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2 **Received Date: 27-Jun-2016**
3 **Revised Date: 06-Sep-2016**
4 **Accepted Date: 18-Oct-2016**
5 **Article Type: Letters**
6 **Article Type: Letter**
7 **Title:** Shifts in outcrossing rates and changes to floral traits are associated with the evolution of
8 herbicide resistance in the common morning glory

9
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11 **Keywords:** outcrossing rate, resistance, human-mediated selection, mating system

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16
17 Number of words in the abstract: 153

18 Number of words in the main text: 5130

19 Number of references: 56

20 Number of figures and tables: 4 figures and 2 tables

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This is the author manuscript accepted for publication and has undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the [Version of Record](#). Please cite this article as [doi: 10.1111/ele.12703](https://doi.org/10.1111/ele.12703)

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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.

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Introduction

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

The plant mating system, or the relative rate of outcrossing versus selfing, is a labile trait (Barrett 1998; Karron *et al.* 2012) that is influenced by human impacts on natural ecosystems (Eckert *et al.* 2010). Habitat fragmentation by deforestation, for example, reduces the outcrossing rate of many forest tree species (Aguilar *et al.* 2006; Eckert *et al.* 2010). Further, plants tolerant to heavy-metal contaminated soils exhibit higher rates of autonomous self-pollination than non-adapted biotypes (Antonovics *et al.* 1971), strongly suggesting that the mating system is either influenced by or concomitantly evolves in response to heavy metal exposure (Antonovics 1968; Antonovics *et al.* 1971). We predict that the mating system of an agricultural weed should co-

92 vary with the level of herbicide resistance in nature, for two main reasons. First, the reproductive
93 assurance hypothesis (Baker 1955, 1974; Goodwillie *et al.* 2005; Pannell *et al.* 2015) would
94 predict resistant individuals, in a mate-limited population following herbicide application, are
95 more likely to produce progeny if they are also highly self-pollinating rather than outcrossing.
96 Second, resistant types that self-pollinate would effectively reduce the influx of non-adapted,
97 susceptible alleles – otherwise known as the ‘prevention of gene flow’ hypothesis (Antonovics
98 1968). Both hypotheses predict that herbicide resistant individuals should self to a higher degree
99 than non-resistant individuals. Interestingly, while many herbicide resistant weed species are
100 reported to be predominantly selfing (Jasieniuk *et al.* 1996), there are no investigations, to our
101 knowledge, that examine the potential for co-variation between mating system and herbicide
102 resistance in nature, a finding that would indicate the mating system may co-evolve when
103 populations respond to the strong selection imparted by herbicides.

104 *Ipomoea purpurea*, an annual weed of agricultural fields and disturbed sites in the
105 southeastern and Midwest US, is a model for examining persistence in stressful and competitive
106 environments (Baucom *et al.* 2011; Chaney & Baucom 2014). As such the species is a
107 particularly relevant candidate for studying how mating systems may evolve under regimes of
108 human-mediated selection. Populations of this agricultural weed have been exposed consistently
109 to the application of glyphosate, the active ingredient in the herbicide RoundUp, since the late
110 1990’s given the widespread adoption of RoundUp Ready crops in the US (NASS 2015).
111 Populations vary for the level of resistance to glyphosate across its North American range; while
112 some populations of *I. purpurea* exhibit 100% survival following application of the field-dose of
113 the herbicide, other populations exhibit high susceptibility (Fig. 1a) (Kuester *et al.* 2015a). In
114 addition, individuals of this mixed-mating, hermaphroditic species (Ennos 1981; Brown & Clegg
115 1984) with a smaller anther-stigma distance (ASD, the distance between the tallest anther and the
116 stigma; Fig. 1b) self-pollinate more often than individuals with a larger ASD (Ennos 1981;
117 Chang & Rausher 1999). Because both anther-stigma distance and glyphosate resistance in this
118 species are heritable and respond to selection (Ennos 1981; Chang & Rausher 1999; Debban *et*
119 *al.* 2015) this common agricultural weed provides a unique opportunity to examine the potential
120 that mating systems and associated reproductive traits evolve in response to extreme regimes of
121 selection imposed by herbicide.

122 Here, we determine if the mating system of *Ipomoea purpurea* co-varies with herbicide
123 resistance, and if reproductive traits associated with self-fertilization are likewise influenced by
124 resistance status. We previously estimated the percent survival of populations following
125 herbicide application using a replicated dose-response greenhouse experiment with individuals
126 sampled as seed from the field in 2012. We used these population-level estimates of survival as
127 each population's resistance level (Fig. 1a) (Kuester *et al.* 2015a), and determined if molecular-
128 marker based estimates of the mating system and measurements of floral morphology co-varied
129 with the level of herbicide resistance in natural populations. Further, our experimental
130 populations were sampled twice, once in 2003 and again in 2012 from the same location (Fig.
131 1a), allowing us to examine the hypothesis that floral traits have changed in these populations
132 over time. We predict that populations with a high level of glyphosate resistance should exhibit
133 evidence of reduced outcrossing and reductions in the anther-stigma distance – either of which
134 would indicate that plant reproductive traits exhibit correlated evolution in response to strong
135 selection from herbicide application.

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138 **Materials and Methods**

139 **Mating system estimates.** We performed a progeny array analysis to estimate the mating system
140 of 24 populations located in the southeastern and Midwestern US (indicated by triangles in Fig.
141 1a). These populations are part of a previous study in which we screened for glyphosate
142 resistance, defined as the proportion each population that survived the application of 1.7 kg
143 a.i./ha RoundUp (Kuester *et al.* 2015a), a rate that is slightly higher than the current
144 recommended field dose. One seed randomly selected from an average of 11 fruits per maternal
145 line from an average of 19 maternal lines per population were used to estimate mating system
146 parameters (see Table S1 in Supporting Information for numbers of maternal lines and progeny
147 per population). Maternal plants were sampled at least two meters apart within each agricultural
148 field. DNA was extracted from seedling cotyledon tissues using a CTAB method (T Culley, pers.
149 comm.). In total, 4798 progeny were genotyped with fifteen previously described microsatellite
150 loci for maternal-line estimates of the mating system (see Kuester *et al.* 2015a) for specific
151 details of the PCR conditions). All sampled genotypes were analyzed using Applied Biosystems
152 PeakScanner 1.0 analytical software (Carlsbad, CA) with a PP (Primer Peaks adjustment) sizing
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153 default, and scoring was double-checked manually for errors in a random sub-sample of 200
154 individuals. We examined our ability to assign parentage using Cervus (Kalinowski *et al.* 2007)
155 and determined population-level estimates of genetic diversity and inbreeding using GenAlEx
156 (Peakall & Smouse 2012). We estimated mating system parameters using BORICE (Koelling *et*
157 *al.* 2012), which is a Bayesian method to estimate the family-level outcrossing rate (t) and
158 maternal line inbreeding coefficients (F) (Koelling *et al.* 2012). BORICE is reported to perform
159 well when either family sizes are small or maternal genotypes are unavailable (Koelling *et al.*
160 2012). We used the following default parameters when estimating mating system parameters: 1
161 million iterations and 99,999 burn-in steps, an outcrossing rate tuning parameter of 0.05, an
162 allele frequency tuning parameter of 0.1, and an initial population outcrossing rate of 0.5. We
163 examined the possibility that null alleles influenced our mating system estimates by re-running
164 all analyses after excluding 4 loci that potentially exhibited null alleles (i.e., loci with ~25% null
165 alleles: IP18, IP1, IP26 and IP42) as indicated by MicroChecker (Van Oosterhout *et al.* 2004).
166 We found no evidence that null alleles impacted estimates across populations (correlation
167 between outcrossing rates for all loci and 4 loci removed: $r = 0.94$, $P < 0.001$) and thus report
168 results using all 15 loci.

