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Title: Shifts in outcrossing rates and changes to floral traits are associated with the evolution of herbicide resistance in the common morning glory

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Statement of authorship

AK collected seeds, performed experiments, analyzed data and wrote the paper; EF collected data; SMC collected data and contributed to the manuscript; RSB designed the study, performed the analyses, and wrote the paper. All authors discussed the results and commented on the manuscript.

Data accessibility

Primary data used in these analyses will be made available in the public github repository https://github.com/rsbaucom/MatingSystem2015, which can be anonymously accessed. Upon acceptance, data will be made available through the Dryad public repository.

Abstract

Human-mediated selection can strongly influence the evolutionary response of natural organisms within ecological timescales. But what traits allow for, or even facilitate, adaptation to the strong selection humans impose on natural systems? Using a combination of lab and greenhouse studies of 32 natural populations of the common agricultural weed, *Ipomoea purpurea*, we show that herbicide resistant populations self-fertilize more than susceptible populations. We likewise show that anther-stigma distance, a floral trait associated with self-fertilization in this species, exhibits a non-linear relationship with resistance such that the most and least resistant populations exhibit lower anther-stigma separation compared to populations with moderate levels of resistance. Overall, our results extend the general finding that plant mating can be impacted by human-mediated agents of selection to that of the extreme selection of the agricultural system. This work highlights the influence of human-mediated selection on rapid responses of natural populations that can lead to unexpected long-term evolutionary consequences.

67 **Introduction**

68 Pesticides are used world-wide to protect agricultural crops from the damaging effects of
69 insects, fungi and weeds (Enserink *et al.* 2013) and are considered vital for maintaining the
70 world's food supply (Lamberth *et al.* 2013). Recently we have begun to recognize that their use
71 can negatively impact the reproduction and mating patterns of natural organisms such as bees
72 (Williams *et al.* 2015), amphibians (Rohr & McCoy 2010), and plants (Pline *et al.* 2003; Thomas
73 *et al.* 2004; Baucom *et al.* 2008; Londo *et al.* 2014) and can have long term evolutionary
74 consequences on these non-target species. In the US, 40% of the pesticides applied across the
75 400 million acres of cropland are herbicides (*US EPA* 2011), which impose extreme selection on
76 naturally occurring agricultural weeds (Jasieniuk *et al.* 1996; Vigueira *et al.* 2013). Strikingly,
77 while herbicide resistance has evolved in over 200 plant species worldwide (Heap 2014), the
78 impact on correlated, non-target traits has been largely unexplored.

79 The plant mating system, or the relative rate of outcrossing versus selfing, is a labile trait
80 (Barrett 1998; Karron *et al.* 2012) that is influenced by human impacts on natural ecosystems
81 (Eckert *et al.* 2010). Habitat fragmentation by deforestation, for example, reduces the outcrossing
82 rate of many forest tree species (Aguilar *et al.* 2006; Eckert *et al.* 2010). Further, plants tolerant
83 to heavy-metal contaminated soils exhibit higher rates of autonomous self-pollination than non-
84 adapted biotypes (Antonovics *et al.* 1971), strongly suggesting that the mating system is either
85 influenced by or concomitantly evolves in response to heavy metal exposure (Antonovics 1968;
86 Antonovics *et al.* 1971). We predict that the mating system of an agricultural weed should co-
87 vary with the level of herbicide resistance in nature, for two main reasons. First, the reproductive
88 assurance hypothesis (Baker 1955, 1974; Goodwillie *et al.* 2005; Pannell *et al.* 2015) would
89 predict resistant individuals, in a mate-limited population following herbicide application, are

90 more likely to produce progeny if they are also highly self-pollinating rather than outcrossing.
91 Second, resistant types that self-pollinate would effectively reduce the influx of non-adapted,
92 susceptible alleles – otherwise known as the 'prevention of gene flow' hypothesis (Antonovics
93 1968). Both hypotheses predict that herbicide resistant individuals should self to a higher degree
94 than non-resistant individuals. Interestingly, while many herbicide resistant weed species are
95 reported to be predominantly selfing (Jasieniuk *et al.* 1996), there are no investigations, to our
96 knowledge, that examine the potential for co-variation between mating system and herbicide
97 resistance in nature, a finding that would indicate the mating system may co-evolve when
98 populations respond to the strong selection imparted by herbicides.

99 *Ipomoea purpurea,* an annual weed of agricultural fields and disturbed sites in the
100 southeastern and Midwest US, is a model for examining persistence in stressful and competitive
101 environments (Baucom *et al.* 2011; Chaney & Baucom 2014). As such the species is a
102 particularly relevant candidate for studying how mating systems may evolve under regimes of
103 human-mediated selection. Populations of this agricultural weed have been exposed consistently
104 to the application of glyphosate, the active ingredient in the herbicide RoundUp, since the late
105 1990's given the widespread adoption of RoundUp Ready crops in the US (NASS 2015).
106 Populations vary for the level of resistance to glyphosate across its North American range; while
107 some populations of *I. purpurea* exhibit 100% survival following application of the field-dose of
108 the herbicide, other populations exhibit high susceptibility (Fig. 1a) (Kuester *et al.* 2015a). In
109 addition, individuals of this mixed-mating, hermaphroditic species (Ennos 1981; Brown & Clegg
110 1984) with a smaller anther-stigma distance (ASD, the distance between the tallest anther and the
111 stigma; Fig. 1b) self-pollinate more often than individuals with a larger ASD (Ennos 1981;
112 Chang & Rausher 1999). Because both anther-stigma distance and glyphosate resistance in this

113 species are heritable and respond to selection (Ennos 1981; Chang & Rausher 1999; Debban *et*
114 *al.* 2015) this common agricultural weed provides a unique opportunity to examine the potential
115 that mating systems and associated reproductive traits evolve in response to extreme regimes of
116 selection imposed by herbicide.

