Identifying the fitness consequences of sex in complex natural environments

Catherine A. Rushworth[1,2,3*], Yaniv Brandvain[3], Tom Mitchell-Olds[4]

1. Department of Evolution and Ecology, University of California, Storer Hall, One Shields Avenue Davis, CA 95616 USA

2. University and Jepson Herbaria, University of California, Berkeley, Berkeley, CA 94720 USA

3. Department of Plant and Microbial Biology, University of Minnesota, St. Paul, MN 55108 USA (ybrandva@umn.edu)

4. Department of Biology and Center for Genomic and Computational Biology, Duke University, Durham, NC 27708 USA (tmo1@duke.edu)

* author for correspondence: catherine.rushworth@gmail.com

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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. Furthermore, correlated biological traits that differ between sexuals and asexuals may alter these costs, or even render the typical costs of sex irrelevant. 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 hybridity, finding that over-winter survival is elevated in asexuals regardless of hybridity, but herbivores target hybrid asexuals. Survival is lowest in homozygous sexual lineages, implicating inbreeding depression as a cost of sex. Our results show that the consequences of sex are shaped by complex natural environments, correlated traits, and the identity of mates, rather than sex itself.

Introduction

Despite over a century of research, sex remains a fundamental mystery in evolutionary biology. Although recombination removes deleterious alleles and unites beneficial ones (Hill & Robertson 1966; Keightley & Otto 2006), enabling organisms to adapt (Felsenstein 1974) and evolve with antagonists (Jaenike 1978), sex has many costs. These typically include the "twofold cost," wherein sexual organisms produce half as many offspring as asexuals due to their investment in male offspring (Maynard Smith 1978), as well as the disruption of co-adapted beneficial loci via recombination (Lehtonen *et al.* 2012). While theory has identified many costs and benefits of sex, these are likely mediated by the genomic composition of asexuals, the patterns of mating among sexuals, and the biotic and abiotic selective environment.

Despite widespread attention to the genetic processes maintaining sex, the ecological context in which sex occurs has received less attention. This is a crucial gap in our knowledge, as the processes governing sexual/asexual dynamics occur in complex natural environments. Our understanding of the ecology of sex stems largely from decades of experimental work on the freshwater snail, which has established sterilizing trematode parasites as the primary selective agent maintaining sex via frequency-dependent selection. This system offers a key example of Red Queen dynamics, wherein sexual reproduction generates novel lineages that avoid infection. Water depth moderates parasite frequency, leading to a striking ecological pattern: sex predominates in shallow water, where secondary hosts occur, while asexuality is widespread in deeper sites (Lively 1987; Jokela & Lively 1995; Lively & Dybdahl 2000; Vergara *et al.* 2014). This

research shows that the abiotic and biotic environment operate in concert to shape sexual/asexual dynamics in the natural world. Critically, both ecology and its interactions with a system's biological features provide the context necessary to pinpoint the real-life costs and benefits of sex (Neiman *et al.* 2018).

Similarly, very few studies have accounted for the widespread observation that sex and asex are often differently associated with fundamental biological and life history traits, despite the critical role these traits play in shaping the costs of sex (Meirmans *et al.* 2012). For example, the cost of males will be minimized in plants with hermaphroditic, self-compatible flowers (Charlesworth 1980; Mogie 1996); as such, frequent inbreeding will further minimize this cost. Additional costs of asexuality may be rooted in the origin and composition of asexual lineages. For example, while polyploidy and hybridity are overrepresented in asexual plants and vertebrates as compared to sexuals (e.g., Vrijenhoek 1978; Asker & Jerling 1992; Lutes *et al.* 2010), the impact of these key traits is rarely considered. Further costs of sex may be related to mate choice (inbreeding or outcrossing) and mate availability for sexual organisms, which have profound phenotypic and genetic consequences (Meirmans *et al.* 2012). Hybridization, or outcrossing between widely divergent taxa, alters numerous fitness-related traits (Yakimowski & Rieseberg 2014), which has repercussions for hybrid asexuals. The genetic impacts of mate choice will similarly influence the evolution of sex. For example, sex via inbreeding can expose a recessive genetic load (Charlesworth & Willis 2009), while asexual reproduction may maintain heterozygosity. Such life history traits likely contribute substantially to sexual/asexual dynamics, but are rarely discussed in studies of the costs and benefits of sex.

99 The mustard genus *Boechera* offers a rare opportunity to study the ecological
100 costs of sex while accounting for multiple sex-associated biological traits. Apomixis
101 (asexual reproduction via seed) is widespread in the genus (Böcher 1951; Schranz *et al.*
102 2005; Aliyu *et al.* 2010). These asexuals may be polyploid or diploid, enabling separate
103 assessment of the effects of polyploidy and asexuality (Kantama *et al.* 2007; Beck *et al.*
104 2012; Mau *et al.* 2015). Furthermore, lineages that reproduce asexually are derived from
105 outcrossing events, which may be either interspecific or intraspecific (Li *et al.* 2017;
106 Rushworth *et al.* 2018), allowing separable examination of hybridity and asexuality.
107 Finally, similar to other asexual systems (e.g., Lutes *et al.* 2010; Fradin *et al.* 2017),
108 asexuals are highly heterozygous, whereas sexual *Boechera* are highly self-fertilizing and
109 thus highly homozygous (Roy 1995; Song *et al.* 2006; Li *et al.* 2017). This particular trait
110 is of broad relevance to all sexual/asexual systems, as all sexual organisms undergo shifts
111 in population size and variable biparental inbreeding. In other words, the consequences of
112 sex will always depend in large part on the identity of available sexual partners. For this
113 reason, the well-characterized mating system of *Boechera* offers an ideal system for
114 studying the evolution of sex.
115 Using a reciprocal transplant experiment in intact native habitat, we identified the
116 fitness components underlying the costs of sex in *Boechera*. We examined female
117 lifetime fitness and insect herbivory of sexual and asexual plants, finding strong over-
118 winter viability selection against sexual lineages, which is lessened in heterozygous
119 sexuals. This survival difference resulted in increased lifetime fitness for asexual
120 lineages, despite elevated insect herbivory on hybrid asexuals. Importantly, the negative
121 impacts of herbivory appeared in later life stages, reducing the probability of survival to a

