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Fitness consequences of occasional outcrossing in a functionally asexual plant (*Oenothera biennis*)

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Abstract. Many clonal organisms occasionally outcross, but the long-term consequences of such infrequent events are often unknown. During five years, representing three to five plant generations, we followed 16 experimental field populations of the forb, *Oenothera biennis,* originally planted with the same 18 original genotypes. *Oenothera biennis* usually self fertilizes, which, due to its genetic system (permanent translocation heterozygosity), results in seeds that are clones of the maternal plant. However, rare outcrossing produces genetically novel offspring (but without recombination or increased heterozygosity). We sought to understand whether novel genotypes produced through natural outcrossing had greater fecundity or different multigenerational dynamics compared to our original genotypes. We further assessed whether any differences in fitness or abundances through time between original and novel genotypes were exaggerated in the presence vs. absence of insect herbivores. Over the course of the experiment, we genotyped >12,500 plants using microsatellite DNA markers to identify and track the frequency of specific genotypes and estimated fecundity on a subset (>3,000) of plants. The effective outcrossing rate was 7.3% in the first year and ultimately 50% of the plants were of outcrossed origin by the final year of the experiment. Lifetime fruit production per plant was on average 32% higher across all novel genotypes produced via outcrossing compared to the original genotypes, and this fecundity advantage was significantly enhanced in populations lacking herbivores. Among 43 novel genotypes that were abundant enough to phenotype with replication, plants produced nearly 30% more fruits than the average of their specific two parental genotypes, and marginally more fruits (8%) than their most fecund parent. Mean per capita fecundity of novel genotypes predicted their relative frequencies at the end of the experiment. Novel genotypes increased more dramatically in herbivore-present compared to suppressed populations (45% vs. 27% of all plants), countering the increased competition from dandelions (*Taraxacum officinale*) that resulted from herbivore suppression. Increased interspecific competition likely also lead to the lower realized fitness of novel vs. original genotypes in herbivore-suppressed populations. These results demonstrate that rare outcrossing and the generation of novel genotypes can create high-fecundity progeny, with the biotic environment influencing the dynamical outcome of such advantages.

Key words: field experiment; Oenothera biennis; *permanent translocation heterozygote; plant fitness; rare outcrossing; sexual reproduction.*

Introduction

Asexual reproduction, the production of progeny genetically identical to a parent, has evolutionary advantages and disadvantages compared to sexual strategies, broadly defined as reproduction involving outcrossing, recombination, and segregation of alleles. Asexual reproduction has evolved repeatedly (Simon et al. 2002, Whitton et al. 2008), and the benefits to asexuality include saving costs associated with mate attraction, avoiding the cost of meiosis, faster growth of asexual vs. sexual populations, and the potential close fit between locally adapted clonal genotypes and their environment (Williams 1975, Maynard-Smith 1978). However, these advantages can be countered by disadvantages that include the loss of genetic variability and the associated lack of adaptability (Weismann 1889, Fisher 1930, Muller 1932, Jaenike 1978, Otto 2009), the inability to keep pace with natural enemies in coevolutionary arms races (the so-called "Red Queen's Hypothesis"; Van Valen 1973, Levin 1975, Bell 1982, Hamilton et al. 1990, Lively 2010), and the disproportionate accumulation of deleterious mutations (i.e., Muller's Ratchet; Muller 1964) that can lead to long-term negative fitness consequences (Lynch and Gabriel 1990, Andersson and Hughes 1996, Hollister et al. 2015, but see Tucker et al. 2013).

Many organisms possess dual reproductive strategies, whereby they predominantly reproduce asexually but can occasionally reproduce sexually, with the extent of outcrossing, recombination, and segregation varying by

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species (Hull et al. 2000, Halket et al. 2005, Molins et al. 2013, Signorovitch et al. 2015). These species may gain the benefits of sexual strategies even though reproduction that generates genetic novelty via outcrossing may only occur rarely (Charlesworth and Charlesworth 1995, D'Souza and Michiels 2010). Indeed, sexual reproduction that occurs at only 5–10% frequency can lead to advantages equivalent to those that occur in populations with 100% sexual reproduction (Charlesworth et al. 1993, Green and Noakes 1995).