117 Here, we determine if the mating system of *Ipomoea purpurea* co-varies with herbicide
118 resistance, and if reproductive traits associated with self-fertilization are likewise influenced by
119 resistance status. We previously estimated the percent survival of populations following
120 herbicide application using a replicated dose-response greenhouse experiment with individuals
121 sampled as seed from the field in 2012. We used these population-level estimates of survival as
122 each population's resistance level (Fig. 1a) (Kuester *et al.* 2015a), and determined if molecular-
123 marker based estimates of the mating system and measurements of floral morphology co-varied
124 with the level of herbicide resistance in natural populations. Further, our experimental
125 populations were sampled twice, once in 2003 and again in 2012 from the same location (Fig.
126 1a), allowing us to examine the hypothesis that floral traits have changed in these populations
127 over time. We predict that populations with a high level of glyphosate resistance should exhibit
128 evidence of reduced outcrossing and reductions in the anther-stigma distance – either of which
129 would indicate that plant reproductive traits exhibit correlated evolution in response to strong
130 selection from herbicide application.

131
132
133 **Materials and Methods**

134 **Mating system estimates**. We performed a progeny array analysis to estimate the mating system
135 of 24 populations located in the southeastern and Midwestern US (indicated by triangles in Fig.
136 1a). These populations are part of a previous study in which we screened for glyphosate

137 resistance, defined as the proportion each population that survived the application of 1.7 kg
138 a.i./ha RoundUp (Kuester *et al.* 2015a), a rate that is slightly higher than the current
139 recommended field dose. One seed randomly selected from an average of 11 fruits per maternal
140 line from an average of 19 maternal lines per population were used to estimate mating system
141 parameters (see Table S1 in Supporting Information for numbers of maternal lines and progeny
142 per population). Maternal plants were sampled at least two meters apart within each agricultural
143 field. DNA was extracted from seedling cotyledon tissues using a CTAB method (T Culley, pers.
144 comm.). In total, 4798 progeny were genotyped with fifteen previously described microsatellite
145 loci for maternal-line estimates of the mating system (see Kuester *et al.* 2015a) for specific
146 details of the PCR conditions). All sampled genotypes were analyzed using Applied Biosystems
147 PeakScanner 1.0 analytical software (Carlsbad, CA) with a PP (Primer Peaks adjustment) sizing
148 default, and scoring was double-checked manually for errors in a random sub-sample of 200
149 individuals. We examined our ability to assign parentage using Cervus (Kalinowski *et al.* 2007)
150 and determined population-level estimates of genetic diversity and inbreeding using GenalEx
151 (Peakall & Smouse 2012). We estimated mating system parameters using BORICE (Koelling *et*
152 *al.* 2012), which is a Bayesian method to estimate the family-level outcrossing rate (t) and
153 maternal line inbreeding coefficients (F) (Koelling *et al.* 2012). BORICE is reported to perform
154 well when either family sizes are small or maternal genotypes are unavailable (Koelling *et al.*
155 2012). We used the following default parameters when estimating mating system parameters: 1
156 million iterations and 99,999 burn-in steps, an outcrossing rate tuning parameter of 0.05, an
157 allele frequency tuning parameter of 0.1, and an initial population outcrossing rate of 0.5. We
158 examined the possibility that null alleles influenced our mating system estimates by re-running
159 all analyses after excluding 4 loci that potentially exhibited null alleles (i.e., loci with ~25% null

160 alleles: IP18, IP1, IP26 and IP42) as indicated by MicroChecker (Van Oosterhout et al. 2004).
161 We found no evidence that null alleles impacted estimates across populations (correlation
162 between outcrossing rates for all loci and 4 loci removed: $r = 0.94$, $P < 0.001$) and thus report
163 results using all 15 loci.
164
165 **Floral phenotypes**. We performed a replicated greenhouse experiment to determine if floral
166 morphology varied according to resistance level and to determine if floral traits differed between
167 two sampling years (2003 and 2012). Seeds were sampled from maternal plants from 32
168 randomly chosen populations located in the Southeast and Midwest US in the fall of 2012 (all
169 populations in Fig. 1a); fifteen of these populations had been previously sampled in 2003 (see
170 Table S2). We planted seeds from between 1-29 maternal lines (average=12.74, median=13; see
171 Table S2 for number of individuals) from each population and each sampling year in 4-inch pots
172 in a completely randomized design at the Matthaei Botanical Gardens at the University of
173 Michigan (Ann Arbor, MI). To increase overall sample size, a second replicate experiment was
174 started two weeks later in the greenhouses for a total of 640 experimental plants. Once plants
175 began flowering, we measured the height of the pistil (cm) and the tallest stamen (cm) to
176 estimate anther-stigma distance (ASD) of an average of 5.5 flowers per plant across 17 sampling
177 dates. An average of 2 flowers were measured from each plant each sampling date;
178 measurements taken on multiple flowers per plant per sampling date were averaged prior to
179 analysis. Over the course of the experiment, we measured 3569 flowers from 622 experimental
180 plants. Of the overall 32 populations sampled in 2012 for floral morphology estimates, 23 were
181 likewise represented in the mating system analyses, presented above.
182