169
170 **Floral phenotypes.** We performed a replicated greenhouse experiment to determine if floral
171 morphology varied according to resistance level and to determine if floral traits differed between
172 two sampling years (2003 and 2012). Seeds were sampled from maternal plants from 32
173 randomly chosen populations located in the Southeast and Midwest US in the fall of 2012 (all
174 populations in Fig. 1a); fifteen of these populations had been previously sampled in 2003 (see
175 Table S2). We planted seeds from between 1-29 maternal lines (average=12.74, median=13; see
176 Table S2 for number of individuals) from each population and each sampling year in 4-inch pots
177 in a completely randomized design at the Matthaei Botanical Gardens at the University of
178 Michigan (Ann Arbor, MI). To increase overall sample size, a second replicate experiment was
179 started two weeks later in the greenhouses for a total of 640 experimental plants. Once plants
180 began flowering, we measured the height of the pistil (cm) and the tallest stamen (cm) to
181 estimate anther-stigma distance (ASD) of an average of 5.5 flowers per plant across 17 sampling
182 dates. An average of 2 flowers were measured from each plant each sampling date;
183 measurements taken on multiple flowers per plant per sampling date were averaged prior to
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184 analysis. Over the course of the experiment, we measured 3569 flowers from 622 experimental
185 plants. Of the overall 32 populations sampled in 2012 for floral morphology estimates, 23 were
186 likewise represented in the mating system analyses, presented above.

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

205
206 We examined whether floral morphology differed according to the level of resistance first using
207 populations sampled in 2012 ($N = 32$). To do so we performed a multivariate ANOVA with sine-
208 transformed values of each floral phenotype (anther-stigma distance, length of the tallest stamen,
209 and pistil height) as dependent variables in the model and the experimental replicate, resistance
210 level of the population, and latitude of the population as fixed, independent variables. Prior to
211 analysis, we removed the influence of sampling date ($N=17$) by performing a MANOVA with
212 transformed variables and retained the residuals for testing our main effects of interest
213 (experimental replicate, resistance level, latitude). We elected to do so because the influence of
214 sampling date on anther-stigma distance was not one of our primary questions and we noted a

215 highly significant influence of this effect on floral morphology. Preliminary analyses indicated a
216 significant influence of population latitude on floral morphology and thus we elected to include
217 this effect in the MANOVA. Further, because a scatterplot of the relationship between anther-
218 stigma distance and resistance indicated the presence of a non-linear relationship, we included a
219 quadratic term (resistance level²) in this and downstream analyses.

220

221 We next ran separate univariate analyses to determine if ASD, height of the tallest stamen and
222 pistil height varied according to resistance level and if they have changed over sampling years
223 using the lm function of R v 3.1.1 (R Core Team 2013). For each model, the sine-transformed
224 floral trait of interest (ASD, height of tallest stamen, pistil height) was the dependent variable
225 with the experimental replicate, resistance level, resistance level², and population latitude as
226 independent variables in the regression models. We again performed analyses using residuals
227 after removing the effect of sampling date. Similar to the mating system analysis, if the non-
228 linear resistance term proved significant, we used the segmented package to perform a piece-
229 wise regression and examine the potential for two different linear relationships as well as the
230 break point between slopes using an initial breakpoint of 0.40 (Muggeo 2008). To determine if
231 floral morphology had changed between sampling years and/or if floral morphology differed
232 between years differently across resistance levels, we performed the same analyses as above
233 using only populations that were sampled in both 2003 and 2012 (N = 15), including a year term,
234 a year by resistance level term, and a year by resistance level² term in each analysis. In these
235 analyses, we used resistance levels from each population each year of sampling (as reported in
236 Kuester *et al.* 2015b).

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243 **Results**

244

245 **Mating system.** The average combined exclusion probabilities across all populations was high,
246 at greater than 99%, indicating that the fifteen microsatellite loci successfully assigned parentage
247 (see Table S1). Thus, the power is sufficient for estimating outcrossing rates among populations.
248 Values of the outcrossing rate varied substantially across populations (range: 0.27-0.8), with an
249 average value (\pm SE) for the species of 0.50 (\pm 0.03) (Table S3).

250

251 We uncovered a strong and striking negative linear relationship between the average
252 family-level outcrossing rate of each population (Table S3) and the level of glyphosate resistance
253 ($\beta = -0.30 \pm 0.09$; Fig. 2a); individuals from highly resistant populations self-pollinate more than
254 individuals from less resistant populations ($F = 10.70$, $P = 0.004$; Table 1). We also uncovered a
255 significant negative quadratic relationship between resistance and the outcrossing rate,
256 suggesting that outcrossing first increases at low levels of resistance and then declines as
257 resistance increases ($F = 5.08$, $P = 0.035$; Table 1). Piece-wise regression analysis indicated,
258 however, that the slope between the outcrossing rate and resistance was positive but not
259 significantly different from zero at low levels of resistance ($\beta = 0.42 \pm 0.36$, 95% CI: -0.34, 1.17)
260 whereas the slope following the estimated break point (0.42 ± 0.07) was negative and
261 significantly different from zero ($\beta = -0.57 \pm 0.14$, 95% CI: -0.28, -0.86). In line with our finding
262 of lower outcrossing in highly resistant populations, we found maternal inbreeding coefficients
263 to increase linearly as the level of resistance increases (Fig. 2b, $F = 6.05$, $P = 0.02$; Table 1). We
264 found no evidence of a nonlinear relationship between the maternal inbreeding coefficients and
265 the level of resistance. Together, these results demonstrate that outcrossing rates were lower
266 and maternal inbreeding coefficients higher in glyphosate resistant compared to susceptible
267 populations.