Studies that have quantified the advantages of rare outcrossing within predominately asexual populations have tracked the frequency of clonal vs. outcrossed genotypes or compared their performance under different conditions in microcosms (Colegrave 2002, Cooper 2007, Becks and Agrawal 2010, 2012, Gray and Goddard 2012, Masri et al. 2013). Among plants, classic work on the grass *Anthoxanthum odoratum* demonstrated that sexually generated progeny had substantially higher fecundity than clonal progeny (Ellstrand and Antonovics 1985, Kelley et al. 1988), and that sexual reproduction was beneficial in reducing herbivory (Schmitt and Antonovics 1986). Few other studies on plants, however, have tested the effects of clonal vs. sexual reproductive strategy on fitness in the presence and absence of enemies (Steets et al. 2007).

Plants have diverse sexual strategies ranging from being primarily clonal (e.g., vegetative reproduction, apomixis) to having mixed mating strategies, to obligately requiring outcrossing (Barrett 2002, Goodwillie et al. 2005). Along this continuum of plant sexual strategies, with varying degrees of outcrossing, recombination, and segregation, is permanent translocation heterozygosity (PTH; Cleland 1972). PTH is a genetic system that occurs in eight plant families and is most diverse in the Onagraceae where it has independently evolved >20 times (Holsinger and Ellstrand 1984, Johnson et al. 2011). In such species, self-fertilization results in clonally related seeds because offspring inherit their parental genomes without meiotic recombination or segregation of chromosomes (Cleland 1972, Rauwolf et al. 2008, Golczyk et al. 2014). Because many of these species were originally formed via the hybridization of two parental species (Cleland 1972, Dietrich et al. 1997), the lack of recombination and segregation results in "permanent" heterozygotes. However, occasional outcrossing in these species results in offspring that inherit one unrecombined haploid chromosome set from each parent. This creates genetic novelty through the formation of new allelic combinations, but without any recombination (since haploid chromosomes are inherited *en masse*; Cleland 1972, Rauwolf et al. 2008). Such rare outcrossing could have a minimal impact on fitness because offspring lack the amount of genetic novelty that typically occurs with sex, and parents are always heterozygous. Alternatively, occasional outcrossing could be advantageous either due to the simple generation of novel genotypes on which selection can operate, or because there is a mechanistic advantage of having a unique genotype, such as what occurs with negative frequency-dependent selection.

Here we document the fitness and multigenerational population-level consequences of rare outcrossing within replicate experimental field populations of common evening primrose, *Oenothera biennis*. *Oenothera biennis* is an annual to biennial semelparous plant, native to eastern North America, and it has a permanent translocation heterozygote genetic system. In 2007, we created experimental populations composed of 18 unique clonal genotypes of *O. biennis* that were either exposed to or protected from ambient insect herbivores (Agrawal et al. 2012, 2013). By genotyping >12,500 plants across replicate experimental populations during five years and multiple generations, we detected the presence of initially rare novel genotypes in each experimental population. This enabled us to ask whether lifetime fecundity differed between the original genotypes and novel genotypes produced via rare outcrossing events. We further determined whether there were different multi-generational dynamics of the original clones vs. novel outcrossed genotypes and whether these dynamics could be predicted from variation among individuals in per capita fecundity. Finally, we assessed whether novel genotypes had greater advantages in the presence vs. absence of herbivores. This comparison is of interest because it sheds light on whether genetic novelty and rare outcrossing is especially beneficial in the face of enemy pressure (Bell 1982).

