table 4). It seems that besides
376 “noise” plasticity, there is evidence of developmental phenotypic plasticity in flower size
377 and herkogamy in *D. stramonium* (Camargo *et al.* 2017). Thus, selection for efficient self-
378 pollination (in terms of number of seeds) is a plausible scenario, since populations from
379 Spain show reduced herkogamy. It must be noted, though, that herkogamy varies widely
380 within and among populations, but the highest positive herkogamy was found in a native
381 population (Table 2). On the other hand, since flower size is as larger than in native

382 populations, is this trait maintained by selection by pollinators? This probably is not the
383 case. Flowers with deep corolla tubes are thought to have evolved in close associations with
384 pollinators, namely but not exclusively, hawkmoths (Nilsson 1988). According to this, it is
385 not expected that large flowers be maintained in Spain by bees, as in the native range.
386 However, as Aigner (2006) has pointed out, in fine-grained environments (e.g., two types
387 of pollinators) an optimal flower phenotype can be consider generalized when it functions
388 well with both specialized and generalized pollinators, or when a broad symmetrical trade-
389 off occurs. It remains to determine if floral phenotypes in the non-native range experience a
390 coarse- or fine-grained pollinator environments (see also Devaux *et al.* 2014).

391 Recent experimental evidence from native populations of *D. stramonium*, indicates
392 that i) there is genetic variation, plasticity and genetic variation in plasticity in floral traits,
393 including herkogamy, in response to variation in the soil nutrient environment; ii) also,
394 there is an interaction between flower position (i.e., ontogeny) and nutrient conditions, and
395 (iii) a systematic trend to reduce herkogamy along flower position in the low nutrient
396 environment, thus increasing the occurrence of self-fertilization (Camargo *et al.* 2017).
397 Thus, phenotypic plasticity appears to be a plausible explanation for the maintenance of
398 large flower sizes in Spain. Most populations of *D. stramonium* from Spain inhabit in
399 cultivated, irrigated fields and riverbeds, which are very rich in water and nutrient
400 resources.

401 During colonisation, inbreeding depression can limit the evolution of selfing in the
402 new environments (Lande & Schemske 1985). However, selfing permits the expression of
403 deleterious recessive alleles that can be purged from colonising populations thus reducing
404 the magnitude of inbreeding depression (Crnokrak & Barrett 2002; Pannell 2015).
405 Furthermore, range expansion may involve the depletion of additive genetic variance

406 necessary for adaptive potential at new environments (Pujol & Pannell 2008); this reduction
407 can be consequence of a genetic bottleneck and further exacerbated by changes in mating
408 systems (Pannell 2015). We did not detect inbreeding depression in seed production in the
409 two Spain populations studied. This implies that populations of Spain may have passed
410 through a genetic bottleneck and lack genetic variation, and/or that selection to purge
411 deleterious alleles has occurred (Pujol & Pannell 2008). Ongoing studies on within-
412 population genetic variation will determine such possibility. In contrast, we found strong
413 inbreeding depression for this fitness component in a Mexican population ($\delta = 0.37$), which
414 indicates that the population maintains a genetic load of deleterious alleles. Ample evidence
415 of inbreeding depression in *D. stramonium*, in fitness related characters (Sosenski 2004)
416 and resistance to herbivores, has been detected in other Mexican populations, together with
417 variation among families in the magnitude of inbreeding depression (Bello-Bedoy &
418 Núñez-Farfán 2010). Moreover, evidence derived from multiple studies point that
419 inbreeding depression increases in stressful as compared with benign environments
420 (Armbruster & Reed 2005). The latter argument reinforces our conclusion given that the
421 detection of inbreeding depression in the native population derived from experiments in the
422 benign environment (greenhouse). Thus, this evidence further supports our hypothesis for
423 the change in mating system of *D. stramonium* in Spain.