268

269 **Floral morphology.** A multivariate analysis of variance indicated that floral morphology related
270 to selfing rates (anther-stigma distance (ASD), height of the pistil and the tallest stamen) was
271 significantly influenced by both the non-linear resistance term and the latitude of the population
272 (resistance level², Approx. $F = 6.66$, $P < 0.001$; latitude, Approx. $F = 2.58$, $P = 0.05$; Table S4).
273 In separate univariate ANOVAs, we found a negative quadratic relationship between ASD and
274 resistance (resistance level²: $F = 5.70$, $P = 0.02$, Table 2, Fig. 3a) and a trend for a negative
275 quadratic relationship between resistance and pistil height (resistance level²: $F = 2.96$, $P = 0.09$,

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276 Table 2, Fig. 3c) but no quadratic relationship between resistance and stamen height (Fig. 3b).
277 No linear relationships between resistance and the three floral traits were uncovered; however, a
278 piece-wise regression analysis of ASD indicated a positive slope between ASD and resistance at
279 low levels of resistance ($\beta = 0.72 \pm 0.32$, 95% CI: 0.06, 1.37) and a negative slope ($\beta = -0.16$
280 ± 0.07 , 95% CI: -0.01, -0.31) after the estimated breakpoint (0.31 ± 0.05).

281 There was no evidence that ASD either increased or decreased between collection years
282 across the subset of populations sampled in both 2003 and 2012 (Year effect in ANOVA: $F_{1,47} =$
283 0.464 , $P = 0.50$). Further, although we again detected a significant quadratic relationship between
284 ASD and resistance using this subset of populations (resistance level²: $F_{1,47} = 5.82$, $P = 0.02$, Fig.
285 4), we uncovered no evidence that this relationship differed between years (Year by resistance
286 level² effect in ANOVA: $F_{1,47} = 1.16$, $P = 0.28$). However, when examining the relationship
287 between resistance level² and ASD separately between collection years, we found a significant
288 negative quadratic relationship for the 2012 sample (solid line in Fig. 4; $\beta = -0.48 \pm 0.23$, $F_{1,22} =$
289 6.07 , $P = 0.02$) but no evidence for a significant relationship among populations sampled in 2003
290 ($\beta = -0.17 \pm 0.19$, $F_{1,22} = 1.08$, $P = 0.31$). Despite the relative stability of ASD values among
291 populations across this nine-year period (i.e., lack of a year effect), both floral traits comprising
292 ASD – height of the tallest stamen and pistil height – showed a significant (or trend for
293 significant) collection year by resistance level interaction (Pistil height: $F_{1,47} = 4.28$, $P = 0.04$;
294 Tallest stamen height: $F_{1,47} = 3.27$, $P = 0.08$). In the 2012 sample, both the pistil and stamen
295 height decreased as the level of resistance increased (2012: pistil $\beta = -0.08 \pm 0.05$, tallest stamen
296 $\beta = -0.08 \pm 0.05$) whereas the pistil and stamen heights from the 2003 sample increased as
297 resistance increased (2003: pistil $\beta = 0.07 \pm 0.04$, tallest stamen $\beta = 0.06 \pm 0.05$).

298
299

300 Discussion

301 In line with our predictions, we demonstrate that the mating system of the agricultural
302 weed, *Ipomoea purpurea*, co-varies with the level of glyphosate resistance. Specifically, we find
303 that outcrossing rates are lower and maternal inbreeding coefficients are higher in resistant
304 compared to susceptible populations. We likewise find that anther-stigma distance, a floral trait
305 associated with self-fertilization in this species, exhibits a nonlinear relationship with resistance

306 such that the most and least resistant populations exhibit lower anther-stigma separation
307 compared to populations with moderate levels of resistance. Further, this relationship was
308 present among populations sampled in 2012 but not 2003, suggesting that reproductive traits may
309 have rapidly evolved in these populations over the course of nine years. Below, we discuss each
310 of our major findings and place them in the broad context of mating system changes associated
311 with human-mediated selection.

312

313 **Plant mating changes associated with resistance**

314 As anticipated by both the reproductive assurance (Baker 1955, 1974; Goodwillie *et al.*
315 2005; Pannell *et al.* 2015) and ‘prevention of gene flow’ (Antonovics 1968) hypotheses, our
316 work finds a significant negative relationship between the outcrossing rate and level of herbicide
317 resistance across natural populations of an agricultural weed indicating that individuals from
318 highly resistant populations self more often than those from susceptible populations. We likewise
319 uncovered a significant negative nonlinear relationship indicating that the outcrossing rate
320 initially increased at low levels of resistance and then declined as resistance increased. When
321 examining the nonlinear relationship using piecewise regression, however, we found that the
322 initial positive slope was not significantly different than zero whereas the negative linear
323 relationship following the estimated breakpoint was significant. Further, we found a positive,
324 linear relationship between maternal inbreeding coefficients and resistance, indicating that
325 maternal individuals from highly resistant populations were more likely to be the product of self-
326 fertilization or mating between close relatives (*i.e.*, biparental inbreeding) than maternal
327 individuals from susceptible populations. These results overall show that the mating system is
328 altered as populations increase in resistance level, indicating that the mating system may co-
329 evolve with resistance.

330 There are currently few examinations of the mating system of xenobiotic tolerant or
331 resistant species for comparison. In the most relevant example to date, metal-tolerant populations
332 of the grass species *Anothoxanthum odoratum* and *Agrostis tenuis* exhibit higher self-fertility
333 compared to nearby susceptible pasture populations (Antonovics 1968; Antonovics *et al.* 1971).
334 Theoretical work by the same authors suggested that higher rates of selfing in the metal-tolerant
335 populations should evolve to reduce the influx of non-adapted genotypes (*i.e.*, the prevention of
336 gene flow hypothesis) (Antonovics 1968); however, no potential mechanism was identified.

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337 Indeed, our findings of lower outcrossing in herbicide resistant populations along with a
338 relationship between floral traits and resistance (*discussed below*) supports the prevention of
339 gene flow hypothesis. Mechanisms that promote the self-pollination of adapted individuals
340 would reduce the level of gene flow from non-adapted individuals (Levin 2010), thus ensuring
341 that the offspring produced in novel or stressful/marginal environments are likewise stress-
342 tolerant (Levin 2010). In this way, the increased self-fertilization of adapted types is
343 hypothesized to lead to reproductive isolation between adapted and non-adapted individuals
344 (Antonovics 1968; McNeilly & Antonovics 1968), and potentially promote niche differentiation
345 (Levin 2010). Such shifts toward a higher propensity to self-fertilize in stressful habitats may not
346 be unusual in nature, as a higher propensity to self has been identified in metal tolerant
347 populations of *Armeria maritime* (Lefebvre 1970) and *Thlaspi caerulescens* (Dubois *et al.* 2003)
348 as well as serpentine tolerant *Mimulus* (Macnair & Gardner 1998). We emphasize, however, that
349 the above cases compare the ability to produce seed autonomously in greenhouse conditions
350 between adapted and non-adapted individuals, which does not always correlate with selfing rates
351 in nature, whereas our work presents estimates of the outcrossing rate of *I. purpurea* sampled
352 from natural conditions.