Methods

Natural history

Oenothera biennis L. (Onagraceae) is common in old fields, lake shores, gravel bars, and other open and recently disturbed habitats. The species requires light to germinate and is a relatively poor competitor, and thus is only usually common in early successional habitats. Plants are attacked by a variety of insect herbivores (Johnson and Agrawal 2005); the principle herbivores in our study were seed and fruit feeders (*Mompha brevivittella*, *M. stellella*, and *Schinia florida*). Plants produce 1-d flowers and seeds produced through selfing (which typically occurs in *O. biennis* before flowers open) are genetic clones of their parent (Cleland 1972, Dietrich et al. 1997, Rauwolf et al. 2008). Despite their propensity for selfing, *O. biennis* maintain high levels of heterozygosity because they were originally created via interspecific hybridization and they lack the ability to undergo recombination and segregation (Cleland 1972, Rauwolf et al. 2008, 2011). In rare cases, cross-pollination (i.e., outcrossing) occurs in *O. biennis*, which results in progeny that consists of a set of the unrecombined paternal haploid chromosomes associated with a maternal haploid set of chromosomes (again, with no recombination), creating seeds that represent a single novel outcrossed genotype. Hereafter, we define "novel genotypes" as plants produced either via de novo outcrossing, or as a result of outcrossed genotypes subsequently producing clonal seeds, as we could not distinguish between these two mechanisms after the first generation of the

experiment. In contrast, we refer to plants that were clones of the original 18 genotypes in our experiment as "original genotypes."

Experimental design and previous results

During 2007, we established experimental populations of *O. biennis* in 16 replicate 13.5-m^2 plots (minimum of 10 m separation) that were located in an old field on Cornell University property outside of Ithaca (Tompkins County), New York, USA. Although evening primrose was not present at the site (the field was successionally advanced), this species is commonly found growing in such fields, especially those that have been recently disturbed. Prior to planting we prepared the field by plowing it, and then spraying twice with the herbicide glyphosate (Roundup; Monsanto, St. Louis, Missouri, USA). Although pollinators could easily move between replicate plots, seeds disperse very locally as *O. biennis* seeds drop directly to the ground from mature fruits. Although there could have been some contamination between plots, steps were taken to minimize this by only entering plots when collecting data and shaking off shoes. Plant population dynamics were expected and observed to be independent between plots, with little movement of seeds. Plots were protected from deer herbivory by 2 m tall mesh fencing.

We selected a subset of 18 phenotypically diverse genotypes from 40 genotypes (all collected within Tompkins County, New York, USA) that had been grown in a common garden during 2006 (Johnson et al. 2009). In the center 1 m^2 of each plot, we planted 60 individual *O. biennis* seedlings composed of three individuals from each of 16 genotypes and six individuals from each of two additional genotypes (18 genotypes total), with the location of each genotype randomly assigned. The latter two genotypes were originally thought to be four distinct genotypes, but extensive additional analysis could distinguish only two genotypes from one another (Agrawal et al. 2013).

Plants were transplanted at the two-leaf stage; see Agrawal et al. (2013) for more details of the experimental set up. Eight of the 16 experimental populations were randomly assigned to an insect herbivore reduction treatment and were sprayed biweekly every year during the growing season (April through October) with esfenvalerate (Asana XL; Dupont, Wilmington, Delaware, USA), a non-systemic insecticide that does not affect *O. biennis* germination, growth, or survival (Agrawal et al. 2012). The remaining experimental populations were sprayed on the same schedule with water, as a control for the water in the insecticide spray. Experimental populations were not weeded or otherwise manipulated after they were established. We estimated the size of each of the 16 *O. biennis* populations in July of each year by counting the number of rosettes and flowering plants within each of nine quadrats (625 cm^2), and then multiplying the mean abundance per square meter by plot area. In 2011 and 2012, as populations declined, all evening primrose plants were counted in all plots.