183 **Statistical analyses**. To determine if mating system parameters (the outcrossing rate (t),
184 maternal inbreeding coefficient (F)) of *I. purpurea* co-varied with resistance, we performed
185 linear regressions using the lm function in R v 3.1.1 (R Core Team 2013) in which each
186 population's mating system estimate was used as the dependent variable with the level of
187 resistance (proportion survival at 1.7 kg a.i./ha) as an independent variable. We included
188 population latitude in preliminary models as an independent variable since previous work
189 (Kuester *et al.* 2015a) indicated a weak trend between resistance and latitude; however, this
190 effect was removed from final models as it was never significant nor did it influence the
191 relationship between mating system and resistance. Preliminary analyses indicated the presence
192 of a nonlinear relationship between the outcrossing rate and resistance, and as such we included a
193 quadratic term in a separate regression for both mating system parameters. Further, because a
194 plot of the outcrossing rate and the quadratic resistance term exhibited a non-linear but not
195 completely convex relationship, we performed a piece-wise regression to examine the potential
196 for two different linear relationships in the data. To do so, we used the segmented package of R
197 (Muggeo 2008) with an initial approximated breakpoint (psi) of 0.40. Each mating system
198 parameter was examined for normality by performing Shapiro-Wilk tests. Neither showed
199 evidence of non-normality and therefore were not transformed prior to analysis.
200
201 We examined whether floral morphology differed according to the level of resistance first using
202 populations sampled in 2012 (N = 32). To do so we performed a multivariate ANOVA with sine-
203 transformed values of each floral phenotype (anther-stigma distance, length of the tallest stamen,
204 and pistil height) as dependent variables in the model and the experimental replicate, resistance
205 level of the population, and latitude of the population as fixed, independent variables. Prior to

206 analysis, we removed the influence of sampling date (N=17) by performing a MANOVA with
207 transformed variables and retained the residuals for testing our main effects of interest
208 (experimental replicate, resistance level, latitude). We elected to do so because the influence of
209 sampling date on anther-stigma distance was not one of our primary questions and we noted a
210 highly significant influence of this effect on floral morphology. Preliminary analyses indicated a
211 significant influence of population latitude on floral morphology and thus we elected to include
212 this effect in the MANOVA. Further, because a scatterplot of the relationship between anther-
213 stigma distance and resistance indicated the presence of a non-linear relationship, we included a
214 quadratic term (resistance level^2) in this and downstream analyses.
215
216 We next ran separate univariate analyses to determine if ASD, height of the tallest stamen and
217 pistil height varied according to resistance level and if they have changed over sampling years
218 using the lm function of R v 3.1.1 (R Core Team 2013). For each model, the sine-transformed
219 floral trait of interest (ASD, height of tallest stamen, pistil height) was the dependent variable
220 with the experimental replicate, resistance level, resistance level^2, and population latitude as
221 independent variables in the regression models. We again performed analyses using residuals
222 after removing the effect of sampling date. Similar to the mating system analysis, if the non-
223 linear resistance term proved significant, we used the segmented package to perform a piece-
224 wise regression and examine the potential for two different linear relationships as well as the
225 break point between slopes using an initial breakpoint of 0.40 (Muggeo 2008). To determine if
226 floral morphology had changed between sampling years and/or if floral morphology differed
227 between years differently across resistance levels, we performed the same analyses as above
228 using only populations that were sampled in both 2003 and 2012 (N = 15), including a year term,

229 a year by resistance level term, and a year by resistance level^2 term in each analysis. In these
230 analyses, we used resistance levels from each population each year of sampling (as reported in
231 Kuester *et al.* 2015b).
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238 **Results**
239
240 **Mating system.** The average combined exclusion probabilities across all populations was high,
241 at greater than 99%, indicating that the fifteen microsatellite loci successfully assigned parentage
242 (see Table S1). Thus, the power is sufficient for estimating outcrossing rates among populations.
243 Values of the outcrossing rate varied substantially across populations (range: 0.27-0.8), with an
244 average value (±SE) for the species of 0.50 (±0.03) (Table S3).
245
246 We uncovered a strong and striking negative linear relationship between the average
247 family-level outcrossing rate of each population (Table S3) and the level of glyphosate resistance
248 (β = -0.30 ±0.09; Fig. 2a); individuals from highly resistant populations self-pollinate more than
249 individuals from less resistant populations (F = 10.70, P = 0.004; Table 1). We also uncovered a
250 significant negative quadratic relationship between resistance and the outcrossing rate,
251 suggesting that outcrossing first increases at low levels of resistance and then declines as
252 resistance increases (F = 5.08, P = 0.035; Table 1). Piece-wise regression analysis indicated,
253 however, that the slope between the outcrossing rate and resistance was positive but not
254 significantly different from zero at low levels of resistance (β = 0.42 ±0.36, 95% CI: -0.34, 1.17)