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

366 Another potential explanation for a relationship between higher selfing and resistance is
367 the ‘segregation effect,’ wherein an allele that causes higher selfing enhances segregation and
368 forms associations with homozygotes for beneficial (and other) alleles. As selfing modifiers
369 become associated with the beneficial mutation, selfing individuals respond quickly to selection,
370 which will then lead to even higher rates of selfing in the population (Uyenoyama & Waller
371 1991). In support of the segregation effect, recent multilocus simulations of the causes and
372 consequences of selfing find a shift from outcrossing to high levels of selfing following the
373 introduction of large-effect beneficial mutations, so long as the beneficial mutations have
374 moderate to large fitness effects (Kamran-Disfani & Agrawal 2014). This dynamic is thus
375 proposed for species that are establishing a new habitat, or following episodes of environmental
376 change (Kamran-Disfani & Agrawal 2014) – both of which are experienced by plants exposed to
377 herbicide application. Regardless of whether the data reported herein are best explained by
378 selection for reproductive isolation (Antonovics 1968), reproductive assurance (Pannell *et al.*
379 2015), or the segregation effect (Kamran-Disfani & Agrawal 2014), the patterns we have
380 uncovered among naturally-occurring populations of this common weed show that the plant
381 mating system can be influenced by and evolve rapidly in response to selection following the
382 application of herbicides.

383

384 **Anther-stigma distance co-varies with herbicide resistance**

385 Initially, and in line with the ‘prevention of gene flow’ hypothesis, we predicted that
386 anther-stigma distance should decrease as the level of herbicide resistance increases, since a
387 lower anther-stigma distance leads to an increased rate of selfing in this species (Chang and
388 Rausher 1997). Strikingly, we found a significant negative quadratic relationship between ASD
389 and resistance but no evidence of a negative linear relationship across all populations as
390 uncovered in the mating system data. Specifically, at very low levels of resistance (e.g. from 0 to
391 30% survival within the population), anther stigma distance increased with the level of
392 resistance, but after 30% resistance, ASD decreased as resistance increased. One interpretation of
393 this pattern is that at low levels of resistance, inbreeding depression in selfed progeny may lead
394 to selection against low-ASD types whereas at higher levels of resistance, increased selfing due
395 to low ASD confers a fitness advantage (i.e., reducing the influx of non-adapted alleles) that is
396 greater than the cost associated with inbreeding.

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

416 Unlike reports from other weedy species (e.g. *Eichhornia paniculata*, Vallejo-Marín &
417 Barrett 2009), we uncovered no evidence that decreases in ASD are the result of increased
418 stamen length, and further, we found only marginal evidence that pistil height differences among
419 populations may explain the pattern between ASD and resistance. Although we cannot identify
420 which component of the composite trait ASD is responsible for the pattern uncovered with
421 resistance, we do have evidence to suggest the relationship is more pronounced in the more
422 recent population sampling (in 2012 versus 2003). While the majority of the populations sampled
423 in 2003 had experienced glyphosate application (Kuester *et al.* 2015b), they further experienced
424 consistent glyphosate application between sampling periods, and on average, populations in 2012
425 exhibit slightly higher levels of resistance compared to the same populations from 2003 (Kuester
426 *et al.* 2015b). Interestingly, we uncovered a significant interaction between sampling year and

427 resistance for pistil height: the relationship between pistil height and resistance was negative in
428 2012 but positive in 2003. This again suggests that perhaps the pistil length is co-evolving with
429 resistance. However, further work targeting the nature of the correlation between these two traits
430 will be required to allow us to draw this conclusion with certainty.

431 As hypothesized above, the responses to selection and subsequent changes to the mating
432 system and associated floral traits that we have identified may be adaptive and due to a genetic
433 basis, or could alternatively be due to plastic changes influenced by the environment (Rick *et al.*
434 1977; Brock & Weinig 2007; van Kleunen 2007; Vallejo-Marín & Barrett 2009). Several lines of
435 evidence suggest that adaptation is the more likely cause for the patterns observed in this study.
436 While we present field estimates of the mating system among populations, we measured floral
437 morphology in a completely randomized greenhouse experiment using replicate maternal lines
438 from many populations, with all individuals experiencing a common environment. Thus, the
439 differences we report in ASD among populations very unlikely to be explained by different
440 environmental factors from different populations. Further, both anther-stigma distance and
441 glyphosate resistance have an additive genetic basis in this species (Chang & Rausher 1998;
442 Baucom & Mauricio 2008; Debban *et al.* 2015), both traits respond rapidly to artificial selection
443 (Chang & Rausher 1998; Debban *et al.* 2015), and populations sampled for the work reported
444 herein were all from agricultural fields with a history of glyphosate application. Taken together,
445 these data suggest that the co-variation we have uncovered between floral phenotypes and
446 resistance across many natural populations of this weed is likely due to adaptive changes
447 following selection rather than plasticity in either the mating system or the level of ASD.

448 449 **Future directions & conclusions**

450 Although anecdotal reports suggest many herbicide resistant plants are predominantly
451 selfing (Jasieniuk *et al.* 1996), ours is the first to identify co-variation between herbicide
452 resistance and estimates of the outcrossing rate, thus providing empirical evidence that the
453 mating or breeding system of a plant may co-evolve with resistance. We note, however, that the
454 results we present cannot address the causal nature of the relationship between resistance and the
455 mating system; although we discuss the dynamic as if the mating system co-evolves in response
456 to the evolution of resistance, it is entirely possible that a highly selfing mating system is

457 responsible for, or has maintained, the high levels of resistance within some populations. Further,
458 like that described in the ‘segregation effect’ hypothesis, the evolution of the two traits may be
459 intertwined such that resistance evolves first, following which selfing modifiers become linked
460 to the beneficial resistance allele which then leads to higher rates of selfing evolving in the
461 population. Future work will thus be required to disentangle the nature of the relationship
462 between resistance and the mating system.

