Identifying the fitness consequences of sex in complex natural environments

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Abstract

In the natural world, sex prevails, despite its costs. While much effort has been dedicated to identifying the intrinsic costs of sex (e.g. the cost of males), few studies have identified the ecological fitness consequences of sex and the relevant environmental context. Furthermore, correlated traits that differ between sexuals and asexuals may alter these costs, and biological traits may render irrelevant the typical costs of sex. Here we use a large-scale transplant experiment of a North American wildflower to show that sex is associated with reduced lifetime fitness, despite lower herbivory. We separate the effects of sex from the associated trait of hybridity, finding that mortality is reduced in asexuals regardless of parentage, and that herbivores target hybrid asexuals. Our results show that the consequences of sex are shaped by complex natural environments, and suggest that some costs of sex are due to correlated traits, rather than sex itself.

Introduction

Even after over a century of research, the predominance of sexual reproduction in nature remains a fundamental mystery in evolutionary biology. At first glance, asexuality appears a simpler method of reproduction, avoiding the metabolic and energetic costs of mate attraction and the inherent risks to sex. Yet asexual reproduction comes with costs, most of which are attributed to the absence of recombination (Lehtonen *et al.* 2012). Over long time scales, the lack of recombination inhibits the removal of deleterious alleles from their genomic background (Müller 1964) and the union of beneficial ones (Hill & Robertson 1966; Keightley & Otto 2006), ultimately resulting in a mutational load that impairs function and/or fitness (Rice 2002). These costs are substantial in the long term; asexual organisms occur only at the tips of eukaryotic phylogenetic trees (Rice 2002), suggesting they are an evolutionary dead end or "blind alley" (Darlington 1939).

Sex is also inherently costly. Much of the focus on the evolution and maintenance of sex has centered on the "twofold cost of sex," wherein sexual organisms produce half as many offspring as asexuals, due to their investment in male offspring (Maynard Smith 1978). However, additional intrinsic costs exist, including that of "genetic slippage," in which recombination reduces fitness via shuffling of beneficial loci (Lehtonen *et al.* 2012). Yet ultimately, through purging of the mutational load, and rapid unification of beneficial mutations into a single genome, sex and recombination likely facilitate adaptation (Felsenstein 1974).

Despite widespread attention to the intrinsic costs of sex and the genetic processes maintaining sex in natural populations, the broader evolutionary and ecological processes

69 that differ among sexual and asexual organisms have received far less attention. This is a
70 crucial gap in our knowledge, as the processes that govern sexual/asexual dynamics occur
71 in complex natural environments. Our best examples come from decades of research on
72 the freshwater snail *Potamopyrgus antipodes*, establishing trematode parasites as the
73 main selective agent maintaining sexual/asexual coexistence via frequency-dependent
74 selection (Lively 1987; Lively & Dybdahl 2000; Vergara *et al.* 2014). At shallow lake
75 depths, sexual reproduction predominates, while in deeper water, asexuality is
76 widespread (Jokela & Lively 1995). Trematodes target common asexual genotypes,
77 leading to repeated cycles of increasing frequency for rare genotypes (Jokela *et al.* 2009;
78 Koskella & Lively 2009). Taken together, this body of work shows that the abiotic and
79 biotic environment work in concert to shape complex sexual/asexual dynamics in the
80 natural world.

81 Critically, both ecology and its interactions with a system's biological features
82 provide the context necessary to pinpoint the real-life costs and benefits of sex (Neiman
83 *et al.* 2018). For example, knowledge of the asexual formation process, and the
84 environments supporting formation and maintenance of asexuality, may identify
85 additional costs or benefits. These costs, such as that of meiotic function or mate choice
86 (inbreeding, outbreeding, and hybridization) are seldom discussed, but may be crucially
87 important to understanding sex/asex dynamics in natural populations (Meirmans *et al.*
88 2012). Indeed, the ecological costs of sex have rarely been identified in the wild, and the
89 majority of evidence comes from studies of *Potamopyrgus* (Neiman *et al.* 2018).

90 A further complication in our understanding of the evolution of sex is the strong
91 association between asexuality and other traits. Two examples are hybridization and

92 polyploidy, which differ intrinsically between numerous closely related sexual and
93 asexual systems. For example, populations of asexual whiptail lizards in the deserts of the
94 southwestern United States are polyploids, resulting from hybridization between sexual
95 species (Case 1990; Lutes *et al.* 2010). Equivalent trait associations are observed in
96 nearly all asexual plant systems (Asker & Jerling 1992). Indeed, by necessity, costs of
97 sex studies generally ignore these intrinsic differences (Gibson *et al.* 2017). But to
98 understand the evolutionary consequences of sex, we must account for the biological
99 differences between reproductive systems that contribute to the cost of sex.

100 The mustard genus *Boechera* offers an ideal system in which polyploidy and
101 hybridization may be accounted for. Apomixis (asexual reproduction via seed) is
102 widespread in the genus (Böcher 1951; Roy & Rieseberg 1989; Roy 1995; Sharbel &
103 Mitchell-Olds 2001; Schranz *et al.* 2005; Schranz *et al.* 2006; Kantama *et al.* 2007).
104 Asexuals may be polyploid or diploid, enabling separate assessment of the effects of
105 polyploidy and asexuality (Sharbel & Mitchell-Olds 2001; Schranz *et al.* 2005; Kantama
106 *et al.* 2007; Beck *et al.* 2012; Mau *et al.* 2015). Further, asexuality is associated with
107 outcrossing events, whether interspecific or intraspecific (Li *et al.* 2017; Rushworth *et al.*
108 2018). This enables comparison of selection on hybrid asexuals separately from non-
109 hybrid asexuals. Finally, we note that because sexual lineages are highly self-fertilizing,
110 heterozygosity differs between sexuals and asexuals. To our knowledge, this trait is
111 inseparable from reproductive system. Nonetheless, this system enables direct assessment
112 of the impacts of reproduction on fitness while accounting for two key confounding
113 factors, offering an extraordinary opportunity to examine the evolution of sex.

114 Here we address a critical gap in the evolution of sex literature, using a large-
115 scale reciprocal transplant experiment in intact native habitat to identify the fitness
116 components underlying the costs of sex. We examine female lifetime fitness and insect
117 herbivory of several thousand sexual and asexual plants, finding strong over-winter
118 viability selection against sexual lineages. This difference in over-winter survival results
119 in elevated lifetime fitness for asexual lineages, even though asexuals experience
120 significantly more herbivory than sexuals. Importantly, the negative impacts of herbivory
121 appear in later life stages, reducing the probability of survival to a second year of life in a
122 manner consistent with a cost of hybridization. Our study offers unprecedented
123 understanding of population-level sexual/asexual dynamics in a flowering plant,
124 identifying the fitness components that underlie both the costs and benefits of sex in the
125 natural environment.
126
127 **Materials and Methods**
128
129 **Study system**
130 *Boechera retrofracta* (Brassicaceae) is a herbaceous short-lived perennial native to
131 western North America. Its range spans a wide elevational gradient from 300-3300m and
132 environments ranging from desert to sagebrush steppe to high elevation mesic and alpine
133 meadows (Al-Shehbaz & Windham 2010). Recently segregated from four other members
134 of the *Boechera holboellii* complex (Windham & Al-Shehbaz 2006), *B. retrofracta* is
135 often confused with closely related species, but distinguished by its stalked, 2-8 rayed
136 trichomes and steeply recurved pedicels. Multiple species of the genus *Boechera* have