424 In plants, success to colonise new environments might depend on the ability to
425 respond to selection rather than physiological plasticity and/or tolerance to the
426 environmental conditions (Lee 2002). Natural selection could proceed in response to
427 environmental climatic factors and biotic interactions. However, response to selection in
428 turn, could be limited by additive genetic variation. In line with this, it is expected that

429 selection, during and after colonisation, might influence plant mating for different reasons
430 (see Pannell 2015), but one important factor is the absence of native pollinators. While *D.*
431 *stramonium* is pollinated by specialised pollinators (*i.e.*, sphingid moths) in its native range,
432 our data revealed that it visited by generalists flower visitors in Spain. Thus, no selection on
433 floral traits that favour outcrossing, like flower size and herkogamy, is expected. In
434 agreement with this, no selection on floral traits was detected in the non-native range.
435 However, in the non-native population La Zubia the selection gradient on corolla size was
436 negative and significant, a result expected, but the whole model was not statistically
437 significant. In contrast, we detected selection to reduce the magnitude of herkogamy in the
438 native population Santo Domingo. Also, in the native population Ticumán, selection to
439 reduce herkogamy was marginally significant but the whole model was not. In contrast with
440 non-native range, in the native range there might be enough genetic variation as selection to
441 occur. It must be bear in mind, though, that selection on these floral traits was assessed
442 considering fitness as seed output. Thus, selection to reduce herkogamy may increase seed
443 output but not necessarily the quality of produced progeny. Independent evidence derived
444 from controlled crosses, selfing and outcrossing, clearly indicated that in the native
445 population outcrossing outperforms selfing (*i.e.*, inbreeding depression), whereas both
446 types of mating produce equivalent amount of seeds (*i.e.*, no inbreeding depression) in non-
447 native populations. These results suggest that in Spain, selection for selfing has occurred,
448 perhaps due to the purge of deleterious alleles. In addition, these results suggest that genetic
449 variation is lower in the non-native populations. In this study and a previous one (Valverde
450 *et al.* 2015), we detected positive phenotypic selection on plant size in *D. stramonium* in
451 Spain. There, plants grow tall, flower and set seeds during the hottest and driest period,
452 when native Mediterranean plants are inactive and cannot compete for space and resources,

453 in comparison with smaller temperate European plants, whose seed output is much lower
454 (J. Arroyo, personal observations). Whether this phenotypic variation to produce larger
455 plants in *D. stramonium* is genetically based or due to plasticity remains to be answered in
456 future studies.

457

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635

636 **Figure legends**

637

638

639 Figure 1. Distribution of outbreeding/inbreeding depression per plant in native (Pedregal)

640 and non-native populations (Hinojos and La Zubia)) of *Datura stramonium* (see Table 6B).

641

Table 1. Locality characteristics of native (Mexico) and non-native (Spain) populations of *Datura stramonium*.

Range	Locality (Province)	Geographic coordinates	Altitude (m a.s.l.)	Mean annual precipitation (mm)	Mean annual temperature (°C)
Native	Pedregal† (Mexico City)	19°19'03.72" N 99°11'26.67" W	2323	803	15.6
	Santo Domingo* (State of Morelos)	19°00'33.93" N 99°03'40.46" W	2050	1463.2	19.9
	Taxco* (State of Guerrero)	18°33'26.35" N 99°35'55.84" W	1746	1417.6	21.6
	Teotihuacán (State of Mexico)	19°40'57.78" N 98°50'34.50" W	2294	563.3	14.9
	Texcoco (State of Mexico)	19°29'19.67" N 98°51'57.72" W	2353	710.3	15.9
	Ticumán (State of Morelos)	18°42'37.62" N 99°07'00.66" W	1210	850	24.1
	Tula* (State of Hidalgo)	20°02'29.70" N 99°19'50.08" W	2081	699.4	17.6
Non-native	Alamillo (Seville)	37° 25'14.59" N 05°59'35.86" W	10	576	18.6
	Cardeña (Cordoba)	38°14'47.51" N 04°12'57.15" W	342	645	17
	Dos Hermanas (Seville)	37°16'19.60" N 05°56'16.90" W	42	591	18.1
	Hinojos†* (Huelva)	37°18'29.31" N 06°23'56.55" W	81	515	18
	La Zubia†* (Granada)	37°08'14.532" N 03°36'4.73" W	681	462	15.2

Populations where inbreeding depression was estimated†, and pollinators recorded*

645 **Table 2.** Average and standard deviations (\pm SD); minimum and maximum values of
 646 corolla length and herkogamy (mm) of individual plants of *Datura stramonium* in natural
 647 populations from native (Mexico) and non-native (Spain) range in 2015-2016. N = sample
 648 size.