463 Overall, we have demonstrated that individuals from herbicide resistant populations self
464 more than those from susceptible populations in natural settings and that low anther-stigma
465 distance may be a potential mechanism underlying this increased rate of selfing. Our work
466 identifies human impacts on plant mating patterns that go beyond the indirect consequences of
467 environmental manipulations such as forest fragmentation and metal contamination. Changes in
468 mating systems can have cascading effects on the effective population size (Nunney 1993), gene
469 flow and the genetic diversity of natural populations (Hamrick & Godt 1996) and can determine
470 the overall evolutionary propensity of these species. Our findings thus highlight the importance
471 of considering the influence of human-mediated selection on correlated responses of natural
472 populations that can lead to long-term evolutionary consequences. Likewise, these results show
473 that associations between highly beneficial traits and plant reproduction can occur rapidly within
474 ecological timescales. The results of our work are thus applicable to other scenarios of strong
475 selection such as climate change or scenarios wherein individuals of colonized populations are
476 mate-limited.

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480 **Acknowledgements** We thank A Wilson for assistance and D. Alvando-Serrano, M. Van Etten,
481 T-L Ashmann, V. Koelling, C. Dick, L. Moyle, S.I. Wright, and S.C.H. Barrett for comments on
482 earlier drafts of the manuscript. This work was funded by USDA NIFA grants 04180 and 07191.
483 The authors declare no competing financial interests. Correspondence and requests for materials
484 should be addressed to RSB (rsbaucom@umich.edu). Primary data used in these analyses will be
485 made available in the public github repository <https://github.com/rsbaucom/MatingSystem2015>,
486 which can be anonymously accessed.

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694 **Tables**

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

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Effect	Outcrossing rate (t)				Maternal inbreeding coefficient (F)			
	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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Table 2. Results of separate ANOVAs testing the influence of population resistance level, resistance level², population latitude and experimental replicate on anther-stigma distance (cm), pistil height (cm) and height of tallest stamen (cm). Significant effects ($P < 0.05$) are bolded whereas an ‘^’ indicates a trend for significance.

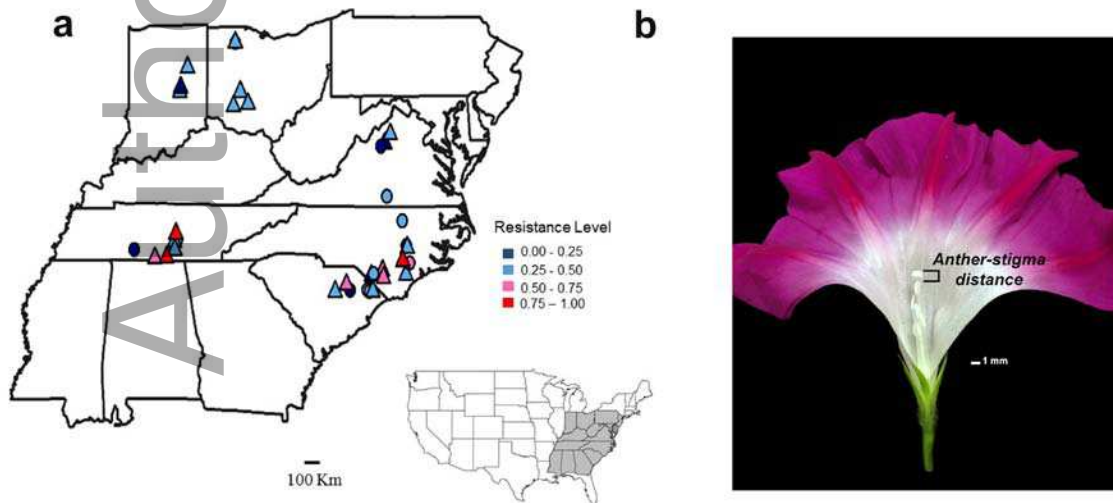
Effect	Anther-stigma distance (cm)			Tallest stamen height (cm)			Pistil height (cm)		
	Df	F	P	Df	F	P	Df	F	P
Resistance Level	1	0.724	0.400	1	0.124	0.727	1	0.860	0.359
Resistance Level ²	1	5.703	0.022	1	0.265	0.610	1	2.955	0.093^
Latitude	1	0.422	0.520	1	3.509	0.068^	1	6.392	0.015
Replicate	1	2.688	0.109	1	6.140	0.017	1	1.238	0.272
Residuals	42			42			42		

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Figures

Figure 1. Map of populations sampled within the USA, **a**, and image of anther-stigma distance in *I. purpurea*, **b**. Floral morphology was measured for all populations (N = 32) whereas the mating system was estimated for populations indicated by triangles (N = 24). The color indicates the resistance level for each population based on proportion survival following application of 1.7 kg ai/ha of herbicide, which is slightly higher than the recommended field dose of herbicide (from Kuester *et al.* 2015a). Sites were sampled at least 5 km apart.