137 proven to be highly self-fertilizing (Hamilton & Mitchell-Olds 1994; Roy 1995; Song *et*
138 *al.* 2006). *B. retrofracta*'s high level of microsatellite homozygosity (mean
139 homozygosity=0.94, Rushworth *et al.* 2018) as well as its minute flowers that self-
140 pollinate prior to anthesis (C. Rushworth, pers. obs.) are likewise consistent with self-
141 fertilization. Nonetheless, *B. retrofracta* has formed hybrids with at least 12 different
142 species across its range (Al-Shehbaz & Windham 2010). Asexuality is tightly associated
143 with outcrossing and hybridization in the genus (Schranz *et al.* 2005; Schranz *et al.* 2006;
144 Kantama *et al.* 2007; Beck *et al.* 2012; Rushworth *et al.* 2018). In this group, apomixis is
145 pseudogamous, requiring fertilization of the endosperm, but not the embryo. Because
146 sexuals are highly selfing and asexuals must produce viable pollen, many of the
147 traditional costs of sex are minimized in this system, allowing investigation of broader
148 ecological and biological factors maintaining sex.
149
150 **Sampling and growth methods**
151 From a collection of 1027 individuals from 129 populations, we selected 53 unique
152 diploid genotypes that reproduce sexually (N=24) and asexually (N=29) for use in a
153 multi-site reciprocal transplant experiment (see Table S1 in Supporting Information). The
154 majority of asexual genotypes resulted from interspecific hybridization events (between-
155 species outcrossing, N=18; "hybrid asexuals"), while the rest were the product of within-
156 species outcrossing events (N=11; "non-hybrid asexuals"). Species and parental species
157 of asexuals were determined based on microsatellite analysis and morphological
158 examination (Rushworth *et al.* 2018) using the *Boechera* Microsatellite Website (Li *et al.*
159 2017). One sexual genotype that was determined not to be *B. retrofracta* was removed

160 from further analyses; all asexual genotypes but one ("A5") involved *B. retrofracta* as a
161 parental species. Other parental species represented were *Boechera sparsiflora*, *Boechera*
162 *exilis*, *Boechera pendulocarpa*, *Boechera stricta*, and *Boechera puberula*. Selected
163 genotypes located within 10km of existing experimental garden sites, so that
164 experimental selection pressures approximated those of the native environment for each
165 genotype. In two cases, genotypes occurred >10km away from the focal population, but
166 were chosen based on similarity of other environmental characteristics.
167 To minimize maternal effects, genotypes were grown for one generation in the
168 greenhouse prior to the experiment. Seeds were germinated on wet filter paper in petri
169 dishes and transplanted as seedlings onto a blend of Fafard 4P Mix and MetroMix 200
170 (Sun Gro Horticulture, Agawam, MA, USA) in Ray Leach Cone-tainers (Steuwe and
171 Sons, Tangent, OR, USA). Plants were grown in the Duke University Greenhouses, then
172 shipped as rosettes for transplanting at six weeks of age. Transplantation into replicated,
173 randomized blocks occurred in fall for data collection the following growing season,
174 allowing synchronization of flowering time with the native vegetation.
175
176 **Transplant experiment**
177 Subsets of the 53 genotypes were planted in two cohorts in the fall of subsequent years
178 (2012 and 2013). Data was collected for two growth seasons for each planting year, for a
179 total of three years of data collection. Planting year 1 consisted of 2000 individuals
180 representing 19 asexual and 22 sexual genotypes, planted at five different experimental
181 garden sites (ALD, 47.791, -114.253, 1993m elevation; CAP, 45.007, -114.026, 1835m;
182 CUM, 45.306, -113.889, 1187m; MIL, 44.337, -113.355, 2257m; and SIL 44.901, -

183 114.397, 1807m; 400 plants/site). In year 2, 2080 individuals representing 18 asexual and
184 21 sexual lineages were transplanted at the same sites as 2012 (520 plants/site; Figure S1).
185 The SIL site was excluded from the second planting cohort because its dense vegetation
186 and close proximity to water are not typical *B. retrofracta* habitat (Al-Shehbaz &
187 Windham 2010). Survival was scored the following spring, and for a second spring.
188 Plants that survived over winter were measured for the following traits: plant width,
189 height, rosette number, fruiting stalk number, flower number, and fruit number. Two sites
190 were removed prior to analyses due to low reproduction: MIL in year 1 (N=34
191 reproducing plants), and CUM in year 2 (N=2 reproducing plants). Leaf damage was
192 quantified visually by trained observers as in Prasad et al. (2012), which has been shown
193 to be a highly accurate assessment of herbivory (Johnson *et al.* 2016). Herbivory was not
194 observed at the CUM site, and it was removed from herbivory analyses. Prior to fruit
195 dehiscence, 1 – 4 fruits were collected from 2 – 3 replicates of each genotype within each
196 garden. Seeds were counted from each fruit and averaged across replicates for an estimate
197 of average seed set per genotype per garden. This average was then rounded to the
198 nearest integer to allow us to appropriately model seed set with a negative binomial
199 model distribution (see below). Fecundity was estimated as individual fruit number
200 multiplied by average seed set for that genotype in that garden, representing maximum
201 seed number for an individual.
202
203 **Pollen viability study**
204 To assess differences in pollen viability between sexuals and asexuals, we grew 51
205 unique genotypes (25 sexual, 26 asexual) in a single growth chamber at the Duke

206 University Phytotron. We collected developing buds, targeting mature buds that have not
207 yet undergone anthesis (stage 12, Smyth *et al.* 1990) from 1 – 3 individuals per genotype
208 (N=112 individual plants). Where more than one replicate was harvested, pollen
209 viabilities were averaged. Pollen was stained using a simplified protocol that
210 differentiates viable from inviable grains (Peterson *et al.* 2010). For each individual, 100
211 pollen grains were randomly counted from a microscopy slide visualized on a Leica
212 MZ7.5 Stereozoom dissecting microscope and imaged using a Canon EOS Rebel T3i
213 digital camera.
214
215 **Statistical analysis**
216 Our primary aim was to evaluate the association between Reproductive System
217 (*RS*, a factor which can take the value of sexual or asexual), and group (*group*, a factor
218 which can take the value of sexual, hybrid asexual, or non-hybrid asexual) with fitness
219 components over time and space.
220 All analyses were performed using R version 3.5.1 (R Core Team 2016). Outliers
221 were visually identified using an adjusted boxplot analysis suitable for far-right skewed
222 data (Hubert & Vandervieren 2008), in the R package robustbase (Maechler *et al.* 2019).
223 To analyze fitness data, we used generalized linear mixed models (GLMMs) in the R
224 package glmmTMB (Brooks *et al.* 2017). Model diagnostics were conducted using the
225 package DHARMa (Hartig 2019) and marginal means were estimated using the
226 command "ggemmeans" in the package ggeffects (Lüdecke 2018). Lifetime fitness was
227 estimated using a zero-inflated negative binomial distribution with the canonical link
228 functions, where structural zeros represent plants that did not survive the winter and the