Range	Population	N	Corolla length (mm)	Herkogamy (mm)	Range Corolla Length (mm)
Native	Santo Domingo	29	88.63 \pm 6.64	-1.77 \pm 2.77	68.63 – 102.44
	Teotihuacán	18	67.48 \pm 9.24	-0.04 \pm 0.09	50.50 – 84.50
	Texcoco	29	79.29 \pm 7.63	-1.45 \pm 1.72	66.16 – 94.84
	Ticumán	25	95.58 \pm 8.78	4.07 \pm 2.91	73.01 – 112.35
Average		101	83.90 \pm 12.53	0.44 \pm 4.24	
Non-native	Alamillo	33	79.52 \pm 7.13	-0.37 \pm 1.70	62.06 – 89.77
	Cardeña	29	86.47 \pm 9.50	-1.84 \pm 1.71	64.61 – 107.32
	Dos Hermanas	35	95.21 \pm 6.64	-0.83 \pm 1.60	73.42 – 109.40
	Hinojos	28	109.89 \pm 5.62	1.14 \pm 1.35	98.50 – 121.29
	La Zubia	30	101.32 \pm 5.08	0.81 \pm 0.81	89.78 – 112.24
Average		155	94.07 \pm 12.62	-0.16 \pm 2.17	

649
 650

651 **Table 3.** Nested analyses of variance of corolla size and herkogamy in native and non-
 652 native populations of *Datura stramonium*.
 653

	Corolla		Herkogamy
Native	Source	% Variance	% Variance
	Population	51.64	43.06
	Plant (Pop.)	22.01	12.04
	Residual	26.35	44.90
Non-native	Source	% Variance	% Variance
	Population	67.14	26.94
	Plant (Pop.)	15.82	15.16
	Residual	17.03	57.89

654
 655

Table 4. (A). Mean number of pollinators' visits per flower (\pm SE) and (B) identity of main pollinators (bold type) in native (Mexico) and non-native (Spain) natural populations of *Datura stramonium*. Different letters are significantly different at $P < 0.002$. N: sample size.

(A)

Range	Population	N	#visits/flower [†]
Native	Santo Domingo	164	3.83 ± 0.21^b
	Tula	50	1.18 ± 0.38^a
	Taxco Viejo	110	4.04 ± 0.25^b
Non-Native	Hinojos	158	1.15 ± 0.19^a
	La Zubia	210	0.91 ± 0.18^a

[†], ANOVA executed using log-transformed values.

(B)

Range	Identity of pollinators
Native	Hawkmoths (<i>Manduca sexta</i> , <i>Hyles</i> sp.) Bees (<i>Apis mellifera</i>)
Non-Native	Bees (<i>Apis mellifera</i> , <i>Anthophora</i> sp.) Carpenter bees (<i>Xylocopa</i> sp.), Bumblebees (<i>Bombus</i> sp.)

659 **Table 5.** Number of seeds (average \pm standard error) of plants under two treatments of
 660 pollination in native (Mexico) and non-native (Spain) populations (A). Average of
 661 outbreeding/inbreeding depression (δ) of populations of *Datura stramonium* (B). $N_f =$
 662 number of flowers.

663 (A)

Range	Population	N_f	Cross-pollination	N_f	Self-pollination	t	P
Native	Pedregal	36	95.19 ± 6.5	40	58.32 ± 6.37	4.03	0.0001
Non-native	Hinojos	19	470.52 ± 18.72	14	470.28 ± 34.99	0.01	0.9949
	La Zubia	16	548.06 ± 25.54	14	520.42 ± 27.30	0.75	0.4560

664

665 (B)

Population	N	δ^*
Pedregal	35	0.371 ± 0.081^a
Hinojos	13	0.058 ± 0.133^{ab}
La Zubia	9	-0.032 ± 0.160^b

666 *Different letters indicate significant differences ($P < 0.05$)