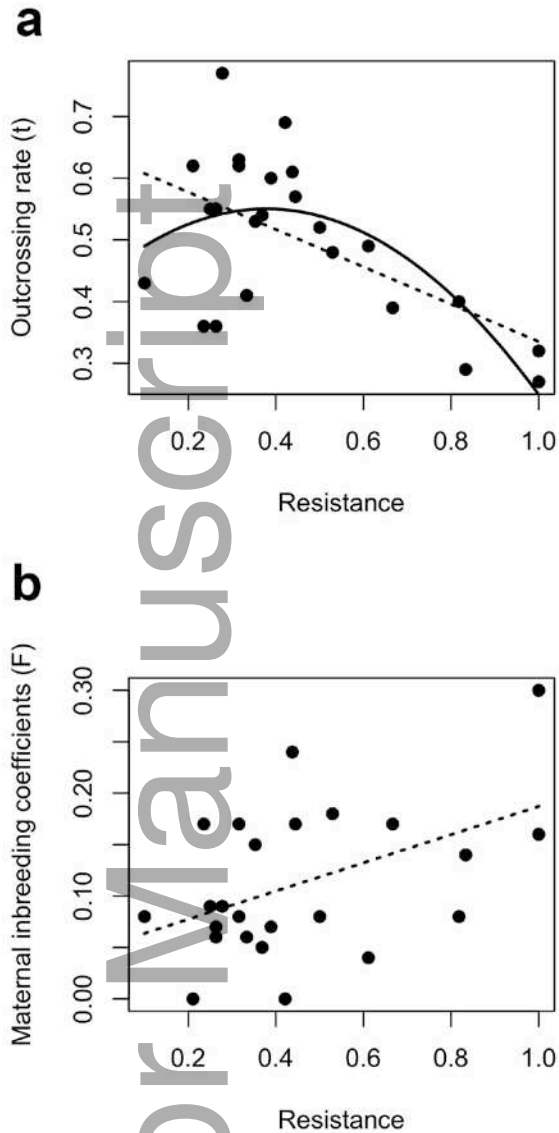


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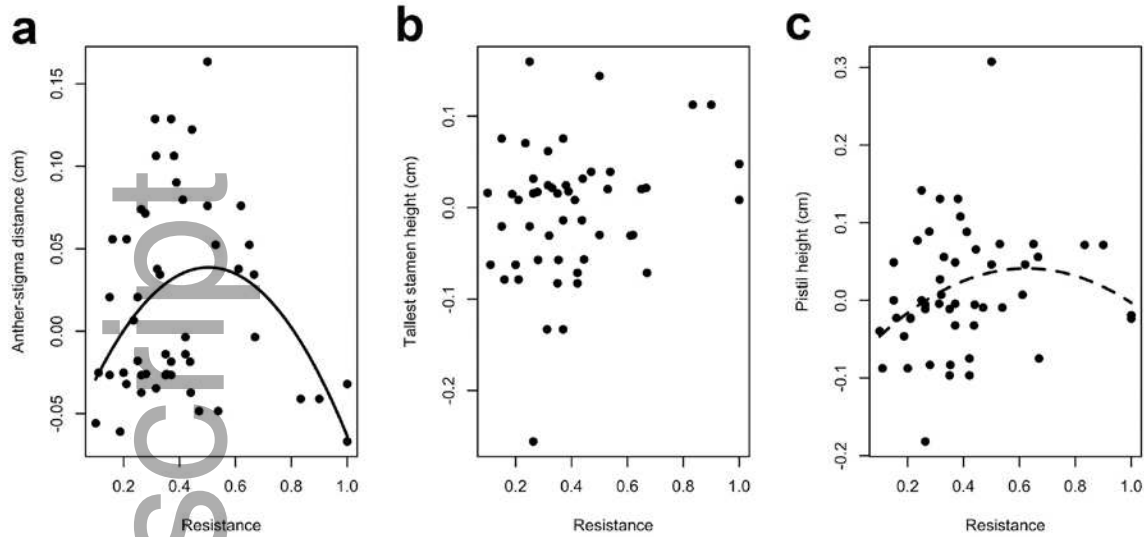
Figure 2. The relationship between mating system parameters and the proportion survival of each population following application of 1.7 kg ai/ha of herbicide in *I. purpurea*. **a**, outcrossing rate (t), **b**, mean inbreeding coefficient of maternal individuals (F). Significance is indicated by a regression 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).

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772 Figure 3. Anther-stigma distance varies non-linearly with the level of resistance. Thirty-two
773 populations sampled as seed in 2012 were used in this analysis to determine if floral traits co-
774 varied with variation in the level of glyphosate resistance. Shown are the residuals, averaged by
775 population, after removing variation due to sampling date for **a**, anther-stigma distance (cm), **b**,
776 height of the tallest stamen (cm), and **c**, pistil height (cm) according to resistance level at 1.7 kg
777 ai/ha. A significant relationship between the trait and resistance is indicated by a solid line ($P <$
778 0.05) whereas a dashed line indicates a trend for significance $P < 0.10$ (see Table 2).

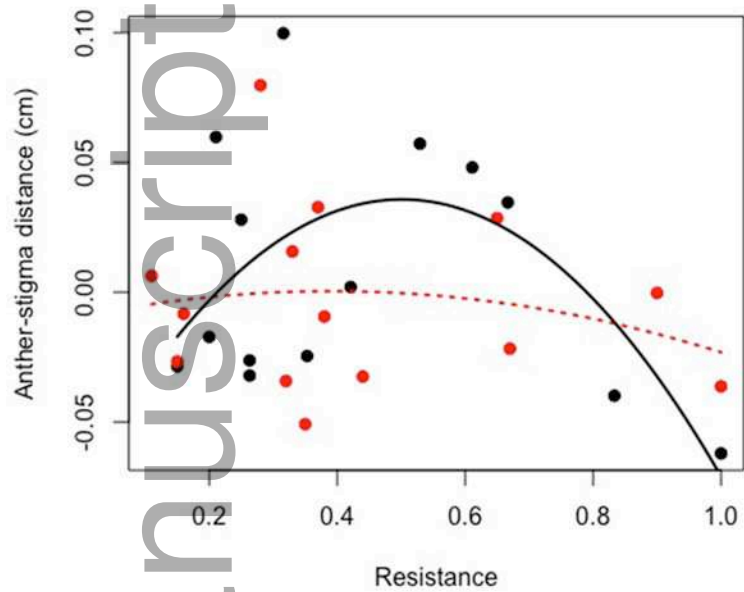


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792 Figure 4. Anther-stigma distance (ASD) varies non-linearly with the level of resistance in 2012
 793 (black dots; solid line) but not 2003 (red dots; dashed line). Fifteen populations sampled as seed
 794 in 2012 and 2003, respectively, were used for this analysis to determine if co-variation between
 795 ASD and glyphosate resistance varied between years. Shown are the residuals of anther-stigma
 796 distance (cm) according to resistance level at 1.7 kg ai/ha (averaged by population after
 797 removing variation due to sampling date). A significant quadratic relationship between ASD and
 798 resistance is present among populations sampled in 2012 (solid black line: $\beta \pm SE$, 2012:

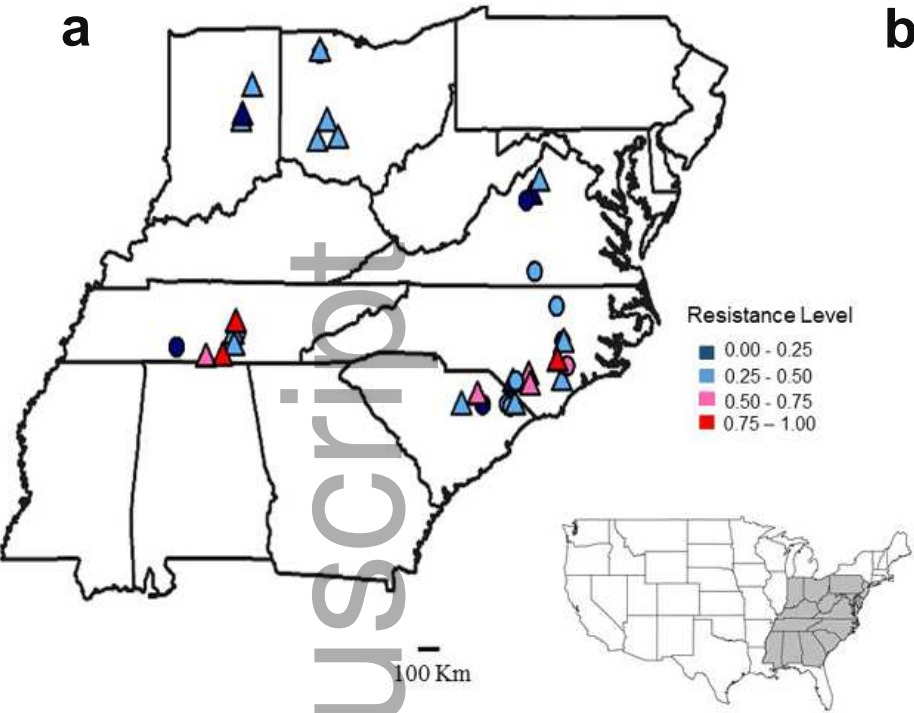
799 -0.48 ± 0.23 ; $F_{1,22} = 6.07$, $P = 0.02$) but not in 2003 (dashed red line: $\beta \pm SE$, 2003: -0.16 ± 0.19 ;
800 $F_{1,22} = 1.08$, $P = 0.31$).