229 conditional portion of the model represents fecundity of plants that survived. Separate
230 fecundity and survival models were also run, using the following error distributions:
231 survival (binomial), fecundity (negative binomial). The model used was:
232

233 $$y = RS + garden + RS*garden + year + RS*year$$

234

235 where y indicates either a fitness component, lifetime fitness, or herbivory. Two random
236 effects were included in all models: block nested in site and genotype nested in *RS*. The
237 same structure was used to model both the zero-inflation and conditional portions of the
238 zero-inflated negative binomial model for lifetime fitness. In all estimable field models,
239 we found that a random effect of genotype nested in reproductive system, crossed with
240 garden, accounted for essentially no variance; it was subsequently removed from our
241 analyses. A scaled covariate for width at planting was included in survival models, while
242 a scaled covariate of plant height was added to all herbivory models. Significance testing
243 for fitness analyses was conducted via likelihood ratio tests (Bolker *et al.* 2009) with
244 Holm corrections (Holm 1979) applied to *P*-values.
245 Leaf damage in the field was log-transformed and analyzed using a linear mixed
246 model (LMM) in the R package lme4 (Bates *et al.* 2014). Significance testing was
247 conducted using F tests with Kenward-Roger approximation of denominator degrees of
248 freedom (Kenward & Roger 1997) using the R package pbkrtest (Halekoh & Højsgaard
249 2014).
250 We next wished to test for differences in fitness between hybrid asexual
251 genotypes, non-hybrid asexual genotypes, and sexual genotypes. We ran the same set of

252 models as above, replacing the *RS* term with the *group* term representing these three
253 categories.
254 To understand the relationship between herbivory and fitness, and how this
255 relationship differs between groups, two additional GLMMs were estimated with log
256 herbivory and its interaction with group and garden as predictors. Each model examined a
257 fitness component: fecundity and survival to a second year, both of which are conditional
258 upon both survival to year 1 and non-zero herbivory. The model specified was:
259
260 *fitness = Group + garden + year + Group*garden + Group*year + herbivory +*
261 *Group*herbivory + garden*herbivory*
262
263 **Results**
264
265 **Asexuals have higher lifetime fitness due to survival advantage**
266 ***Asexuals have higher fitness:*** Lifetime fitness, the product of survival and reproduction,
267 was higher for asexuals than sexuals. The main effect of RS was significant (χ^2=11.90,
268 df=2, *P*=7.82e-03), as were the interactions between RS and year (χ^2=34.74, df=2,
269 *P*=1.72e-07) and *RS* and garden (χ^2=23.45, df=8, *P*=0.016; Table S2). To disentangle the
270 effects of hybridization and sex, we analyzed the same data with the group term. This
271 model similarly shows that the main effect of group was significant (χ^2=13.33, df=4,
272 *P*=0.039), as were the interactions between group and year (χ^2=36.87, df=4, *P*=1.53e-06)
273 and group and garden (χ^2=66.70, df=16, *P*=3.00e-07; Table 1, Table S2, Appendix S1).
274 Estimated lifetime fitness for each group varies across year and site, with higher lifetime

275 fitness most often observed for hybrid asexuals (Figure 1). Estimating marginal means
276 for a zero-inflated model without accounting for interactions is not recommended, and
277 thus we do not predict overall lifetime fitness for each group. However, raw data show
278 higher overall fitness in hybrid asexuals, although years vary slightly (Year 1 means:
279 sexual, 211.54; hybrid asexual, 289.40; non-hybrid asexual, 299.93; Year 2 means:
280 sexual, 76.29; hybrid asexual, 181.57; non-hybrid asexual, 145.55; Figure S2).
281
282 ***Asexuals, particularly hybrids, have higher survival:*** Our next question was whether
283 fitness disparities are attributable to differences in survival or fecundity. The zero-
284 inflation portion of a zero-inflated negative binomial GLMM addresses structural zeros,
285 or the probability of not surviving to reproduce. Over-winter survival was significantly
286 elevated in asexual genotypes compared to sexuals. In year 1, 81% of asexuals survived
287 to censusing, while 64.3% of sexuals survived. In year 2, 56.5% of asexuals survived,
288 compared with only 23.8% of sexuals.
289 The survival difference between reproductive systems was significant (χ^2=11.82,
290 df=1, *P*=2.35e-03; Table S3A). Survival also varied significantly across year (χ^2=9.16,
291 df=1, *P*=0.03; Table S3A), and garden site (χ^2=46.73, df=4, *P*=6.92e-09; Table S3A).
292 The interaction between year and RS was significant, although in both years asexuals had
293 higher survival (χ^2=22.88, df=1, *P*=8.60e-06; Table S3A). The interaction between *RS*
294 and garden was also significant (χ^2=16.23, df=4, *P*=0.016). Similar results were seen in a
295 separate model of survival (Table S4A). Here, plant size also had a significant impact on
296 survival, with larger plants surviving more often (χ^2=54.20, df=1, *P*=3.62e-13; Table
297 S4A).

298 Over-winter survival was strongly affected by group (χ^2=12.22, df=2, *P*=0.01;
299 Table 1, Table S3B), with both groups of asexuals surviving better than sexuals. The
300 effects of group differed both temporally (group × year interaction, χ^2=23.56, df=2,
301 *P*=5.36e-05) and spatially (group × site interaction, χ^2=42.82, df=8, *P*=6.66e-06; Figure
302 1B, Table 1, Table S3B, Appendix S1). Significance results were likewise reflected in the
303 separate model of survival, where group, group × garden, and group × year significantly
304 predicted mortality (group: χ^2=28.99, df=2, *P*=3.56e-06; group × garden: χ^2=23.54, df=8,
305 *P*=0.01; group × year: χ^2=9.40, df=2, *P*=0.027; Table S4B, Appendix S1). Hybrid
306 asexuals were larger than other groups at planting; rosette size, included as a covariate,
307 predicts survival (χ^2=53.79, df=1, *P*=3.62e-13; Table S4B). Both groups of asexuals had
308 higher survival in both cohorts (Figure 2A). Across garden sites, hybrid asexuals had
309 higher predicted survival than sexuals, while non-hybrid asexual survival was more
310 variable (Figure 2B, Appendix S1). In three of five sites (ALD, CAP, and MIL), survival
311 was roughly equivalent in both asexual groups. In the remaining two sites (CUM and
312 SIL), survival was similarly low for sexuals and non-hybrid asexuals.
313
314 ***Fecundity does not differ by group or RS:*** In the greenhouse, we found no difference in
315 pollen viability between sexuals and asexuals (pooled t-test, *t*=0.599 on 49 df, *P*=0.55),
316 which suggests that any intrinsic difference in seed set is due either to maternal
317 investment or to pollen quantity.
318 We next examined the conditional portion of the zero-inflated negative binomial
319 GLMM, which analyzes fecundity (seed set conditional on survival). In total, 995
320 individuals set seed (597 asexuals and 398 sexuals); removing nine outliers left a dataset