667

668

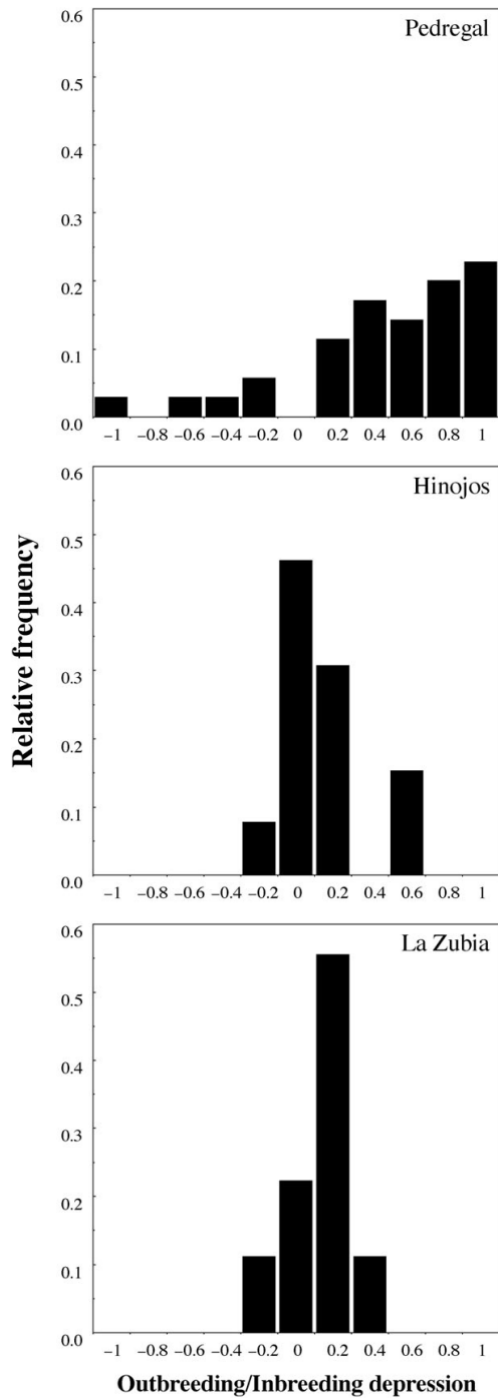
669

Table 6. Directional selection gradients β (\pm SE) obtained from partial regression analyses on flower traits in native (Mexico) and non-native (Spain) populations of *Datura stramonium*. F and R^2 are reported for full model.

Range	Population	Trait	β (\pm SE)	t (df)	P	F	R^2
Native	Santo Domingo	Stem	0.19 (0.03)	1.03	0.314	3.05*	0.19
		Corolla length	0.16 (0.02)	0.89	0.381		
		Herkogamy	-0.41 (0.07)	-2.20	0.037		
	Teotihuacán	Stem	0.27 (0.03)	1.05	0.317	1.43 ^{NS}	0.08
		Corolla length	-0.43 (0.03)	-1.67	0.124		
		Herkogamy	0.04 (2.49)	0.15	0.880		
	Texcoco	Stem	0.75 (0.02)	4.67	0.000	9.21***	0.48
		Corolla length	0.03 (0.02)	0.20	0.842		
		Herkogamy	-0.06 (0.08)	-0.36	0.721		
	Ticumán	Stem	0.27 (0.05)	1.22	0.238	2.54 ^{NS}	0.19
		Corolla length	0.06 (0.03)	0.28	0.780		
		Herkogamy	-0.41 (0.09)	-1.97	0.065		
Non-native	Alamillo	Stem	0.74 (0.02)	6.01	0.000	12.6***	0.52
		Corolla length	0.09 (0.01)	0.71	0.486		
		Herkogamy	0.05 (0.04)	0.43	0.673		
	Cardeña	Stem	0.57 (0.03)	3.45	0.002	4.94**	0.31
		Corolla length	-0.16 (0.02)	-0.96	0.346		
		Herkogamy	-0.03 (0.08)	-0.16	0.877		
	Dos Hermanas	Stem	0.47(0.01)	3.08	0.004	6.63**	0.29
		Corolla length	-0.00 (0.02)	-0.01	0.989		
		Herkogamy	-0.27 (0.08)	-1.48	0.148		
	Hinojos	Stem	0.69 (0.03)	5.56	0.000	12.8***	0.53
		Corolla length	0.16 (0.02)	1.31	0.202		
		Herkogamy	-0.15 (0.08)	-1.24	0.226		
	La Zubia	Stem	-0.12 (0.03)	-0.72	0.478	1.93 ^{NS}	0.08
		Corolla length	-0.39 (0.02)	-2.16	0.039		
		Herkogamy	0.09 (0.16)	0.46	0.650		

*** $P < 0.0005$; ** $P < 0.01$; * $P < 0.05$; NS: non significant.

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674 **Outbreeding/Inbreeding depression**
 675 Fig. 1. Distribution of outbreeding/inbreeding depression per plant in native
 676 (Pedregal) and non-native (Hinojos and La Zubia) populations of *Datura*
 677 *stramonium* (see Table 5B).
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