255 whereas the slope following the estimated break point (0.42 ±0.07) was negative and
256 significantly different from zero (β = -0.57 ±0.14, 95% CI: -0.28, -0.86). In line with our finding
257 of lower outcrossing in highly resistant populations, we found maternal inbreeding coefficients
258 to increase linearly as the level of resistance increases (Fig. 2b, F = 6.05, *P* = 0.02; Table 1). We
259 found no evidence of a nonlinear relationship between the maternal inbreeding coefficients and
260 the level of resistance. Together, these results demonstrate that outcrossing rates were lower
261 and maternal inbreeding coefficients higher in glyphosate resistant compared to susceptible
262 populations.
263
264 **Floral morphology.** A multivariate analysis of variance indicated that floral morphology related
265 to selfing rates (anther-stigma distance (ASD), height of the pistil and the tallest stamen) was
266 significantly influenced by both the non-linear resistance term and the latitude of the population
267 (resistance level^2, Approx. F = 6.66, *P* < 0.001; latitude, Approx. F = 2.58, *P* = 0.05; Table S4).
268 In separate univariate ANOVAs, we found a negative quadratic relationship between ASD and
269 resistance (resistance level^2: F = 5.70, *P* = 0.02, Table 2, Fig. 3a) and a trend for a negative
270 quadratic relationship between resistance and pistil height (resistance level^2: F = 2.96, *P* = 0.09,
271 Table 2, Fig. 3c) but no quadratic relationship between resistance and stamen height (Fig. 3b).
272 No linear relationships between resistance and the three floral traits were uncovered; however, a
273 piece-wise regression analysis of ASD indicated a positive slope between ASD and resistance at
274 low levels of resistance (β = 0.72 ±0.32, 95% CI: 0.06, 1.37) and a negative slope (β = -0.16
275 ±0.07, 95% CI: -0.01, -0.31) after the estimated breakpoint (0.31±0.05).
276 There was no evidence that ASD either increased or decreased between collection years
277 across the subset of populations sampled in both 2003 and 2012 (Year effect in ANOVA: $F_{1,47}$ =

278 0.464, P = 0.50). Further, although we again detected a significant quadratic relationship between
279 ASD and resistance using this subset of populations (resistance level^2: $F_{1,47}$ = 5.82, P = 0.02, Fig.
280 4), we uncovered no evidence that this relationship differed between years (Year by resistance
281 level^2 effect in ANOVA: $F_{1,47}$ = 1.16, P = 0.28). However, when examining the relationship
282 between resistance level^2 and ASD separately between collection years, we found a significant
283 negative quadratic relationship for the 2012 sample (solid line in Fig. 4; β = -0.48 ±0.23, $F_{1,22}$ =
284 6.07, P = 0.02) but no evidence for a significant relationship among populations sampled in 2003
285 (β = -0.17 ±0.19, $F_{1,22}$ = 1.08, P = 0.31). Despite the relative stability of ASD values among
286 populations across this nine-year period (i.e., lack of a year effect), both floral traits comprising
287 ASD – height of the tallest stamen and pistil height – showed a significant (or trend for
288 significant) collection year by resistance level interaction (Pistil height: $F_{1, 47}$ = 4.28, *P* = 0.04;
289 Tallest stamen height: $F_{1, 47}$ = 3.27, P = 0.08). In the 2012 sample, both the pistil and stamen
290 height decreased as the level of resistance increased (2012: pistil β = -0.08 ±0.05, tallest stamen
291 β = -0.08 ±0.05) whereas the pistil and stamen heights from the 2003 sample increased as
292 resistance increased (2003: pistil β = 0.07 ±0.04, tallest stamen β = 0.06 ±0.05).
293
294
295 **Discussion**
296 In line with our predictions, we demonstrate that the mating system of the agricultural
297 weed, *Ipomoea purpurea*, co-varies with the level of glyphosate resistance. Specifically, we find
298 that outcrossing rates are lower and maternal inbreeding coefficients are higher in resistant
299 compared to susceptible populations. We likewise find that anther-stigma distance, a floral trait

300 associated with self-fertilization in this species, exhibits a nonlinear relationship with resistance
301 such that the most and least resistant populations exhibit lower anther-stigma separation
302 compared to populations with moderate levels of resistance. Further, this relationship was
303 present among populations sampled in 2012 but not 2003, suggesting that reproductive traits may
304 have rapidly evolved in these populations over the course of nine years. Below, we discuss each
305 of our major findings and place them in the broad context of mating system changes associated
306 with human-mediated selection.
307
308 **Plant mating changes associated with resistance**
309 As anticipated by both the reproductive assurance (Baker 1955, 1974; Goodwillie *et al.*
310 2005; Pannell *et al.* 2015) and 'prevention of gene flow' (Antonovics 1968) hypotheses, our
311 work finds a significant negative relationship between the outcrossing rate and level of herbicide
312 resistance across natural populations of an agricultural weed indicating that individuals from
313 highly resistant populations self more often than those from susceptible populations. We likewise
314 uncovered a significant negative nonlinear relationship indicating that the outcrossing rate
315 initially increased at low levels of resistance and then declined as resistance increased. When
316 examining the nonlinear relationship using piecewise regression, however, we found that the
317 initial positive slope was not significantly different than zero whereas the negative linear
318 relationship following the estimated breakpoint was significant. Further, we found a positive,
319 linear relationship between maternal inbreeding coefficients and resistance, indicating that
320 maternal individuals from highly resistant populations were more likely to be the product of self-
321 fertilization or mating between close relatives (*i.e.*, biparental inbreeding) than maternal
322 individuals from susceptible populations. These results overall show that the mating system is

323 altered as populations increase in resistance level, indicating that the mating system may co-
324 evolve with resistance.