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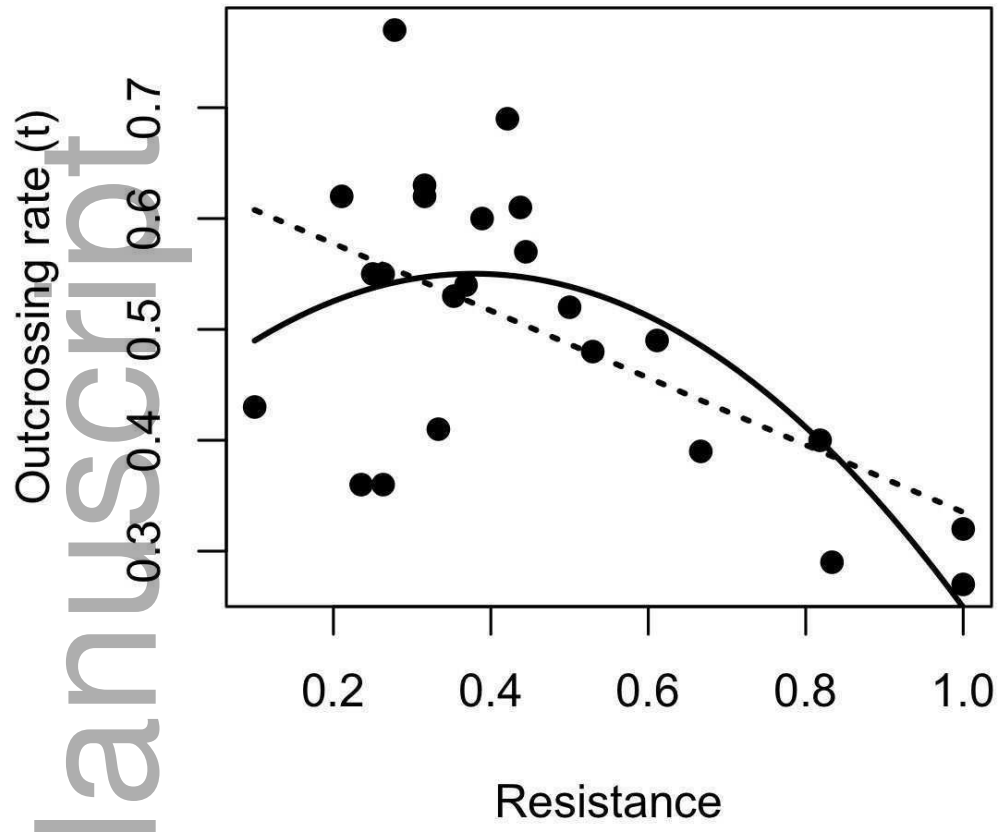
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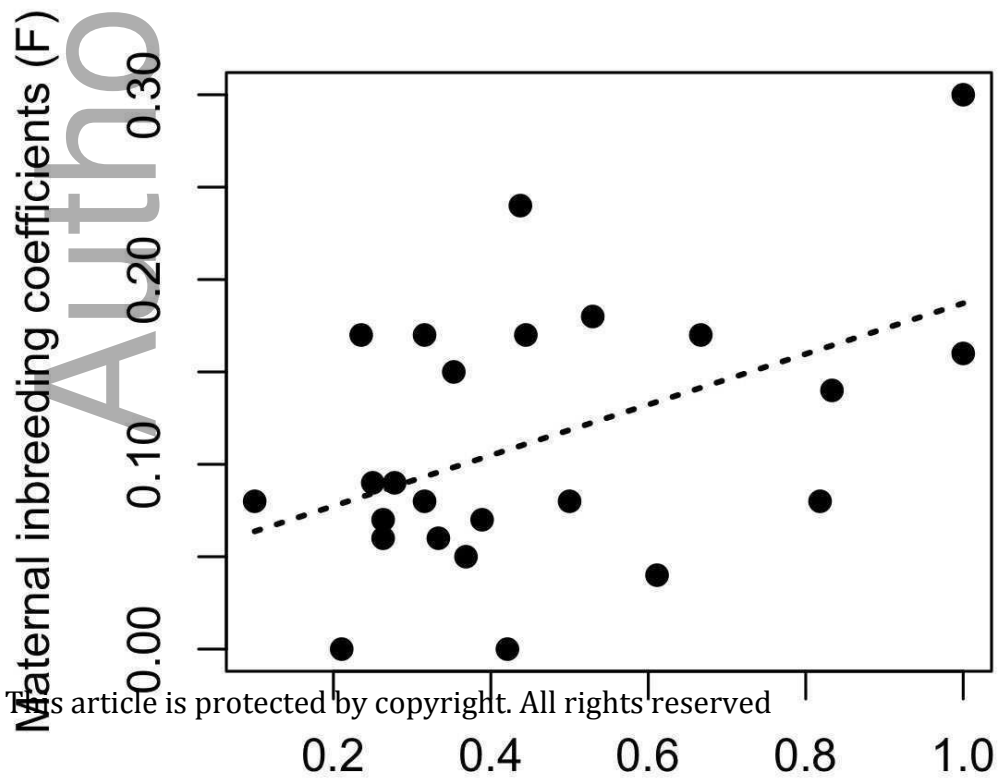
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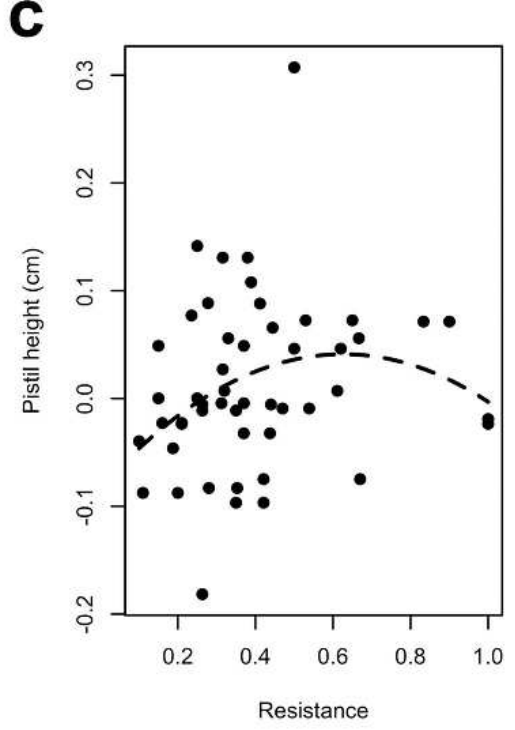