Most plants were annual in 2007 and thereafter plants were a mix of annual and biennial. Evening primrose abundance increased after initial planting and peaked during 2009, when averages ($\pm$SE) were 4,167 $\pm$ 625 and 1,971 $\pm$ 396 individuals in herbivore-present and insecticide-treated populations, respectively (Agrawal et al. 2012). Thereafter, experimental populations began to decline as *O. biennis* was outcompeted by colonizing vegetation in each plot (Agrawal et al. 2012). The difference in peak *O. biennis* density between herbivore-present and insecticide-treated populations was initially unexpected, but arose from altered interspecific competitive dynamics driven by the common dandelion (*Taraxacum officinale*) that was released from suppression by the insecticide treatment (Agrawal et al. 2012). Due to the non-intuitive result that plants declined more in populations protected from herbivores compared to those exposed to herbivores, the effects of insect suppression on *O. biennis* genotypes reported here must be interpreted carefully. We specifically incorporate plant density in some analyses to address its possible role vs. that of herbivores in the evolutionary dynamics.

Genotyping

We genotyped rosettes and flowering plants according to their relative proportions estimated per plot per year in the quadrat sampling described in subsection *Experimental design and previous results*. Each year we genotyped at least 190 individuals from each plot (or all plants in the plot if <190 plants) using four microsatellite DNA markers (Larson et al. 2008) that distinguished the 18 genotypes (Agrawal et al. 2012, 2013). Details of the genotyping procedure are provided in Appendix S1.

Outcrossing rate

Novel outcrossed progeny were recognized by multilocus microsatellite genotypes that did not match those of the original genotypes, which breed true without segregation of heterozygous loci when self fertilized (Rauwolf et al. 2008). Less than 0.05% of the >12,500 genotyped individuals could not be assigned as clonal original vs. novel genotypes; these cases were checked with repeated PCR ruling out PCR error and were considered possible mutants. We identified the original parents of novel outcrossed genotypes (hereafter referred to as "novel genotypes" for simplicity) by determining all possible microsatellite haplotypes that comprised the Renner complexes of the original 18 genotypes. This analysis was facilitated by the very high heterozygosity associated with the PTH genetic system (Cleland 1972, Hollister et al. 2015). For example, consider the following idealized case of two parents with the following microsatellite allelic combinations at loci A through D: A_1/A_2, B_1/B_2, C_1/C_2, D_1/D_2 (parental genotype I: allele 1, maternal allele; allele 2, paternal allele) and A_3/A_4, B_3/B_4, C_3/C_4, D_3/D_4 (parental genotype II: allele 3, maternal allele; allele 4 , paternal allele). If we observed genotypes A_1/A_4, B_1/B_4, C_1/C_4, $D_1/$

D_4 or A_3/A_2, B_3/B_2, C_3/C_2, D_3/D_2 among any progeny in the next generation, we were able to conclude that these new genotypes arose by cross-fertilization between parental genotypes I and II. Outcrossing rate was estimated only in the first generation (produced during 2007), as the frequency of outcrossed plants could not be distinguished from clonal reproduction of the novel genotypes created by outcrossing during subsequent generations.

Genotype abundance in each plot (i.e., replicate population) was estimated as the product of a genotype's frequency in the 190 individuals genotyped from each plot and the total number of individuals estimated within each plot. As an estimate of individual performance, in both 2007 and 2008, we counted all fruits produced by the original 60 plants in the center 1 m^2 of each plot. Beginning in 2009, populations were too large to sample every plant, so we subsampled populations by counting fruits on at least 50 randomly chosen individuals from each plot (or all bolting individuals if <50 were present) and identified their genotypes. These data enabled us to estimate genotype-specific fecundity. We estimated the total per plot fruit production for each genotype by multiplying genotype-specific estimates of fruit production by the estimated number of reproductive individuals of that genotype. Seed counts were impractical in this experiment, but fruit number is a good proxy for total fecundity (Agrawal et al. 2013). Fruit size of novel genotypes, a correlate of seed number (Agrawal et al. 2013) did not differ significantly from that of original genotypes (data not shown).