325 There are currently few examinations of the mating system of xenobiotic tolerant or
326 resistant species for comparison. In the most relevant example to date, metal-tolerant populations
327 of the grass species *Anothoxanthum odoratum* and *Agrostis tenuis* exhibit higher self-fertility
328 compared to nearby susceptible pasture populations (Antonovics 1968; Antonovics *et al.* 1971).
329 Theoretical work by the same authors suggested that higher rates of selfing in the metal-tolerant
330 populations should evolve to reduce the influx of non-adapted genotypes (*i.e.*, the prevention of
331 gene flow hypothesis) (Antonovics 1968); however, no potential mechanism was identified.
332 Indeed, our findings of lower outcrossing in herbicide resistant populations along with a
333 relationship between floral traits and resistance (*discussed below*) supports the prevention of
334 gene flow hypothesis. Mechanisms that promote the self-pollination of adapted individuals
335 would reduce the level of gene flow from non-adapted individuals (Levin 2010), thus ensuring
336 that the offspring produced in novel or stressful/marginal environments are likewise stress-
337 tolerant (Levin 2010). In this way, the increased self-fertilization of adapted types is
338 hypothesized to lead to reproductive isolation between adapted and non-adapted individuals
339 (Antonovics 1968; McNeilly & Antonovics 1968), and potentially promote niche differentiation
340 (Levin 2010). Such shifts toward a higher propensity to self-fertilize in stressful habitats may not
341 be unusual in nature, as a higher propensity to self has been identified in metal tolerant
342 populations of *Armeria maritime* (Lefebvre 1970) and *Thlaspi caerulescens* (Dubois *et al.* 2003)
343 as well as serpentine tolerant *Mimulus* (Macnair & Gardner 1998). We emphasize, however, that
344 the above cases compare the ability to produce seed autonomously in greenhouse conditions
345 between adapted and non-adapted individuals, which does not always correlate with selfing rates

346 in nature, whereas our work presents estimates of the outcrossing rate of *I. purpurea* sampled
347 from natural conditions.

348 Our data are likewise consistent with the reproductive assurance hypothesis. Originally
349 proposed by Baker (1955), this hypothesis predicts greater selfing ability in colonizing species
350 since they are likely mate-limited when arriving to new areas. Reproductive assurance through
351 self-fertility is broadly, although not ubiquitously, supported by empirical research in other plant
352 systems, such as small or pollinator-limited populations of *Capsella* (Foxe *et al.* 2009; Guo *et al.*
353 2009), *Leavanworthia* (Busch *et al.* 2011) and *Clarkia* (reviewed in Busch & Delph 2012).
354 Agricultural weeds that experience selection from herbicide application or population reduction
355 *via* other means, such as tilling, are analogous to species that colonize novel or new habitats. For
356 example, in a scenario in which resistance alleles are at low frequency in the population and
357 strong selection *via* herbicide application significantly reduces the population size, individuals
358 that survive and re-colonize crop fields will then likely be mate limited. Resistant individuals
359 with a higher propensity to self-pollinate would thus be at a relative advantage compared to those
360 that cannot self-pollinate.

361 Another potential explanation for a relationship between higher selfing and resistance is
362 the 'segregation effect,' wherein an allele that causes higher selfing enhances segregation and
363 forms associations with homozygotes for beneficial (and other) alleles. As selfing modifiers
364 become associated with the beneficial mutation, selfing individuals respond quickly to selection,
365 which will then lead to even higher rates of selfing in the population (Uyenoyama & Waller
366 1991). In support of the segregation effect, recent multilocus simulations of the causes and
367 consequences of selfing find a shift from outcrossing to high levels of selfing following the
368 introduction of large-effect beneficial mutations, so long as the beneficial mutations have

369 moderate to large fitness effects (Kamran-Disfani & Agrawal 2014). This dynamic is thus
370 proposed for species that are establishing a new habitat, or following episodes of environmental
371 change (Kamran-Disfani & Agrawal 2014) – both of which are experienced by plants exposed to
372 herbicide application. Regardless of whether the data reported herein are best explained by
373 selection for reproductive isolation (Antonovics 1968), reproductive assurance (Pannell *et al.*
374 2015), or the segregation effect (Kamran-Disfani & Agrawal 2014), the patterns we have
375 uncovered among naturally-occurring populations of this common weed show that the plant
376 mating system can be influenced by and evolve rapidly in response to selection following the
377 application of herbicides.
378
379 **Anther-stigma distance co-varies with herbicide resistance**
380 Initially, and in line with the 'prevention of gene flow' hypothesis, we predicted that
381 anther-stigma distance should decrease as the level of herbicide resistance increases, since a
382 lower anther-stigma distance leads to an increased rate of selfing in this species (Chang and
383 Rausher 1997). Strikingly, we found a significant negative quadratic relationship between ASD
384 and resistance but no evidence of a negative linear relationship across all populations as
385 uncovered in the mating system data. Specifically, at very low levels of resistance (e.g. from 0 to
386 30% survival within the population), anther stigma distance increased with the level of
387 resistance, but after 30% resistance, ASD decreased as resistance increased. One interpretation of
388 this pattern is that at low levels of resistance, inbreeding depression in selfed progeny may lead
389 to selection against low-ASD types whereas at higher levels of resistance, increased selfing due
390 to low ASD confers a fitness advantage (i.e., reducing the influx of non-adapted alleles) that is
391 greater than the cost associated with inbreeding.

392 An alternative explanation for the pattern between ASD and resistance is that low-ASD
393 may be favored in both low and high resistant populations due to some other agent of selection
394 that is potentially similar between the two types of populations. This explanation is less likely
395 than the first, however, since low-ASD populations that are low- or high- resistance are both
396 found in very different areas of the landscape – both R and S populations are from TN and
397 NC/SC – where edaphic factors such as elevation and rainfall are very different (see Fig. 1).
398 Further, although we found no evidence of a negative linear relationship between ASD and
399 resistance across all sampled populations, the ASD of the least resistant populations was twice as
400 large as the ASD from the most resistant populations (ASD, <20% resistant, N = 6: 1.00 ±0.10
401 (mm); >80% resistant, N = 4: 0.50 ±0.20 (mm)). Thus, despite the initial increase in ASD with
402 resistance, the most resistant populations exhibit significantly lower ASD than the least resistant
403 populations. As above, we note that for the mating system and associated floral traits to evolve in
404 response to resistance evolution, the benefit of producing selfed progeny should outweigh any
405 associated cost of inbreeding. While previous work has shown evidence of inbreeding depression
406 in a single population of this species (Chang and Rausher 1999a), the level of inbreeding
407 depression discovered was not strong enough to counteract the transmission advantage of selfing
408 (i.e., delta < 0.50; Chang and Rausher 1999a). Further work will thus be required to determine if
409 the costs of inbreeding relative to the benefits of selfing are responsible for the changes to the
410 mating system and ASD that we describe herein.
411 Unlike reports from other weedy species (e.g. *Eichhornia paniculata,* Vallejo-Marín &
412 Barrett 2009), we uncovered no evidence that decreases in ASD are the result of increased
413 stamen length, and further, we found only marginal evidence that pistil height differences among