321 of 986 individuals. We saw no main effect of RS, showing that seed set does not differ
322 intrinsically between sexuals and asexuals. However, interaction terms were significant.
323 Asexual fecundity was higher than sexual in year 1, and sexual fecundity was higher in
324 year 2 (RS × year interaction: χ^2=11.86, df=1, *P*=1.72e-03; Table S3A). The conditional
325 group model produced similar results: although the main effect of group was not
326 significant, the influence of group on fecundity varied by year (χ^2=13.32, df=2, *P*=5.13e-
327 03; Table 1, Table S3B, Appendix S1). In both years, non-hybrid asexual fecundity was
328 lowest; in year 1, hybrid fecundity was highest, while sexual fecundity was highest in
329 year 2 (Figure 1C). The influence of group on fecundity also varied by garden (χ^2=23.70,
330 df=8, *P*=0.01; Table 1, Table S3B, Appendix S1). Across gardens, hybrid asexual
331 fecundity was highest of the three groups in two sites: CAP and MIL (Figure 1D). At SIL
332 and CUM, sexual fecundity was highest or among the highest groups, respectively. At
333 ALD, sexual and non-hybrid asexual fecundity were higher than non-hybrid asexual
334 (Figure 1D). Substantial environmental affects on seed set were visible in the significant
335 main effect of garden for both models (RS: χ^2=139.46, df=4, *P*=4.05e-28; group: χ^2=
336 138.92, df=4, *P*=4.80e-28; Table S3). Similar results were found in the independent
337 models of fecundity (Table S5).
338 Together, these results show that asexuals, whether derived from hybridization or
339 outcrossing events, have higher over-winter survival compared to sexuals, although this
340 advantage varies spatially and temporally. This early life fitness advantage is due either
341 to asexuality or to a trait shared by numerous asexual lineages.
342
343 **Herbivores target hybrid asexuals more than other groups**

344 33% of the experiment (1379 of 4080 planted individuals) experienced leaf herbivory.
345 The removal of 29 high-herbivory outliers resulted in a dataset of 1350 individuals (809
346 asexuals, 541 sexuals). Asexual herbivory was higher than sexual, with an average of
347 5.45%±0.002se leaf area damaged per asexual individual (estimated marginal mean
348 2.14%±0.048se, 95%CI 1.72 – 2.66%). By comparison, mean sexual leaf damage across
349 both experimental years was 3.16%±0.002se (estimated marginal mean 1.23%±0.05se,
350 95%CI 0.98 – 1.55%). This difference is statistically significant (F=22.55, df=1, 48.29,
351 P=9.33e-05; Table 2A, Figure 3, Appendix S1). Herbivory was also spatially variable
352 (garden term: F=6.40, df=3, 62.99, P=1.50e-03; Table 2A, Figure S3, Appendix S1),
353 consistent with spatial variation in herbivore communities. To account for a link between
354 plant size and herbivore attraction, a covariate of maximum plant height was included in
355 both the RS and group models. This term was significant in both models (RS: F=7.52,
356 df=1, 1318.25, P=0.019; group: F=6.96, df=1, 1320.42, P=0.019).
357 Group had a significant effect on herbivory (F=18.08, df=2, 42.39, P=1.26e-05;
358 Table 2B), with both hybrid and non-hybrid asexuals experiencing higher leaf herbivory
359 than sexuals (Figure 3, Appendix S1). Importantly, lifetime fitness was highest in
360 asexuals despite increased herbivory (Figures 1 and 2).
361
362 **Herbivory negatively impacts survival to subsequent years**
363 What are the effects of herbivory on fitness, and do these effects differ between sexuals
364 and asexuals? We next predicted two separate fitness components, fecundity and survival
365 to a second year, using models incorporating a main effect of log-transformed herbivory,
366 and its interaction with both group and garden.

367 We note that >90% of fitness occurred in the first year of life for this experiment.
368 In both years, more asexuals than sexuals survived to a second year. In experimental year
369 1, only 8.15% of plants survived for a second year (N=163: 108 asexuals, 55 sexuals).
370 Similarly, 5.9% of plants in year 2 survived for a second year (N=123: 104 asexuals, 19
371 sexuals). Just over 1% of plants reproduced in their second year (Year 1: 1.4%, N=28: 24
372 asexuals, 4 sexuals; Year 2: 1.3%, N=26: 25 asexuals, 1 sexual). Nevertheless, survival to
373 subsequent years could substantially increase lifetime fitness.
374 In temperate montane environments, herbivory and seed set co-occur during the
375 short growing season; we thus expected herbivory to directly reduce seed set. However,
376 the effect of herbivory on fecundity was non-significant (χ^2=0.28, df=1, *P*=0.598; Table
377 S6, Figure S4, Appendix S1). In contrast, herbivory had a significant negative impact on
378 survival to the second year (χ^2=21.26, df=1, *P*=8.02e-06), as did group (χ^2=32.88, df=2,
379 *P*=5.79e-07; Table S7, Appendix S1), with more hybrid asexuals than non-hybrids or
380 sexuals surviving to a second year (Year 1: 82 hybrid asexuals, 17 non-hybrid asexuals,
381 41 sexuals; Year 2: 75 hybrid asexuals, 28 non-hybrid asexuals, 17 sexuals). In all three
382 groups, herbivory and probability of survival were negatively correlated (Figure 4,
383 Appendix S1). Although the interaction between group and herbivory was non-significant,
384 the negative relationship was strongest in hybrid asexuals (Figure 4), consistent with the
385 possibility of a limiting role for herbivory in hybrid asexual fitness.
386
387 **Discussion**
388

389 Despite extensive theoretical work on the evolution of sex, few researchers have
390 examined its ecology: identifying ecologically-relevant traits associated with sex, and the
391 selective processes acting in natural environments. Such efforts could elucidate the
392 phenotypes, and ultimately the precise mechanisms, underlying the cost of sex in wild
393 populations, a fundamental question in evolutionary biology. Here we present the results
394 of what is, to our knowledge, the first large-scale, multi-site transplant experiment
395 assessing lifetime fitness of sexual and asexual plants in their native habitat. We find that
396 strong viability selection against sex results in higher lifetime fitness for asexual lineages.
397 Insect herbivory is higher in asexuals, and negatively impacts survival to a second year of
398 life. We further disentangle the influence of a key correlated trait, hybridization, on these
399 patterns, showing that hybrid and non-hybrid asexuals differ in multiple selection
400 components. This work suggests a dynamic relationship between reproductive system,
401 hybridity, and the complex natural environment, which collectively shape patterns of
402 reproductive variation in the wild.
403
404 **Intrinsic costs and benefits of sex have limited applicability**
405 Although this study does not definitively pinpoint the mechanisms underlying the
406 cost of sex, it does suggest—and rule out—several possibilities. *Boechera* avoids many
407 of the traditional costs of sex via the production of hermaphroditic flowers, including the
408 time and metabolic investment in finding mates, the risk of infection, and the production
409 of separate males. Hermaphroditic systems could exhibit unique costs of sex, such as
410 reducing costly investment in the quantity or quality of male gamete production
411 (Meirmans *et al.* 2012). Indeed, relaxed selection on asexual male function is predicted to

412 result in the production of fewer, or fewer functional, male gametes (Whitton *et al.* 2008).
413 However, selection to maintain male function may not differ when asexuals require
414 endosperm fertilization to trigger parthenogenesis, as in this system. Despite reports of
415 reduced pollen viability in previous studies of apomictic *Boechera* (Schranz *et al.* 2006),
416 we saw no difference in pollen viability between sexual and asexual lineages. It is
417 important to note that fecundity is highly environmentally dependent, and pollen viability
418 may vary in field conditions. Nonetheless, our results are consistent with no cost of sex
419 via differences in male functionality.