122 second year, consistent with a cost of hybridization. Numerous lines of evidence suggest
123 that the cost of sex in this system is due at least in part to inbreeding depression in highly
124 homozygous sexual lineages.
125
126 **Materials and Methods**
127
128 **Study system**
129 *Boechera retrofracta* (Brassicaceae) is a short-lived perennial wildflower native to
130 western North America. Multiple *Boechera* species are highly self-fertilizing (Hamilton
131 & Mitchell-Olds 1994; Roy 1995; Song *et al.* 2006), and *B. retrofracta*'s high
132 microsatellite homozygosity (mean homozygosity=0.94, Rushworth *et al.* 2018) and
133 small flowers that self-pollinate before anthesis (C. Rushworth, pers. obs.) suggest
134 frequent self-fertilization. Nonetheless, *B. retrofracta* forms hybrids with at least 12
135 species (Windham & Al-Shehbaz 2006). Asexuality is tightly associated with outcrossing
136 and hybridization in *Boechera* (Schranz *et al.* 2005; Kantama *et al.* 2007; Beck *et al.*
137 2012; Rushworth *et al.* 2018). Apomixis in this system is pseudogamous, requiring
138 endosperm (but not embryo) fertilization (Böcher 1951). Because sexuals are highly
139 selfing and asexuals must also produce a small amount of viable pollen, several of the
140 traditional costs of sex are minimized in this system, allowing investigation of broader
141 ecological and biological factors maintaining sex.
142
143 **Sampling and growth methods**

144 We used 53 wild-collected diploid genotypes (24 sexual, 29 asexual) in a multi-site
145 reciprocal transplant experiment (see Table S1 in Supporting Information). Species (or
146 parental species, in the case of asexuals) were determined based on microsatellites and
147 morphology (Rushworth *et al.* 2018) using the *Boechera* Microsatellite Website (Li *et al.*
148 2017). Heterozygosity, estimated from microsatellites, was calculated for each maternal
149 lineage. 21 sexual genotypes were entirely homozygous, and three were not
150 (heterozygosity ranging from 0.08 – 0.42); heterozygosity of all but one asexual
151 genotypes was >0.5 (Figure S1). Eighteen asexual genotypes are "hybrid asexuals,"
152 resulting from interspecific hybridization events, while eleven are "non-hybrid asexuals,"
153 resulting from within-species outcrossing. One sexual genotype was not *B. retrofracta*,
154 and was not analyzed; all asexual genotypes but one ("A5") involved *B. retrofracta* as a
155 parental species. Other parental species represented in hybrids were *Boechera*
156 *sparsiflora*, *Boechera exilis*, *Boechera pendulocarpa*, *Boechera stricta*, and *Boechera*
157 *puberula*. To ensure that experimental selection pressures approximated those of each
158 genotype's native environment, we selected lines based on proximity or similarity of
159 collection and garden environments. All but two genotypes were collected within 10km
160 of an experimental garden.

161 To minimize maternal environmental effects, genotypes were grown in controlled
162 conditions for one generation before the experiment. Seeds were germinated on wet filter
163 paper in petri dishes and transplanted as seedlings onto a blend of Fafard 4P Mix and
164 MetroMix 200 (Sun Gro Horticulture, Agawam, MA, USA) in Ray Leach Cone-tainers
165 (Steuwe and Sons, Tangent, OR, USA) in the Duke University greenhouses, and shipped
166 as rosettes for transplanting at six weeks of age. Transplantation into replicated,

167 randomized blocks occurred in fall for data collection the following season, allowing
168 synchronization of flowering time with the native vegetation.
169
170 **Transplant experiment**
171 Subsets of the 53 genotypes were planted in two cohorts of replicated, randomized blocks
172 in the fall of 2012 and 2013. Data was collected for two growth seasons for each planting
173 year, totaling three years of data collection. Planting year one consisted of 2000
174 individuals representing 19 asexual and 22 sexual genotypes, planted in five gardens with
175 400 plants/site (ALD, CAP, CUM, MIL, and SIL; Table S1). In year two, 2080
176 individuals representing 18 asexual and 21 sexual lineages were transplanted at the same
177 sites (520 plants/site; Figure S2). However, the SIL site was excluded from year two
178 because its dense vegetation and high humidity are atypical *B. retrofracta* habitat. Over-
179 winter survival was scored the following spring. Plants that survived were measured for
180 width, height, flower number, and fruit number. Due to low reproduction, MIL in year
181 one (N=34 reproducing plants) and CUM in year two (N=2) were excluded from
182 analyses. Leaf damage was quantified visually by trained observers, as in Prasad et al.
183 (2012). The CUM site was excluded in herbivory analyses, as no herbivory
184 measurements were made there. Prior to fruit dehiscence, 1 – 4 fruits were collected from
185 2 – 3 replicates of each genotype within each garden. Seeds were counted from each fruit
186 and averaged across replicates to estimate average seed set per genotype per garden.
187 Fecundity was estimated as average seed set per fruit multiplied by individual fruit
188 number. This average was rounded to the nearest integer to allow us to model seed set as
189 a negative binomially distributed random variable.