414 populations may explain the pattern between ASD and resistance. Although we cannot identify
415 which component of the composite trait ASD is responsible for the pattern uncovered with
416 resistance, we do have evidence to suggest the relationship is more pronounced in the more
417 recent population sampling (in 2012 versus 2003). While the majority of the populations sampled
418 in 2003 had experienced glyphosate application (Kuester *et al.* 2015b), they further experienced
419 consistent glyphosate application between sampling periods, and on average, populations in 2012
420 exhibit slightly higher levels of resistance compared to the same populations from 2003 (Kuester
421 *et al.* 2015b). Interestingly, we uncovered a significant interaction between sampling year and
422 resistance for pistil height: the relationship between pistil height and resistance was negative in
423 2012 but positive in 2003. This again suggests that perhaps the pistil length is co-evolving with
424 resistance. However, further work targeting the nature of the correlation between these two traits
425 will be required to allow us to draw this conclusion with certainty.

426 As hypothesized above, the responses to selection and subsequent changes to the mating
427 system and associated floral traits that we have identified may be adaptive and due to a genetic
428 basis, or could alternatively be due to plastic changes influenced by the environment (Rick *et al.*
429 1977; Brock & Weinig 2007; van Kleunen 2007; Vallejo-Marín & Barrett 2009). Several lines of
430 evidence suggest that adaptation is the more likely cause for the patterns observed in this study.
431 While we present field estimates of the mating system among populations, we measured floral
432 morphology in a completely randomized greenhouse experiment using replicate maternal lines
433 from many populations, with all individuals experiencing a common environment. Thus, the
434 differences we report in ASD among populations very unlikely to be explained by different
435 environmental factors from different populations. Further, both anther-stigma distance and
436 glyphosate resistance have an additive genetic basis in this species (Chang & Rausher 1998;

437 Baucom & Mauricio 2008; Debban *et al.* 2015), both traits respond rapidly to artificial selection
438 (Chang & Rausher 1998; Debban *et al.* 2015), and populations sampled for the work reported
439 herein were all from agricultural fields with a history of glyphosate application. Taken together,
440 these data suggest that the co-variation we have uncovered between floral phenotypes and
441 resistance across many natural populations of this weed is likely due to adaptive changes
442 following selection rather than plasticity in either the mating system or the level of ASD.
443

444 **Future directions & conclusions**

445 Although anecdotal reports suggest many herbicide resistant plants are predominantly
446 selfing (Jasieniuk *et al.* 1996), ours is the first to identify co-variation between herbicide
447 resistance and estimates of the outcrossing rate, thus providing empirical evidence that the
448 mating or breeding system of a plant may co-evolve with resistance. We note, however, that the
449 results we present cannot address the causal nature of the relationship between resistance and the
450 mating system; although we discuss the dynamic as if the mating system co-evolves in response
451 to the evolution of resistance, it is entirely possible that a highly selfing mating system is
452 responsible for, or has maintained, the high levels of resistance within some populations. Further,
453 like that described in the 'segregation effect' hypothesis, the evolution of the two traits may be
454 intertwined such that resistance evolves first, following which selfing modifiers become linked
455 to the beneficial resistance allele which then leads to higher rates of selfing evolving in the
456 population. Future work will thus be required to disentangle the nature of the relationship
457 between resistance and the mating system.

458 Overall, we have demonstrated that individuals from herbicide resistant populations self
459 more than those from susceptible populations in natural settings and that low anther-stigma

distance may be a potential mechanism underlying this increased rate of selfing. Our work identifies human impacts on plant mating patterns that go beyond the indirect consequences of environmental manipulations such as forest fragmentation and metal contamination. Changes in mating systems can have cascading effects on the effective population size (Nunney 1993), gene flow and the genetic diversity of natural populations (Hamrick & Godt 1996) and can determine the overall evolutionary propensity of these species. Our findings thus highlight the importance of considering the influence of human-mediated selection on correlated responses of natural populations that can lead to long-term evolutionary consequences. Likewise, these results show that associations between highly beneficial traits and plant reproduction can occur rapidly within ecological timescales. The results of our work are thus applicable to other scenarios of strong selection such as climate change or scenarios wherein individuals of colonized populations are mate-limited.

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689 **Tables**

690 Table 1. Results of separate linear and quadratic regressions testing the influence of resistance on
691 the outcrossing rate (t) and the maternal inbreeding coefficient (F). Coefficients from the
692 quadratic regressions included the linear term whereas coefficients from the linear regressions
693 were determined without the quadratic term. Effects that are significant ($P < 0.05$) are bolded.