420 Genetic slippage is also less relevant in a self-fertilizing system.
421 Recombination may disrupt beneficial allelic combinations, negatively impacting the
422 fitness of sexual organisms. However, self-fertilization obviates the cost of genetic
423 slippage by rapid assembly and fixation of locally adaptive combinations, accelerating
424 adaptation to the immediate environment (Glémin & Ronfort 2012; Hartfield *et al.* 2017).
425 Additionally, although recombination occurs in selfers, high homozygosity leads to fewer
426 novel genetic combinations, limiting the opportunities for functional disruption (Hartfield
427 *et al.* 2017).
428

429 **The exposure of deleterious recessive alleles may underlie the cost of sex in *Boechera***

430 Despite the limited relevance of the costs of sex that are laid out in the literature,
431 we find a pattern of reduced sexual fitness, consistent with a short-term cost of sex.
432 Following several lines of evidence, we hypothesize that the observed increase in sexual
433 mortality is due to inbreeding depression rather than a cost of sex. However, we note that

434 our study does not definitively rule out a benefit of asexuality itself, and additional work
435 is needed.
436 First, there are stark differences in homozygosity between selfing sexuals and
437 asexuals. In this system, as in many other sexual/asexual systems, transitions to
438 asexuality co-occur with intra- or interspecific outcrossing, resulting in highly
439 heterozygous asexuals (Beck *et al.* 2012; Lovell *et al.* 2013; Li *et al.* 2017; Rushworth *et*
440 *al.* 2018). Through drift, genetic load of many slightly deleterious alleles may fix in
441 selfing populations, although purging of highly deleterious alleles in selfers has been both
442 predicted (Barrett & Charlesworth 1991; Wang *et al.* 1999) and shown empirically
443 (Arunkumar *et al.* 2015; Roessler *et al.* 2019).
444 Second, we find over-winter survival of asexuals is higher than sexuals,
445 regardless of hybridity (Figure 2A). In other systems such as inbred maize, the masking
446 of numerous mildly deleterious alleles via heterosis has profound effects on quantitative
447 traits such as fitness (Springer & Stupar 2007; Yang *et al.* 2017). It is reasonable to
448 expect fitness in *Boechera* populations may increase with masking of deleterious alleles
449 through outcrossing, although further work is needed.
450 Third, inbreeding depression is predicted to vary spatially and temporally
451 (Armbruster & Reed 2005; Cheptou & Donohue 2010), and the fitness effects of mildly
452 deleterious alleles will vary across environments. Consistent with this prediction, we find
453 that the intensity of viability selection varies spatially and temporally, although its
454 direction is consistent across years and gardens (Figure 2, Table 1, Table S4). Inbreeding
455 depression is predicted to increase in stressful environments (Cheptou & Donohue 2010),
456 and multiple studies have confirmed this prediction, although the pattern is not universal

457 (Armbruster & Reed 2005; Fox & Reed 2011). Previously, we showed that asexuality
458 was associated with anthropogenic disturbance (Rushworth *et al.* 2018), as shown in
459 other asexual systems (Glesener & Tilman 1978). Unfortunately, each of our garden sites
460 is relatively disturbed, and it is possible that the uniformly low sexual survival that we
461 observe, if underlain by inbreeding depression, is caused by the stress of disturbance.
462 Were we to include more diverse sites, we may observe a different pattern.
463
464 **Hybridization influences biotic selection on asexuality**
465 Hybridization is strongly linked with asexuality in numerous systems (Vrijenhoek
466 1978; Kearney 2005; Lutes *et al.* 2010; Gutekunst *et al.* 2018), but to our knowledge, has
467 previously been impossible to tease apart from asexuality. We find that hybrid asexuals
468 experience greater herbivory than both sexuals and non-hybrid asexuals (Table 2B,
469 Figure 3), showing that interspecific hybridization alters insect herbivore feeding patterns.
470 An ample body of research shows the profound impacts of hybridization on plant defense
471 and herbivore preference (Strauss 1994; Fritz 1999; Whitham *et al.* 1999; Orians 2000),
472 and our results support this pattern. In contrast, viability selection favors both inter- and
473 intraspecific asexuals, implicating shared causative traits such as heterozygosity or
474 asexuality itself (Figure 2A). As a pair, these results suggest that, although survival of
475 adverse abiotic conditions may result from either asexuality itself or associated
476 heterozygosity, hybridization plays a singular role in the interaction between asexuality
477 and the biotic environment.
478 Substantial research has shown that the effects of herbivory on fitness are direct
479 and negative. Indeed, this is the case in *Boechera stricta* (Prasad *et al.* 2012), where

480 fitness and herbivory are negatively correlated. Insect herbivory can impact multiple
481 fitness components, ranging from biomass to photosynthetic capability to root growth
482 (Marquis 1992). Here we see that, although herbivory has little effect on seed set, it is
483 negatively correlated with survival to a second year of life (Figure 4). This phenomenon
484 is not limited to plants; one recent study of Soay sheep showed that fecundity and
485 parasite load collectively reduce overwinter survival (Leivesley *et al.* 2019). If the
486 consequences of herbivory manifest at later life stages, they may not be readily apparent
487 within the normal scope of a short field experiment. Indeed, we find that the relationship
488 between second-year survival and herbivory is strongest in hybrid asexuals, suggesting
489 that hybridization may limit the spread of asexuality via herbivory. However, more
490 hybrids survive to a second year of life overall. Further work, including long term
491 experiments and herbivore exposure manipulations, are needed to understand the
492 interplay between fitness and herbivory in this system.

493 Few field studies examine the role of complex environments in the evolution of
494 sex, despite the multiplicity of selective forces in nature, their vital role in the evolution
495 of sex (Neiman *et al.* 2018), and a clear need for more field-based evolution of sex
496 studies (Agrawal 2006, 2009). Here we show a clear role for both abiotic and biotic
497 factors in the maintenance of sex in nature. Extensive research in freshwater snails has
498 revealed the importance of biotic interactions in shaping sexual/asexual dynamics, where
499 sterilizing parasites target common asexual genotypes in lower depths of a freshwater
500 lake. Indeed, a recent set of mesocosm experiments representing natural parasite loads
501 corroborates a two-fold cost of sex when asexuals are rare (Gibson *et al.* 2017). In sharp
502 contrast to our rich understanding of snails, few researchers have experimentally assessed