190

191 **Pollen viability**

192 To evaluate how sexual system impacted pollen viability, we grew 25 sexual and 26
193 asexual genotypes in a growth chamber at the Duke University Phytotron. We collected
194 mature developing buds (stage 12, Smyth *et al.* 1990) from 1 – 3 individuals per
195 genotype (N=112 individual plants), and stained pollen to differentiate viable from
196 inviable grains (Peterson *et al.* 2010), averaging pollen viabilities across replicates. For
197 each individual, 100 pollen grains were randomly counted from a microscopy slide
198 visualized on a Leica MZ7.5 Stereozoom dissecting microscope and imaged using a
199 Canon EOS Rebel T3i digital camera.

200

201 **Statistical analyses**

202 Our primary aim was to evaluate the association between Reproductive System
203 (*RS*, a factor which takes the value of sexual or asexual), and group (*group*, a factor
204 which can take the value of sexual, hybrid asexual, or non-hybrid asexual) with fitness
205 components over time and space. Because sexuals and asexuals differ intrinsically in
206 heterozygosity, any observed fitness differences may stem from inbreeding or
207 outbreeding depression. We conducted additional analyses to address this possibility.

208 All analyses were performed using R version 3.5.2 (R Core Team 2018). Outliers
209 were visually identified using an adjusted boxplot analysis suitable for far-right skewed
210 data (Hubert & Vandervieren 2008), with robustbase (Maechler *et al.* 2019). To analyze
211 fitness data, we used generalized linear mixed models (GLMMs) in glmmTMB (Brooks
212 *et al.* 2017). Model diagnostics were conducted using DHARMa (Hartig 2019) and

marginal means were estimated using the package ggeffects (Lüdecke 2018). Lifetime fitness was estimated using a zero-inflated negative binomial distribution with the canonical link functions, where structural zeros represent plants that did not survive the winter and the conditional portion of the model represents fecundity of survivors. Separate fecundity and survival models were also run, using the negative binomial and binomial error distributions, respectively. The model used was:

$$y \sim RS + garden + RS*garden + year + RS*year$$

where y indicates a fitness component, lifetime fitness, or herbivory. Two random effects were included in all models: block nested in site and genotype nested in *RS*. The same structure was used for the zero-inflation and conditional portions of the zero-inflated negative binomial model for lifetime fitness. In all estimable models, a random effect of genotype nested in reproductive system, crossed with garden, accounted for essentially no variance, and was therefore removed from analyses. A scaled covariate for width at planting was included in survival models, while a scaled covariate of plant height was added to all herbivory models to account for a link between plant size and insect attraction.

We next tested for fitness differences between hybrid asexual genotypes, non-hybrid asexual genotypes, and sexual genotypes, by running the same models as above, replacing the *RS* term with the *group* term. To assess the effects of heterozygosity on survival, we used fixed-effects only models to analyze survival of heterozygous and homozygous sexual lineages, and complementary analyses (heterozygous sexuals vs.

236 asexuals; homozygous sexuals vs. asexuals) were also conducted. Significance testing for
237 fitness analyses was conducted via likelihood ratio tests (Bolker *et al.* 2009) with Holm
238 corrections (Holm 1979).

239 Leaf damage was log-transformed and analyzed using a linear mixed model
240 (LMM) in lme4 (Bates *et al.* 2015). Significance testing was conducted using *F* tests with
241 Kenward-Roger approximation of denominator degrees of freedom (Kenward & Roger
242 1997) using pbkrtest (Halekoh & Højsgaard 2014).

243 To understand the relationship between herbivory and fitness, and how it differs
244 among groups, two additional models were estimated with log herbivory and its
245 interaction with group and garden as predictors. Each model examined a fitness
246 component: fecundity and survival to a second year, both of which are conditional upon
247 survival to year one and non-zero herbivory. The model specified was:

248

249 $$y \sim group + garden + year + group*garden + group*year + herbivory +$$
250 $$group*herbivory + garden*herbivory$$

251

252 **Results**

253

254 **Higher asexual lifetime fitness due to survival advantage**

255 ***Higher asexual lifetime fitness:*** Lifetime fitness, the product of survival and
256 reproduction, was higher in asexuals than sexuals (χ^2=11.90, df=2, P=7.82×10^{-3}). The
257 effect of RS depended on year (χ^2=34.74, df=2, P=1.72×10^{-7}) and garden (χ^2=23.45,
258 df=8, P=0.016; Table S2). To disentangle the effects of hybridization and sex, we

259 analyzed the same data with the group term. This model similarly showed an effect of
260 group (χ^2=13.33, df=4, P=0.039), and its interaction with both year (χ^2=36.87, df=4,
261 P=1.53×10^{-6}) and garden (χ^2=66.70, df=16, P=3.00×10^{-7}; Table 1, Table S2). Estimated
262 lifetime fitness for each group varied across year and site, but was elevated in both
263 asexual groups, with consistently high hybrid asexual fitness (Figure S3). While our
264 model design did not allow us to predict overall lifetime fitness for each group without
265 accounting for interactions, raw summaries showed higher overall fitness in both groups
266 of asexuals, although years varied slightly (Year 1 means: sexual, 211.54; hybrid asexual,
267 289.40; non-hybrid asexual, 299.93; Year 2 means: sexual, 76.29; hybrid asexual, 181.57;
268 non-hybrid asexual, 145.55; Figure S4). Estimated marginal means for all models are in
269 Appendix S1.
270
271 ***Higher asexual survival:*** We next asked if fitness disparities were attributable to
272 differences in survival or fecundity. The zero-inflation portion of a zero-inflated negative
273 binomial GLMM addresses structural zeros, or the probability of not surviving to
274 reproduce. Over-winter survival was significantly elevated in asexual genotypes
275 compared to sexuals. In year 1, 81% of asexuals survived, while 64.3% of sexuals
276 survived. In year 2, 56.5% of asexuals survived, compared with only 23.8% of sexuals.
277 Survival differed by reproductive system (χ^2=11.82, df=1, P=2.35×10^{-3}), and
278 across year (χ^2=9.16, df=1, P=0.04) and garden (χ^2=46.73, df=4, P=1.21×10^{-8}; Table
279 S3A). In both years asexuals had higher survival; the interactions between year and RS,
280 and garden and RS, were also significant (year: χ^2=22.88, df=1, P=8.60×10^{-6}; garden:
281 χ^2=16.23, df=4, P=0.016; Table S3A). Similar results were seen in a separate model of