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	Outcrossing rate (t)				Maternal inbreeding coefficient (F)			
Effect	Coefficient (SE)	Df	F	P	Coefficient (SE)	Df	F	P
Resistance Level	-0.30 (0.09)	1, 22	10.694	**0.004**	0.14 (0.06)	1, 22	6.051	**0.022**
Resistance Level^2	-0.77 (0.35)	1, 21	5.084	**0.035**	0.13 (0.22)	1, 21	0.335	0.569

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714 Table 2. Results of separate ANOVAs testing the influence of population resistance level,
715 resistance level^2, population latitude and experimental replicate on anther-stigma distance (cm),
716 pistil height (cm) and height of tallest stamen (cm). Significant effects ($P < 0.05$) are bolded
717 whereas an '^' indicates a trend for significance.
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	Anther-stigma distance (cm)			Tallest stamen height (cm)			Pistil height (cm)		
Effect	Df	F	P	Df	F	P	Df	F	P
Resistance Level	1	0.724	0.400	1	0.124	0.727	1	0.860	0.359
Resistance Level^2	1	5.703	**0.022**	1	0.265	0.610	1	2.955	0.093^
Latitude	1	0.422	0.520	1	3.509	0.068^	1	6.392	**0.015**
Replicate	1	2.688	0.109	1	6.140	**0.017**	1	1.238	0.272
Residuals	42			42			42		

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741 **Figures**

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743 Figure 1. Map of populations sampled within the USA, **a**, and image of anther-stigma distance in

744 *I. purpurea,* **b**. Floral morphology was measured for all populations (N = 32) whereas the mating

745 system was estimated for populations indicated by triangles (N = 24). The color indicates the

746 resistance level for each population based on proportion survival following application of 1.7 kg

747 ai/ha of herbicide, which is slightly higher than the recommended field dose of herbicide (from

748 Kuester *et al.* 2015a). Sites were sampled at least 5 km apart.

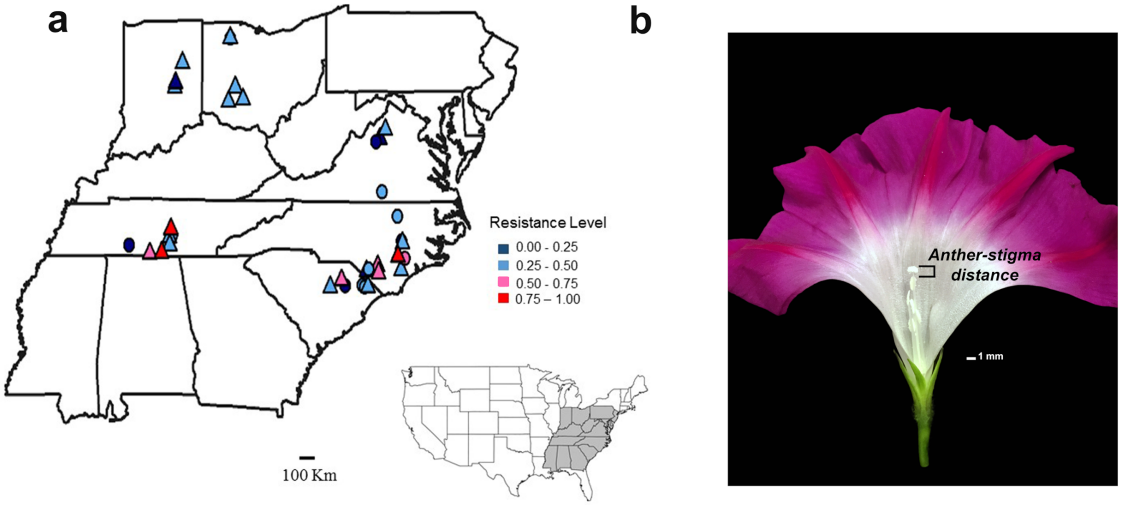

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Figure 2. The relationship between mating system parameters and the proportion survival of each population following application of 1.7 kg ai/ha of herbicide in *I. purpurea*. **a**, outcrossing rate (t), **b**, mean inbreeding coefficient of maternal individuals (F). Significance is indicated by a regression line. A significant negative linear (dashed line) and quadratic (solid line) relationship was detected for the outcrossing rate whereas a significant positive linear relationship was uncovered for the maternal inbreeding coefficients (Table 1).