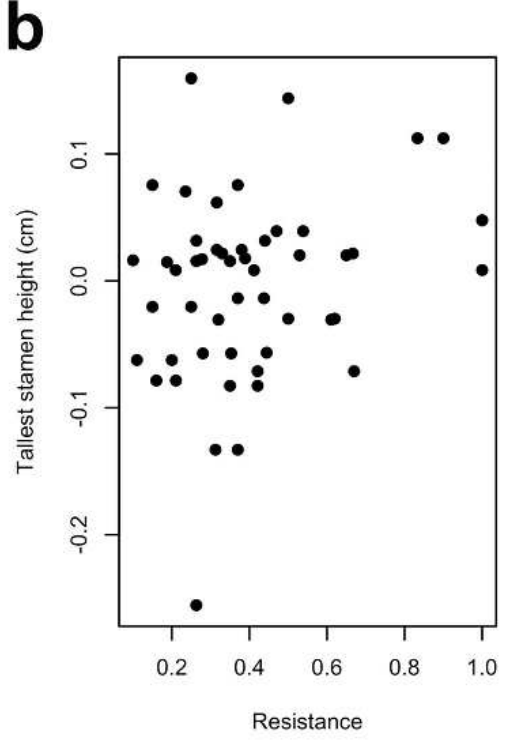
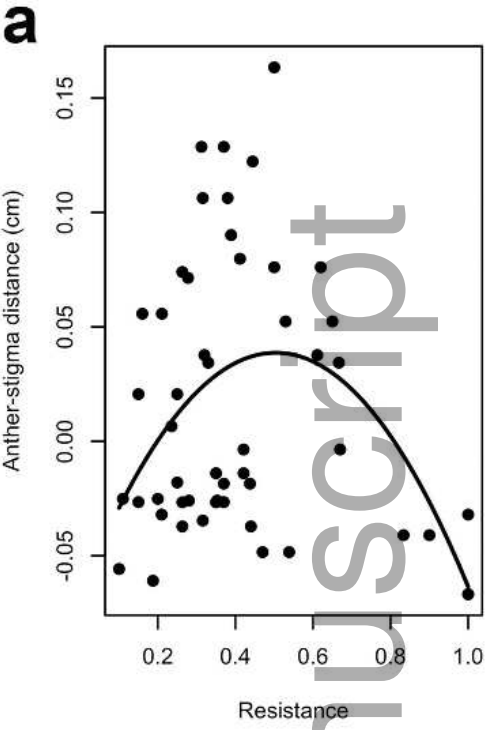
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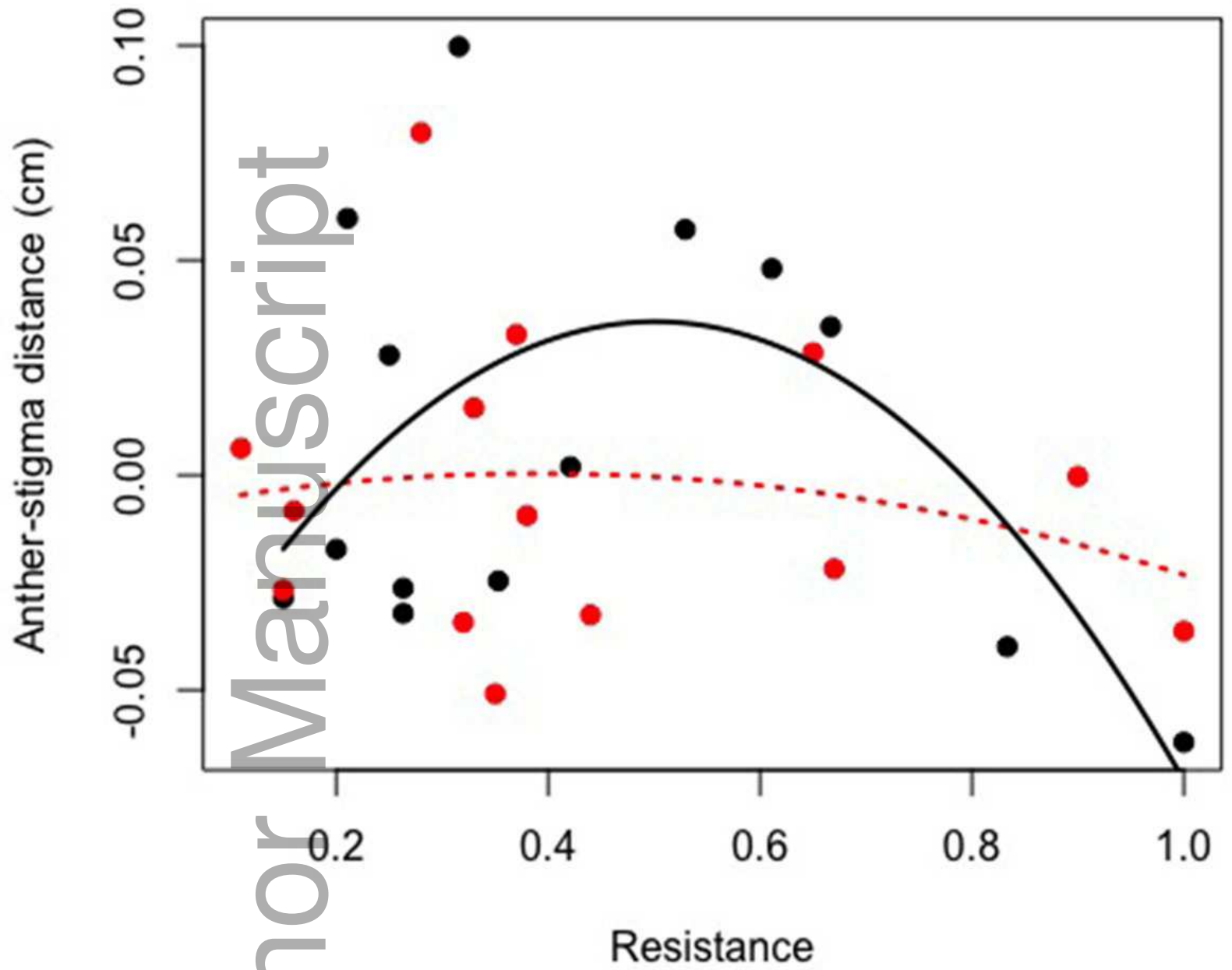


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