282 survival (Table S4A). Plant size also increased plant survival (χ^2=54.20, df=1,
283 P=7.24×10^{-13}).
284 Over-winter survival differed by group (χ^2=12.22, df=2, P=0.01; Table 1, Table
285 S3B), with both types of asexuals surviving better than sexuals in both cohorts (Figure
286 1A). The effects of group differed temporally (group × year interaction, χ^2=23.56, df=2,
287 P=5.36×10^{-5}) and spatially (group × site interaction, χ^2=42.82, df=8, P=6.66×10^{-6}; Figure
288 1B, Table 1, Table S3B). A separate model of survival showed similar results (Table
289 S4B). Hybrid asexuals were larger than other groups at planting; rosette size (χ^2=53.79,
290 df=1, P=7.24×10^{-13}; Table S4B). Across gardens, hybrid asexuals had higher predicted
291 survival than sexuals, while non-hybrid asexual survival was more variable (Figure 1B).
292 In three of five sites, survival was roughly equivalent in both asexual groups. In two sites,
293 survival was similarly low for sexuals and non-hybrid asexuals.
294 Heterozygosity also impacted survival. Survival of heterozygous sexuals
295 exceeded that of homozygous sexuals (χ^2=7.07, df=1, P=0.024; Figure S5, Table S5), but
296 did not result in increased total fitness for heterozygous sexuals (Table S6). Asexual and
297 heterozygous sexual survival did not differ (χ^2=0.47, df=1, P=1). Together, these results
298 are consistent with inbreeding depression manifesting as reduced over-winter survival in
299 homozygous sexual lineages.
300
301 ***No differences in fecundity:*** We next examined the conditional portion of the zero-
302 inflated negative binomial GLMM, which analyzed fecundity (seed set conditional on
303 survival). In total, 995 individuals set seed (597 asexuals and 398 sexuals); removing
304 nine outliers left a dataset of 986 individuals. Seed set did not differ intrinsically between

305 sexuals and asexuals, but the influence of RS varied by year (χ^2=11.86, df=1, P=1.72×10^{-}
306 ^{3}; Table S3A). The conditional group model produced similar results: although the main
307 effect of group was not significant, non-hybrid asexual fecundity was lowest in both
308 years (Figure S6A). The influence of group varied by year (χ^2=13.32, df=2, P=5.13×10^{-3};
309 Table 1, Table S3B) and by garden (χ^2=23.70, df=8, P=0.01; Table 1, Table S3B, Figure
310 S6B). There were substantial environmental effects on seed set (Table S3). Similar results
311 were found in the separate fecundity models (Table S7). Pollen viability assays revealed
312 no difference between sexuals and asexuals (pooled t-test, t=0.599 on 49 df, P=0.55),
313 suggesting that any differences in seed set may be due to either maternal investment or
314 pollen quantity. Heterozygosity had no impact on sexual fecundity (χ^2=1.24, df=1,
315 P=0.26; Table S8).
316
317 **Herbivores targeted hybrid asexuals**
318 33% of the experiment (1379 of 4080 plants) experienced leaf herbivory. The removal of
319 29 high-herbivory outliers resulted in a dataset of 1350 individuals (809 asexuals, 541
320 sexuals). Asexual herbivory was higher than sexual, with an average of 5.45%±0.002se
321 leaf area damaged per asexual individual (estimated marginal mean 2.14%±0.048se,
322 95%CI 1.72 – 2.66%). By comparison, mean sexual leaf damage across years was
323 3.16%±0.002se (estimated marginal mean 1.23%±0.05se, 95%CI 0.98 – 1.55%), a
324 significant difference (F=22.55, df=1, 48.29, P=9.33e-05; Table 2A, Figure 2). Herbivory
325 varied by garden (F=6.40, df=3, 62.99, P=2.99×10^{-3}; Table 2A, Figure S7), consistent
326 with spatial variation in herbivore communities. Herbivory was highest in hybrid
327 asexuals (Figure 2). Group had a significant effect on herbivory (F=18.08, df=2, 42.39,

328 P=1.26×10^{-5}; Table 2B), with both hybrid and non-hybrid asexuals experiencing higher
329 leaf herbivory than sexuals (Figure 2). Plant height was significant in both models (Table
330 2).
331
332 **Herbivory negatively impacted survival to subsequent years**
333 What are the effects of herbivory on fitness, and do these effects differ between sexuals
334 and asexuals? We next predicted two separate fitness components, fecundity and survival
335 to a second year, using models incorporating a main effect of log-transformed herbivory,
336 and its interaction with both group and garden.
337 Over 90% of experimental fitness occurred in the first year of life. In both years,
338 more asexuals than sexuals survived to a second year. In experimental year 1, 8.2% of
339 plants survived for a second year (N=163: 108 asexuals, 55 sexuals). Similarly, 5.9% of
340 plants in year 2 survived for a second year (N=123: 104 asexuals, 19 sexuals). Roughly
341 1% of plants reproduced in their second year (Year 1: 1.4%, N=28: 24 asexuals, 4
342 sexuals; Year 2: 1.3%, N=26: 25 asexuals, 1 sexual). Nevertheless, survival to subsequent
343 years could substantially increase lifetime fitness.
344 In temperate montane environments, herbivory and seed set co-occur during the
345 short growing season, and may thus influence one another. We found no effect of
346 herbivory on fecundity (χ^2=0.28, df=1, P=0.598; Table S9), but herbivory decreased
347 survival to a second year (χ^2=21.26, df=1, P=8.02×10^{-6}; Table S10). Group also affected
348 subsequent survival (χ^2=32.88, df=2, P=5.79×10^{-7}; Table S10), with more hybrid
349 asexuals than non-hybrids or sexuals surviving (Year 1: 82 hybrid asexuals, 17 non-
350 hybrid asexuals, 41 sexuals; Year 2: 75 hybrid asexuals, 28 non-hybrid asexuals, 17