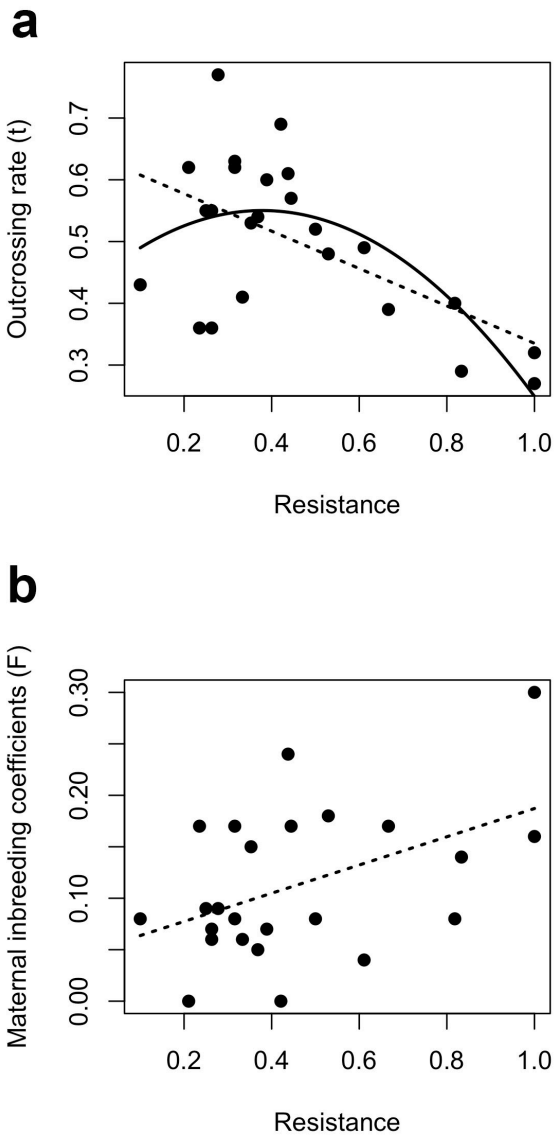

Figure 3. Anther-stigma distance varies non-linearly with the level of resistance. Thirty-two populations sampled as seed in 2012 were used in this analysis to determine if floral traits co-varied with variation in the level of glyphosate resistance. Shown are the residuals, averaged by population, after removing variation due to sampling date for **a**, anther-stigma distance (cm), **b**, height of the tallest stamen (cm), and **c**, pistil height (cm) according to resistance level at 1.7 kg ai/ha. A significant relationship between the trait and resistance is indicated by a solid line ($P < 0.05$) whereas a dashed line indicates a trend for significance $P < 0.10$ (see Table 2).

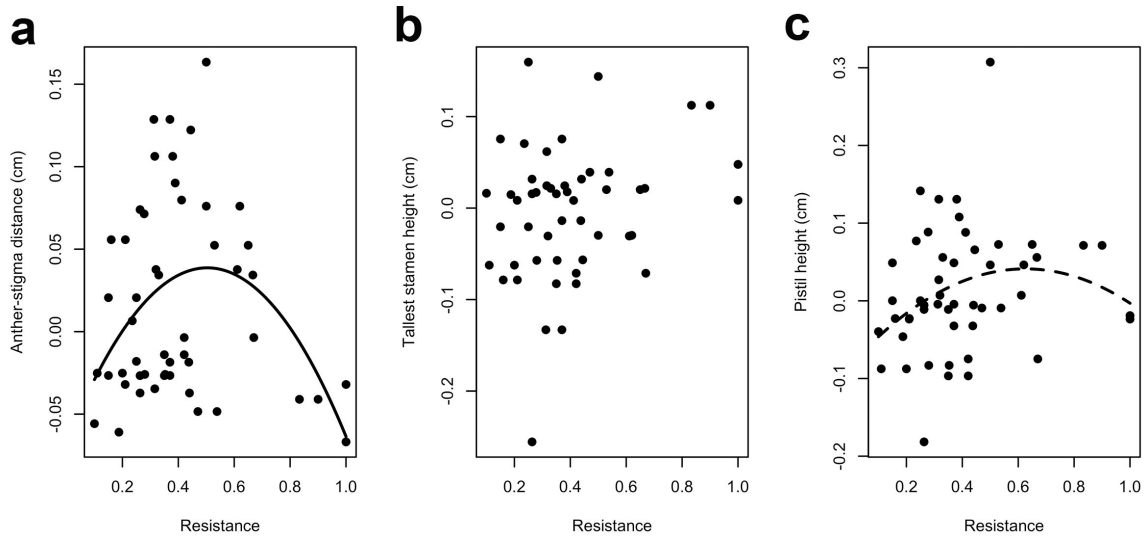
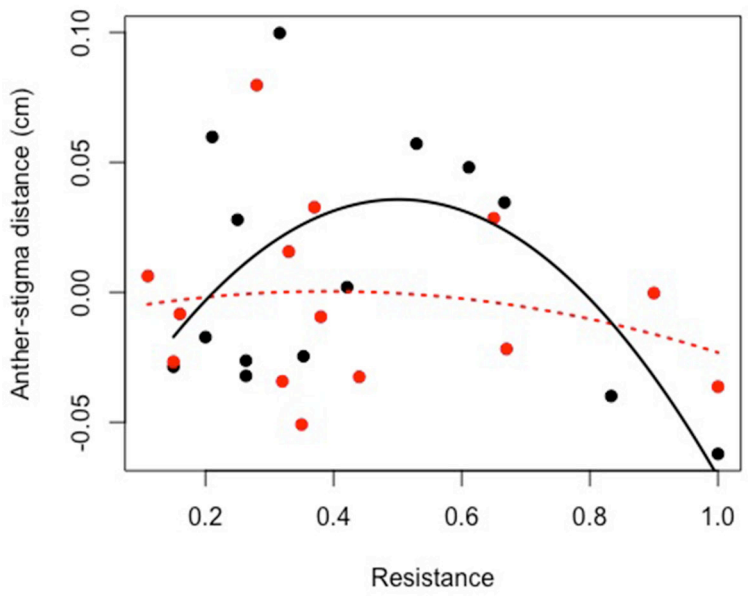
Figure 4. Anther-stigma distance (ASD) varies non-linearly with the level of resistance in 2012 (black dots; solid line) but not 2003 (red dots; dashed line). Fifteen populations sampled as seed in 2012 and 2003, respectively, were used for this analysis to determine if co-variation between ASD and glyphosate resistance varied between years. Shown are the residuals of anther-stigma distance (cm) according to resistance level at 1.7 kg ai/ha (averaged by population after removing variation due to sampling date). A significant quadratic relationship between ASD and resistance is present among populations sampled in 2012 (solid black line: $\beta \pm$ SE, 2012: -0.48±0.23; $F_{1,22} = 6.07$, $P = 0.02$) but not in 2003 (dashed red line: $\beta\pm$ SE, 2003: -0.16±0.19; $F_{1,22} = 1.08$, $P = 0.31$).