503 fitness of sexual and asexual plants in their native habitat. A series of studies examining
504 vernal sweet grass, *Anthoxanthum odoratum*, highlighted the importance of genetic
505 variation (and thus sexual reproduction) for fitness (Antonovics & Ellstrand 1984;
506 Ellstrand & Antonovics 1985; Schmitt & Antonovics 1986a; Schmitt & Antonovics
507 1986b). In a later study examining fitness of sexually and asexually-produced propagules,
508 sexual progeny were found to have a nearly 1.5-fold fitness advantage (Kelley *et al.*
509 1988). Importantly, further work showed that asexual tillers were more frequently
510 targeted by a common virus, as were common sexual genotypes, highlighting the
511 importance of both abiotic and biotic factors in the evolution of sex (Kelley 1994). These
512 few studies provide much of our understanding of the benefits and detriments of sex in
513 natural plant populations.
514
515 **The coexistence of sexual and asexual reproduction in *Boechera***
516 If herbivory does not limit the spread of asexuality in plant populations, what
517 does? Ecological differentiation between sexuals and asexuals limits competitive
518 interactions, and can render irrelevant the need for a balance between costs of sex and
519 asex. Numerous plant and animal systems show sexual/asexual ecological differentiation,
520 and sexual and asexual *Boechera* are also ecologically differentiated (Rushworth *et al.*
521 2018). Despite this differentiation, roughly one-third of documented populations
522 comprise both sexual and asexual lineages, suggesting limiting costs are relevant in this
523 group. Importantly, differentiation occurs repeatedly along the same environmental
524 variables (disturbance, precipitation and temperature seasonality, and slope), offering
525 avenues for manipulative experiments in the future (Rushworth *et al.* 2018).

526 Our study suggests that herbivory could potentially limit the spread of asexuality.
527 First, herbivory may have negative consequences that we failed to observe within the
528 scope of the work presented here. This relationship may be elucidated by inclusion of
529 additional sites or years, more individuals or lineages, or measurement of additional
530 fitness traits. Additionally, the strength of the negative correlation between herbivory and
531 fitness is highly dependent on numerous factors, including the resources available in a
532 given environment, which will limit both fitness and herbivory; the timing of herbivory
533 events; or the abundance and identity of the insect community (Zangerl & Bazzaz 1992).
534 Each of these factors vary strongly with the environment, and may result in different
535 experimental outcomes.

536 Fitness loss due to herbivory may not be the only cost of asexuality in this system.
537 Previous research has shown that mutations accumulate more rapidly in asexual lineages
538 of *Boechera* (Lovell *et al.* 2017), which could ultimately reduce both function and fitness.
539 Alternatively, the costs of asexuality in this system may be due to outcrossing itself
540 (Meirmans *et al.* 2012), which may introduce maladaptive alleles to locally adapted
541 populations (Kirkpatrick & Barton 1997; Lenormand 2002). The fitness consequences of
542 such alleles may covary with genetic distance of the parents, with some optimal distance
543 between relieving inbreeding depression and avoiding outbreeding depression (e.g. Willi
544 *et al.* 2005; Wei & Zhang 2018). Although evidence for an optimal mating distance
545 between parents is scant (Edmands 2007), it is clear that interspecific matings may result
546 in lower fitness for multiple reasons. Additionally, the formation of asexual lineages via
547 outcrossing may itself be difficult due to rarity of outcrossing, crossing barriers, or
548 developmentally complex transitions to asexuality.

549 Here we've shown that natural selection and environmental variation act upon
550 asexual lineages representing different parental backgrounds to shape sexual/asexual
551 dynamics. We see that the impacts of sex and hybridization on fitness in the natural
552 environment are separable and unique, and that selection in early life is consistent with a
553 short-term cost of sex that may be underlain by inbreeding depression. Despite strong
554 pressure from biotic agents of selection, the tolerance response of both asexuals and
555 sexuals shows a complex role for herbivory in sexual/asexual coexistence. Future work is
556 needed to characterize linkage between phenotypes relevant to growth and defense, and
557 the effects of hybridization and heterozygosity on their expression. This study offers
558 strong evidence that ecological variation and natural selection drive the evolution of sex
559 in the wild, and highlights the importance of field-based studies to address this central
560 question in evolutionary biology.
561

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572 **Figures and Tables**

573

574 **Table 1**. **Reproductive system (sexual vs. asexual) and hybridity predict lifetime**
575 **fitness via spatially- and temporally-variable selection.** Results for the main effect of
576 group and the interactions between group and garden and group and year for zero-inflated
577 negative binomial GLMMs of lifetime fitness. Left, the zero-inflation portion models
578 structural zeros or plants that did not survive to reproduce. Center, the conditional portion
579 models seed set in plants that survived (fecundity). Right, significance estimates for the
580 overall model, incorporating both portions. Group indicates sexuals, "sex" vs. asexual
581 hybrids, "hyb" vs. asexual non-hybrids, the reference category. Estimates come from
582 conditional models, while test statistics (χ^2 deviance, degrees of freedom, and *P* values)
583 come from likelihood ratio tests for each overall effect. Full results from each separate
584 model are in Tables S3, S4, and S5.

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			Zero-inflation				*Conditional*				*Overall model*		
term	condition	df	estimate	se	χ^2	*P*	estimate	se	χ^2	*P*	df	χ^2	*P*
Group	sex	2	0.339	0.468	12.22	**0.01**	-0.329	0.254	1.12	0.57	4	13.33	**0.039**
	hyb		-0.528	0.487			-0.357	0.250					
Group* Garden	sex*CAP	8	0.129	0.342	42.82	**6.66e-06**	0.048	0.221	23.70	**0.01**	16	66.70	**3.00e-07**
	hyb*CAP		0.977	0.349			0.485	0.208					
	sex*CUM		-0.596	0.495			0.377	0.319					
	hyb*CUM		0.376	0.517			0.682	0.322					
	sex*MIL		-0.278	0.394			0.052	0.233					
	hyb*MIL		1.063	0.402			0.435	0.228					
	sex*SIL		-1.167	0.661			1.843	0.481					
	hyb*SIL		-1.746	0.663			1.895	0.463					
Group* Year	sex*2	2	1.103	0.378	23.56	**5.36e-05**	0.594	0.194	13.32	**5.16e-03**	4	36.87	**1.53e-06**
	hyb*2		-0.402	0.407			0.071	0.193					

598 **Table 2**. **Herbivory varies by reproductive system, hybridity, and garden.** (A) Model
599 results for reproductive system ("RS", sexual vs. asexual) LMM and (B) group (sexual,
600 "sex" vs. asexual hybrid, "hyb" vs. asexual non-hybrid, the reference category) LMM.
601 Significant *P*-values are in bold. Estimates apply to the conditional models, while test
602 statistics (*F* statistics, degrees of freedom, and *P* values) come from significance tests for
603 each overall effect.
604 A.

term	**condition**	**estimate**	**se**	**df**	***F***	***P***
	intercept	-1.633	0.071	-	-	-
RS	sexual	-0.299	0.079	1, 48.29	22.55	**9.33-05**
Garden	CAP	-0.002	0.074	3, 62.99	6.40	**1.50e-03**
	MIL	0.340	0.095			
	SIL	-0.002	0.103			
RS*Garden	sexual*CAP	0.049	0.080	3, 1280.95	1.74	0.372
	sexual*MIL	0.059	0.115			
	sexual*SIL	0.234	0.105			
Year	2	-0.045	0.076	1, 67.59	0.38	1
RS*Year	sexual*2	-0.013	0.084	1, 1175.63	0.02	0.876
Plant height	-	0.045	0.016	1, 1318.25	7.52	**0.019**

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B.