351 sexuals). In all groups, herbivory and probability of survival were negatively correlated
352 (Figure 3). Although the interaction between group and herbivory was non-significant,
353 the negative relationship was strongest in hybrid asexuals (Figure 3), consistent with
354 herbivory limiting hybrid asexual fitness.
355
356 **Discussion**
357 Here we show that both abiotic and biotic factors play key roles in the maintenance of sex
358 in nature. While viability selection favors both inter- and intraspecific asexuals,
359 implicating shared causative traits such as asexuality or heterozygosity, biotic
360 interactions are especially influenced by hybridization. Together, these results suggest
361 that, although survival of adverse abiotic conditions is facilitated by asexuality or
362 associated heterozygosity, hybridization plays a singular role in the interaction between
363 asexuality and herbivory. The precise targets of selection are currently unknown, but our
364 results suggest that the costs of sex depends on ones' sexual partners, and may be
365 mediated by interactions with biological antagonists.
366
367 **The complex ecology of sex**
368 Despite extensive theoretical work and recurrent calls for field-based studies
369 (Agrawal 2006, 2009), few researchers have examined the ecology of sex. Identifying
370 ecologically-relevant traits associated with sex, and the selective processes acting in
371 natural environments, could elucidate the phenotypes and ultimately the molecular
372 mechanisms underlying the cost of sex in wild populations.

373 Although numerous selective agents are likely important to the evolution of sex
374 (Neiman *et al.* 2018), we have little understanding of how these biotic and abiotic
375 selection pressures maintain sex in the wild. Previous work shows that sex may permit
376 survival during abiotic environmental shifts (Stross & Hill 1965) or enable avoidance of
377 fitness-reducing biotic interactions (Lively 1987). We found that strong viability
378 selection early in life results in higher lifetime fitness for asexual lineages, but increased
379 insect herbivory may provide an upper limit on asexual fitness. That different agents act
380 at different life stages suggests that long-term studies are key to understanding the
381 evolution of sex.
382 Many of the best-known asexual systems are polyploid (e.g., Fox *et al.* 1996;
383 Thompson & Whitton 2006), hybrid (e.g., Xu *et al.* 2015) or both (e.g., Lutes *et al.* 2010;
384 Gutekunst *et al.* 2018). Yet these biological features are rarely considered in either
385 studies of these systems or in broader discussions of the costs of sex. We explicitly
386 considered traits correlated with reproductive mode in assessing patterns of selection on
387 sexual reproduction, finding that viability is especially reduced in homozygous sexual
388 lineages, consistent with inbreeding depression acting as a cost of sex. Hybrid asexuals
389 were more prone to herbivory, suggesting that the costs of asexuality may be related to
390 the correlated trait of hybridity. Clearly, selective processes that influence the evolution
391 of sex are shaped by both the origin and composition of asexual lineages and patterns of
392 mating in sexuals.
393
394 **Mating system influences the evolution and ecology of sex**

395 The fundamental role of sex in all organisms is to reshuffle variation across the
396 genome through recombination and segregation. But the effects of these processes are
397 tempered by the efficacy of such shuffling. When sexual partners resemble one another
398 due to population structure, limited population genomic variation and/or inbreeding, the
399 traditional costs and benefits of sex can change dramatically (De Visser & Elena 2007).
400 For example, self-fertilization cannot unite beneficial mutations arising in different
401 lineages. Practically speaking, the costs and benefits of sex are always a function of mate
402 availability and identity.

403 Despite the widespread existence of asexual reproduction in flowering plants,
404 little is known about the costs of sex in angiosperms. Researchers studying the evolution
405 of sex in natural populations have largely focused on animal systems, where the existence
406 of separate sexes provide clear predictions about the relevant costs and benefits
407 (Lehtonen *et al.* 2012). Although hermaphroditic asexuals are still predicted to have a
408 1.5-fold fitness advantage, sex via self-fertilization muddles these predictions
409 (Charlesworth 1980), as do additional biological traits common in plants (Mogie 1996).
410 For example, both selfing and pseudogamous apomictic *Boechera* must produce
411 sufficient pollen for self-fertilization, reducing the applicability of any cost of males.
412 Additionally, the strong link between hybridization and asexuality in numerous vertebrate
413 and plant taxa (Asker & Jerling 1992; Kearney 2005) suggests that recurrent independent
414 hybridization events generate novel asexuals. In *Boechera*, both sexual and asexual
415 lineages have limited variation, but high diversity among asexual lineages may be
416 generated by multiple independent origins of asexuality. Such cases require consideration
417 of additional costs and benefits of sex absent from most models.

418 We hypothesize that inbreeding depression manifests as the primary cost of sex in
419 sexual *Boechera*. Our experiment showed fitness costs and benefits associated with sex
420 that follow predictions for inbreeding depression. Sexuals had reduced overwinter
421 survival (Figure 1), particularly in homozygous lineages (Figure S4), that varied both
422 spatially and temporally (Figure 1, Table 1, Table S4), consistent with inbreeding
423 depression (Armbruster & Reed 2005; Cheptou & Donohue 2010). Our experiment
424 contained few heterozygous sexuals, and was thus underpowered to detect a strong
425 relationship between heterozygosity and fitness. Nonetheless, our results suggest that the
426 main cost of sex in *Boechera* may be related to deleterious load, and that outcrossing may
427 be sufficient to increase survival in this system. This pattern, known as a heterozygosity-
428 fitness correlation or HFC, is widespread in wild systems (Szulkin *et al.* 2010). Despite
429 reduced survival in homozygous sexual lineages, we saw no evidence that survival
430 differed between asexuals and heterozygous sexuals (Table S5), consistent with the
431 hypothesis that the benefit of heterozygosity is the masking of genome-wide recessive
432 load.

433 Although inbreeding likely poses a main cost of sex in this system, other factors
434 may also be at play. First, asexual lineages likely arise frequently and those found in
435 nature have survived selection, potentially resulting in an overrepresentation of high-
436 fitness asexual genotypes in the field. Indeed, the majority of de novo *Boechera* hybrids
437 have lower fitness than their selfed progenitors (Rushworth & Mitchell-Olds 2020),
438 suggesting that fitness variation may be more common in the wild than our experiment
439 suggests. Additionally, the recurrent origins of asexual lineages (Sharbel & Mitchell-Olds
440 2001) suggest that asexuals comprise a range of ages. With deleterious mutations

441 accumulating over the lifespan of a given asexual lineage, older lineages may have
442 reduced fitness. Lastly, reproductive isolation that varies across populations may also
443 result in fitness that depends on interactions between parental genotypes. Each of these
444 scenarios is plausible and provides avenues for further research.

445 Self-fertilization also has clear benefits. Sex and recombination enable adaptation
446 to novel environments (Felsenstein 1974), and inbreeding may speed local adaptation. In
447 situations where environmental changes are substantial, fixation of beneficial recessive
448 alleles through self-fertilization results in rapid adaptation (Glémin & Ronfort 2012).
449 Self-fertilization will also reduce the potential for reshuffling of beneficial allelic
450 combinations, as high homozygosity limits opportunities for functional disruption
451 (Hartfield *et al.* 2017). Our results showed that sexual lineages experienced less insect
452 herbivory than asexuals (Figure 2). Local adaptation underlies variation in chemical
453 defense both among populations of *Boechera stricta* (Prasad *et al.* 2012) and between
454 species (Windsor *et al.* 2005). We thus hypothesize that interactions between divergent
455 alleles in outcrossed asexuals disrupt chemical defenses, consistent with outbreeding
456 depression.

457

458 **Herbivory as an ecological cost of asexuality**

459 Classic literature posits multiple costs of asexuality, including the accumulation of
460 deleterious mutations and the extinction of asexual clones with the highest mutation load
461 (Müller 1964; Felsenstein 1974). While asexual *Boechera* do show elevated putatively
462 deleterious mutations (Lovell *et al.* 2017), the heterozygous state of these mutations, the
463 higher fitness of asexuals observed here, and the replenishment of asexuals by novel

464 hybridization events, suggests that these intrinsic genetic costs have relatively little to do
465 with the costs of asexuality in *Boechera*.

466 Rather, we found evidence for a short-term ecological cost associated with
467 asexuality in this system – a 73% increase in asexual insect herbivory (Figure 2),
468 consistent with predictions from theoretical literature (Stearns 1987). This pattern may be
469 underlain by asexuality, heterozygosity, hybridization, or some combination of all three.
470 In functionally asexual *Oenothera*, generalist herbivory is elevated across multiple
471 species (Johnson *et al.* 2009), supporting a role for asexuality in the macroevolution of
472 plant defense. Microevolutionary patterns suggest the same; viral infection is elevated in
473 asexual and common sexual grass genotypes (Kelley 1994). Although elevated in both
474 asexual groups, insect herbivory is highest in hybrid asexuals (Figure 2). Ample research
475 also shows that hybridization strongly alters plant defense and herbivore preference
476 (Strauss 1994; Fritz 1999; Whitham *et al.* 1999; Orians 2000), and hybrid taxa may
477 produce greater or lesser quantities of chemical compounds, and/or novel ineffective
478 compounds (Orians 2000). Disentangling the influence of asexuality and hybridization on
479 defense offers a fruitful avenue for future research.

480 While the costs of herbivory were insufficient to overcome the other benefits of
481 asexuality in our two-year study, these costs could manifest in other traits or at later life
482 stages (Marquis 1992). We found that herbivory decreased survival to subsequent years
483 (Figure 3), consistent with the negative correlation between fitness and herbivory
484 documented in *Boechera stricta* (Prasad *et al.* 2012). Similar patterns have been
485 identified in animals, suggesting that downstream consequences of parasitism or
486 herbivory are broadly applicable. One recent study of Soay sheep showed that fecundity

487 and parasite load collectively reduce overwinter survival (Leivesley *et al.* 2019).
488 Additionally, we found that the relationship between second-year survival and herbivory
489 is strongest in hybrid asexuals, suggesting that hybridization may limit the spread of
490 asexuality via herbivory. However, more hybrids survive to a second year of life overall.
491 Further work, including herbivore exposure experiments, are needed to understand the
492 interplay between fitness and herbivory in this system.
493 Collectively, the work presented here shows that sexual/asexual dynamics in wild
494 plant populations are largely shaped by environmental complexity and correlated traits,
495 including mating system. Asexual *Boechera* have consistently higher fitness and higher
496 herbivory damage than self-fertilizing sexual lineages, and survival is lowest in
497 homozygous sexuals. Our results highlight the importance of both the biotic and abiotic
498 environment in the evolution of sex, and suggest that inbreeding and outbreeding
499 depression underlie the ecological costs and benefits of sex in this system. Collectively,
500 this work shows that the complex natural environment and the choice of a sexual partner
501 work in tandem to shape patterns of reproductive variation in the wild.
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722 **Tables and Figures**