term	condition	estimate	se	df	*F*	*P*
	intercept	-1.719	0.095	-	-	-
Group	sex	-0.210	0.010	2, 42.39	18.08	**1.26e-05**
	hyb	0.141	0.102			
Garden	CAP	-0.035	0.098	3, 62.98	6.37	**1.49e-03**
	MIL	0.332	0.122			
	SIL	0.239	0.149			
Group*Garden	CAP*sex	0.083	0.102	6, 1273.93	1.99	0.065
	CAP*hybrid	0.060	0.102			
	MIL*sex	0.067	0.139			
	MIL*hybrid	0.016	0.132			
	SIL*sex	-0.005	0.150			
	SIL*hybrid	-0.324	0.152			
Year	2	-0.087	0.099	1, 66.98	0.30	1
Group*Year	sex*2	0.023	0.105	2, 1056.46	0.45	0.77
	hybrid*2	0.092	0.105			
Plant height	-	0.042	0.016	1, 1320.42	6.96	**0.019**

623 **Figure 1. Predicted lifetime fitness of asexuals is higher than that of sexuals.**

624 Estimated marginal means for lifetime fitness, including both survival and seed set, by
625 group across experimental years (Year 1, top row; Year 2, bottom row) and gardens (left
626 to right). Lifetime fitness is composed of survival and fecundity: sexual fecundity is not
627 sufficiently high to overcome temporally- and spatially-variable viability selection
628 favoring both groups of asexuals. Note that SIL was not planted in year 2, but predicted
629 fitness from the model is shown. Lifetime fitness is shown on a log scale. Bars show 95%
630 confidence intervals.

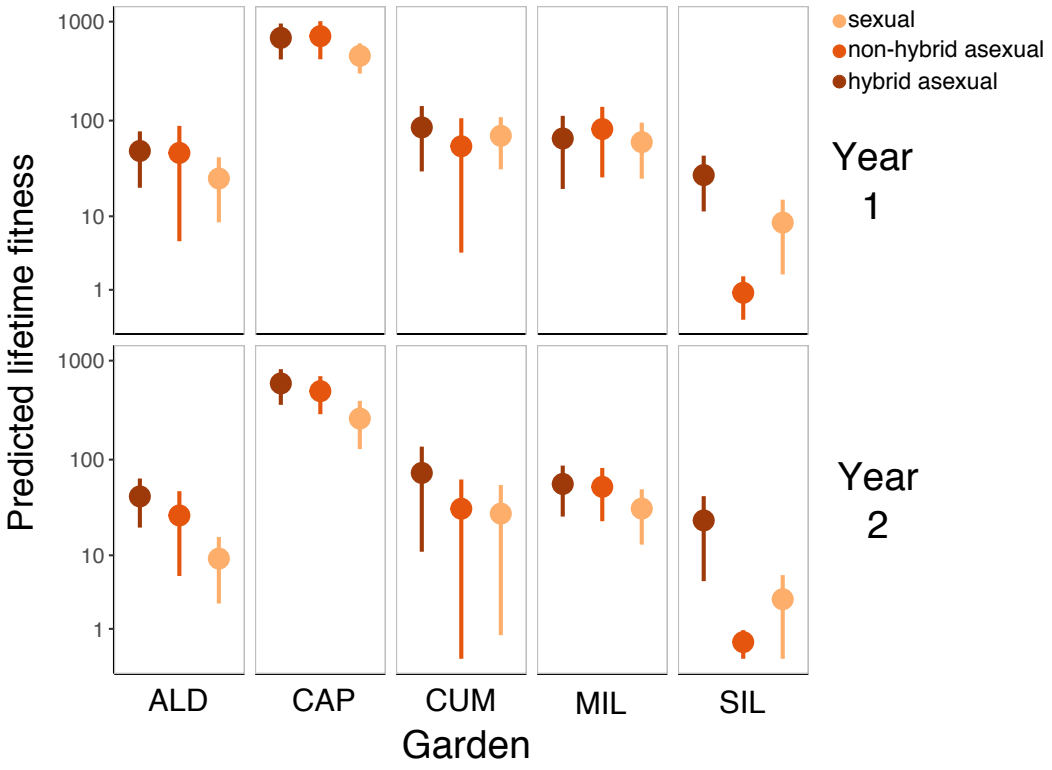

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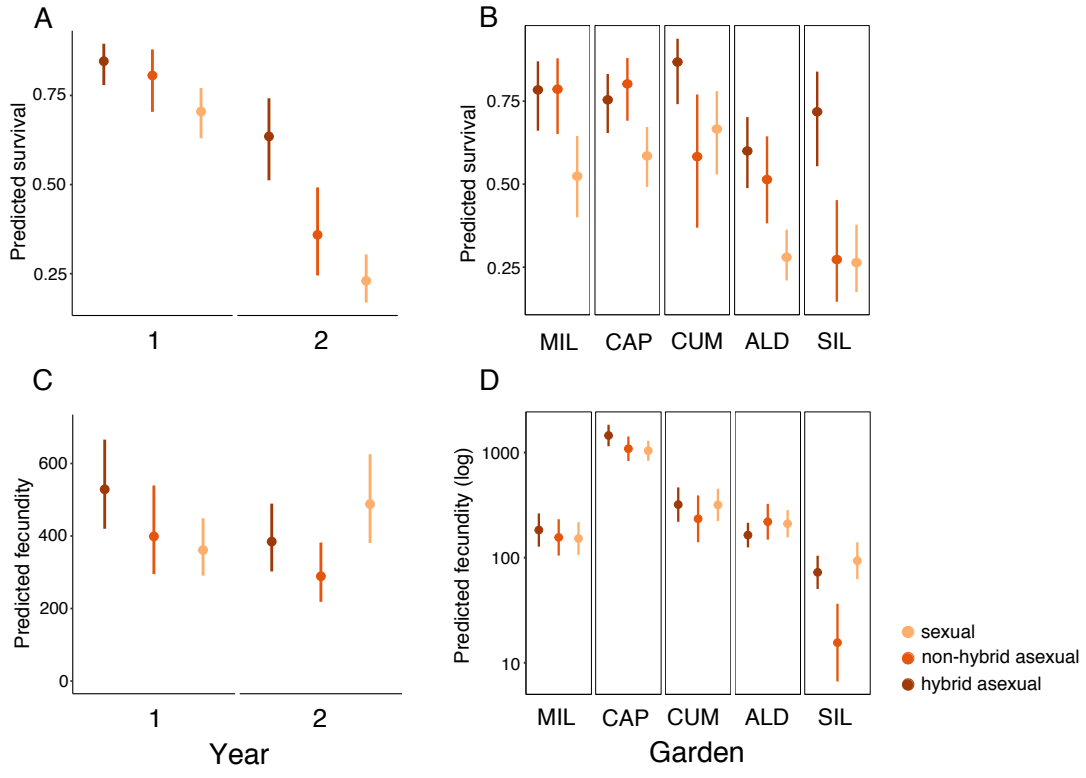
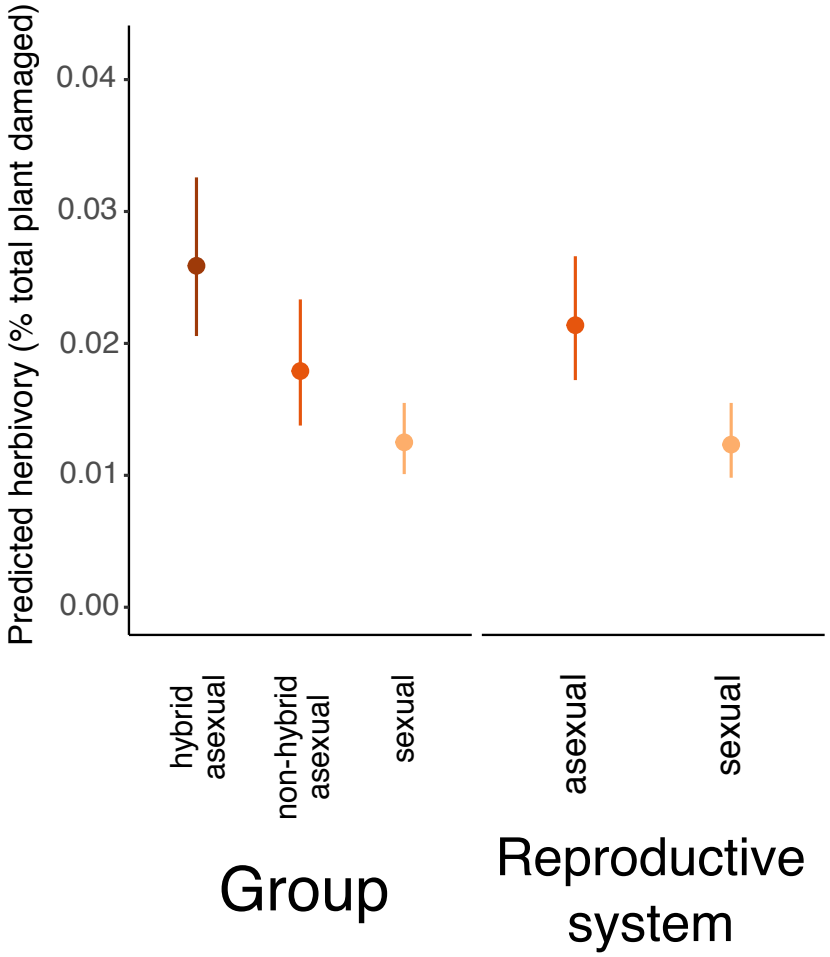
Figure 2. **Predicted survival is highest for asexuals, while temporally-variable fecundity selection favors asexuals in year 1 and sexuals in year 2.** Estimated marginal means for survival (A and B) and fecundity (C and D) show substantial temporal variation (A and C, group by year interaction) and spatial variation (B and D, group by garden interaction). (A) Survival is reduced in sexuals in both experimental years. (B) In all sites, hybrid asexuals have higher over-winter survival than sexuals; non-hybrid asexual survival is variable. (C) Fecundity varies temporally, with higher hybrid asexual fecundity in year 1 and higher sexual fecundity in year 2. (D) In contrast to survival, sexual fecundity (shown on log scale) is higher than asexual in some garden sites. Bars show 95% confidence intervals.