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724 **Table 1**. **Reproductive system (sexual vs. asexual) and hybridity predict lifetime**
725 **fitness via spatially- and temporally-variable selection.** Results for the main effect of
726 group and two interactions (group and garden, and group and year) for zero-inflated
727 negative binomial GLMMs of lifetime fitness. Left, the zero-inflation portion models
728 structural zeros or plants that did not survive to reproduce. Center, the conditional portion
729 models seed set in plants that survived (fecundity). Right, significance estimates for the
730 overall model, incorporating both portions. Group indicates sexuals, "sex" vs. asexual
731 hybrids, "hyb" vs. asexual non-hybrids, the reference category. Estimates come from
732 conditional models, while test statistics (χ^2 deviance, degrees of freedom, and *P* values)
733 come from likelihood ratio tests for each overall effect. Significant *P*-values are in bold.
734 Full results from each separate model are in Supplementary Information.

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			Zero-inflation				*Conditional*				*Overall model*		
term	condition	df	coef	se	χ^2	*P*	coef	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	**6.66×10^{-6}**	0.048	0.221	23.70	**0.01**	16	66.70	**3.00×10^{-7}**
	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	**5.36×10^{-5}**	0.594	0.194	13.32	**5.16×10^{-3}**	4	36.87	**1.53×10^{-6}**
	hyb*2		-0.402	0.407			0.071	0.193					

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

A.

term	condition	estimate	se	df	*F*	*P*
	intercept	-1.633	0.071	-	-	-
RS	sexual	-0.299	0.079	1, 48.29	22.55	**9.33×10^{-5}**
Garden	CAP	-0.002	0.074	3, 62.99	6.40	**2.99×10^{-3}**
	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**

765 B.

term	condition	estimate	se	df	*F*	*P*
	intercept	-1.719	0.095	-	-	-
Group	sex	-0.210	0.010	2, 42.39	18.08	**1.26×10^{-5}**
	hyb	0.141	0.102			
Garden	CAP	-0.035	0.098	3, 62.98	6.37	**2.99×10^{-3}**
	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**

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773 **Figure 1**. **Asexuals survival is higher than sexual.** Estimated marginal means for
774 survival show substantial temporal (A, group by year interaction) and spatial variation (B,
775 group by garden interaction). (A) Survival is reduced in sexuals in both experimental
776 years. (B) In all sites, hybrid asexuals have higher over-winter survival than sexuals; in
777 three of five sites, non-hybrid asexual survival is similarly high. Bars show 95%
778 confidence intervals. SIL was only included in year one, but fitness was predicted at this
779 site.
780
781 **Figure 2**. **Herbivory is elevated in hybrid asexuals.** Estimated marginal means for
782 herbivory from LMMs show the main effects of group (sexual vs. non-hybrid asexual vs.
783 hybrid asexual, left) and reproductive system (sexual vs. asexual, right). Bars show 95%
784 confidence intervals.
785
786 **Figure 3**. **Plants with less herbivory are more likely to survive to the next year.**
787 Herbivory has a significant impact on survival to a second year, suggesting that the
788 detrimental consequences of herbivory may not be observed in a single growing season.
789 Although the correlation between year 2 survival and herbivory differs qualitatively by
790 group (sexual vs. non-hybrid asexual vs. hybrid asexual), the interaction is not
791 significant. Estimated marginal means are shown averaged across five different values of
792 log herbivory. Points are jittered for ease of reading. Bars show 95% confidence
793 intervals.
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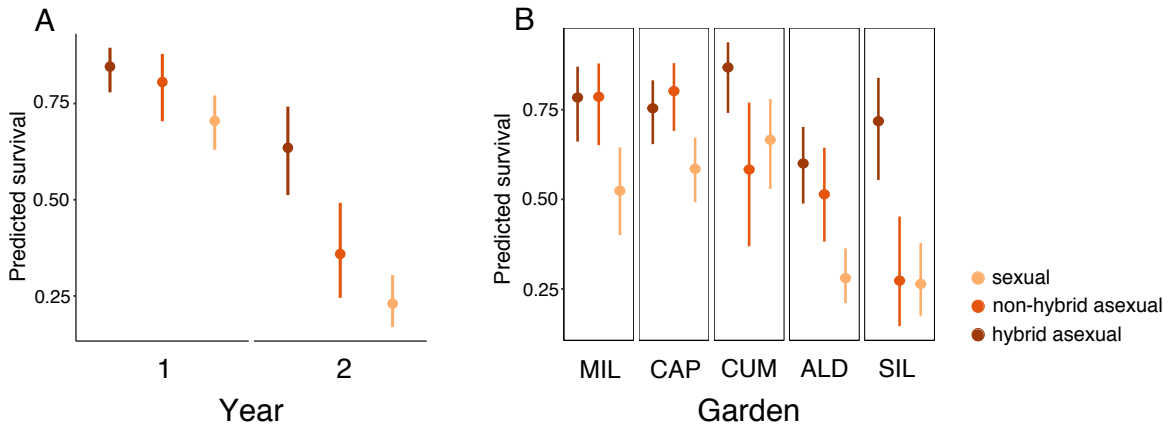
A
Predicted survival
0.75
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1
2
Year
B
Predicted survival
0.75
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MIL
CAP
CUM
ALD
SIL
Garden
sexual
non-hybrid asexual
hybrid asexual

Figure 1

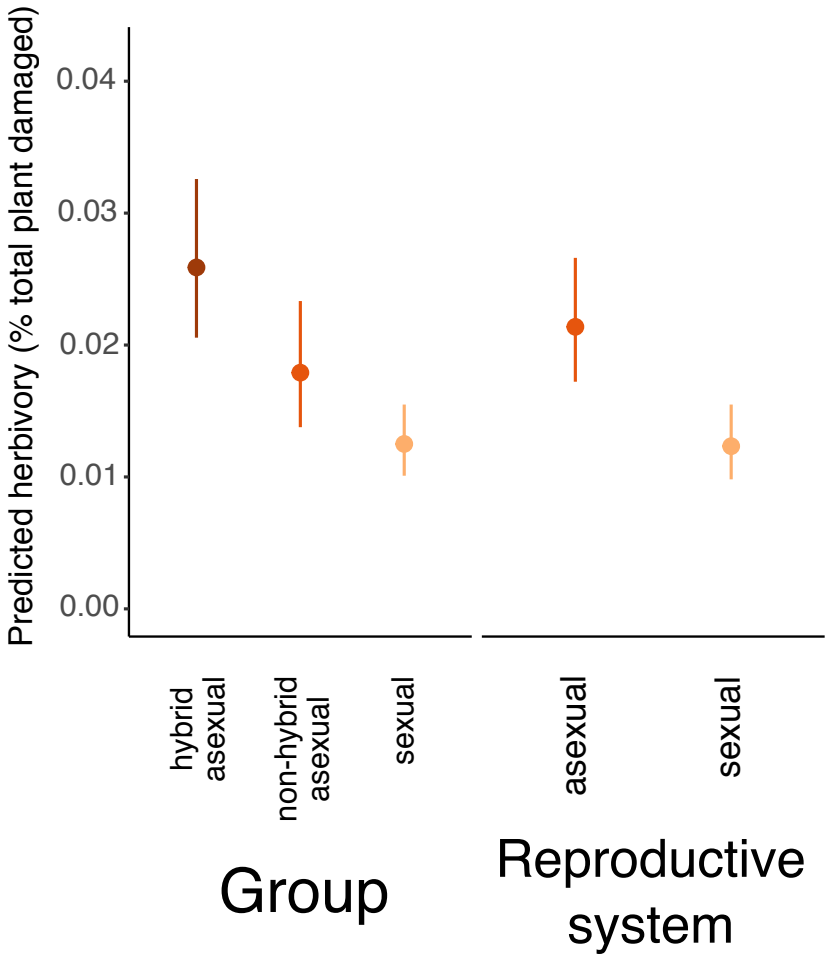
Predicted herbivory (% total plant damaged)
0.04
0.03
0.02
0.01
0.00
hybrid asexual
non-hybrid asexual
sexual
asexual
sexual
Group
Reproductive system

Figure 2

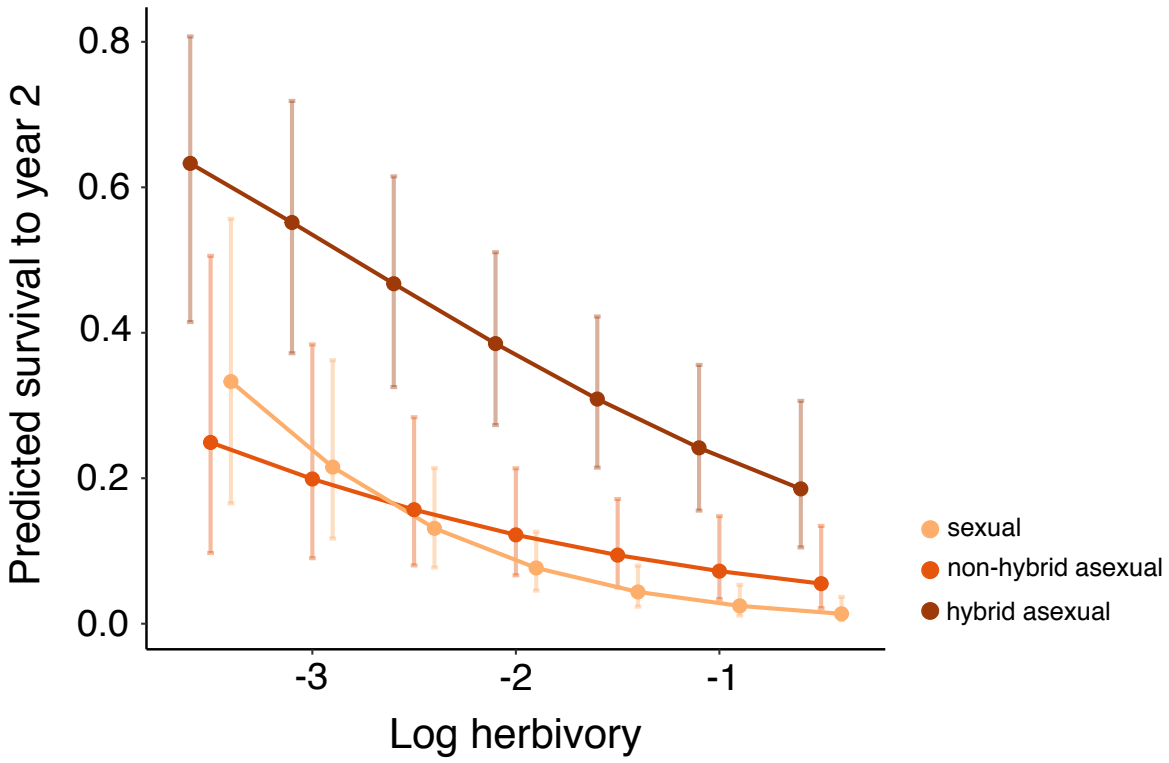
Predicted survival to year 2
0.8
0.6
0.4
0.2
0.0
-3
-2
-1
Log herbivory
sexual
non-hybrid asexual
hybrid asexual

Figure 3