649 **Figure 3**. **Herbivory is elevated in hybrid asexuals.** Estimated marginal means for
650 herbivory from LMMs show the main effects of group (sexual vs. non-hybrid asexual vs.
651 hybrid asexual, left) and reproductive system (sexual vs. asexual, right). Bars show 95%
652 confidence intervals.



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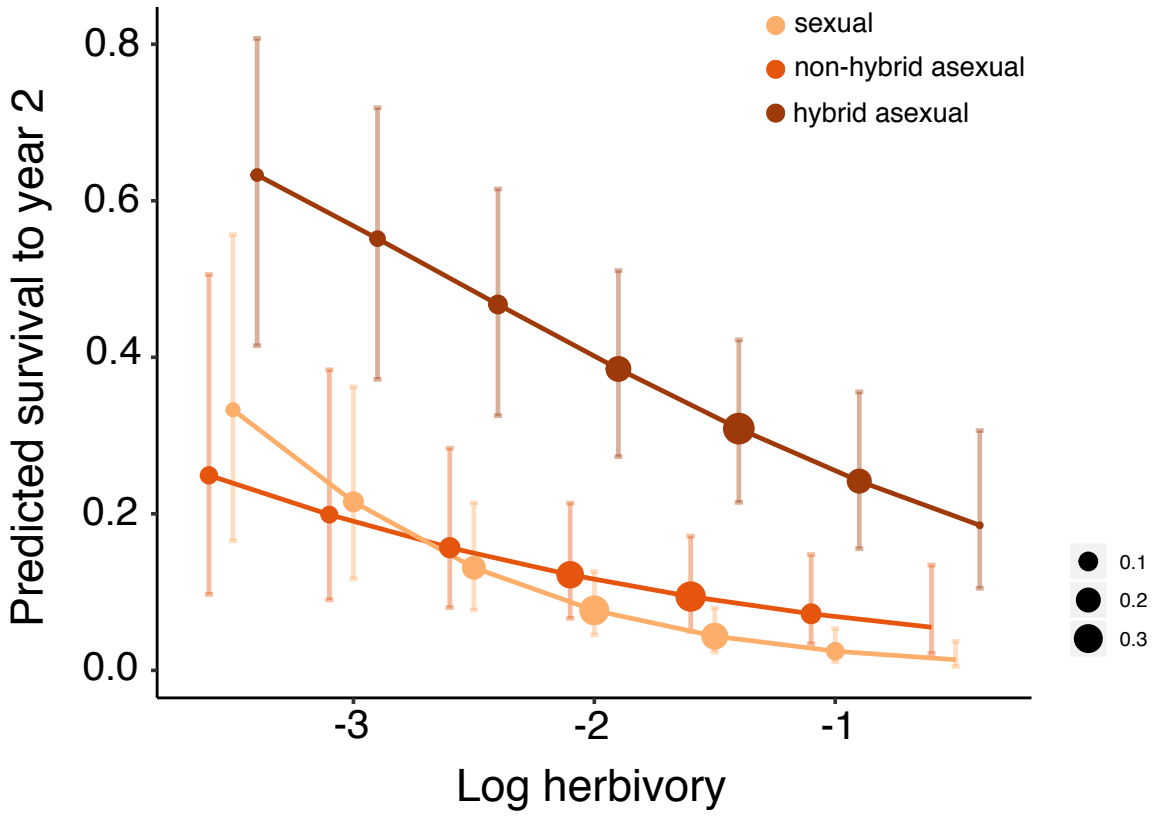
659 **Figure 4**. **Herbivory is lower in plants that survive to a second year of life.** Herbivory
660 has a significant impact on survival to a second year (P = 8.02e-06), suggesting that the
661 detrimental consequences of herbivory may not be observed in a single growing season.
662 Although the correlation between year 2 survival and herbivory differs qualitatively by
663 group (sexual vs. non-hybrid asexual vs. hybrid asexual), the interaction is not significant
664 (Table S7). Estimated marginal means are shown averaged across five different values of
665 log herbivory. To convey the relative contribution of each level of herbivory to total year
666 2 survival, data point size is proportional to the number of plants in each group that
667 experienced this level of herbivory. Points are jittered for ease of reading. Bars show 95%
668 confidence intervals.



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