Analyses

We performed a mixed-model ANOVA to examine how per capita fruit production within each plot varied between original and novel genotypes. In this analysis, year, insecticide treatment, and mode of reproduction were fixed factors, and plant genotype (nested within mode of reproduction) and plot (nested within treatment) were random factors. Fruit production data were log-normally distributed, and analyses were conducted on log-transformed data, with back-transformed means presented in figures. In this analysis, all two-factor interactions were tested (the three-way interaction was not significant and was removed from the model). We compared novel genotypes to that of their clonal parents (mean or maximum fecundity of the two parents) using correlation and paired *t* tests. In analyses of fecundity, we used adjusted genotype means from a model with year, plot (treatment), and treatment, to control for environmental differences across plots and years. Analyses were conducted in either Systat (v. 11, Systat Software, Inc., San Jose, CA, USA) or JMP Pro (v. 11, SAS Software, Cary, NC, USA).

Results

Generation of novel genotypes by occasional outcrossing

During the first year of the experiment, 85% (of the 960 individuals) of plants were annual, with a mix of annual and biennial individuals thereafter. Flowering frequency was particularly low in 2008, presumably because of the high density of newly recruited plants. Novel genotypes represented >20% of the >12,500 plants genotyped during all years. In total, 106 novel genotypes were identified (most were rare) and the estimated effective outcrossing rate was 7.3% in the first generation. The rate of outcrossing did not differ with insecticide application ($F_{1,14} = 0.035$, $P = 0.854$). The most abundant novel genotype (new60) was initially detected in only two of the 16 experimental populations during 2008, but occurred in 11 populations during 2009, and all 16 populations during 2010 and 2011. Novel genotypes that composed >1% of all plants initially arose independently in about one-half of the populations by 2009, and eventually occupied at least two-thirds of populations by the end of the experiment in 2012. The phenotypes of novel genotypes correlated strongly with the average of their two original clonal parents for probability of flowering in the first year ($n = 8$, $r = 0.789$, $P = 0.019$) and fruit ellagitannin chemistry ($n = 8$, $r = 0.987$, $P < 0.001$), but not plant growth rate ($n = 8$, $r = 0.453$, $P = 0.626$) (based on data from Agrawal et al. 2012). In contrast, fruit production of novel genotypes correlated only weakly with the average of the two parents ($n = 43$, $r = 0.300$, $P < 0.050$, see below and Fig. 2).

Fecundity of original vs. novel genotypes

Lifetime per capita fruit production was 50% higher for plants in populations where insects were suppressed compared to herbivore-present populations, although this effect varied among years (treatment $F_{1,16.66} = 1.21$, $P = 0.287$, treatment × year $F_{4,\ 3151} = 3.29$, $P = 0.011$; Fig. 1A). Although novel genotypes produced 32% more fruits, on average, than the original genotypes (main effect of reproductive type $F_{1,80.88} = 12.67$, $P < 0.001$), the effects of outcrossing were of greatest magnitude during the peak of plant abundance, in 2010 (year × reproductive type interaction: $F_{4,\ 2168} = 2.74$, $P = 0.027$; Fig. 1B). This result did not depend on whether the analysis included the most abundant novel genotype (new60).

Forty-three novel genotypes provided sufficient data to address the effects of outcrossing on per capita fruit production compared to their original parental genotypes. Novel genotypes were 29% more fecund than the average of their clonal parents (df = 42, paired $t = 6.201$, $P < 0.001$, Fig. 2A), and fecundity of novel genotypes was marginally (8%) higher than the most fit parent (df = 42, paired $t = 1.769$, $P = 0.084$, Fig. 2B). The frequency of the novel genotypes at the end of the experiment varied positively with their mean per capita fruit production estimated across years (Fig. 3).

Effects of insect suppression on fecundity of original and novel genotypes

Overall, insect suppression enhanced fecundity more strongly for novel genotypes (71% fitness increase) than

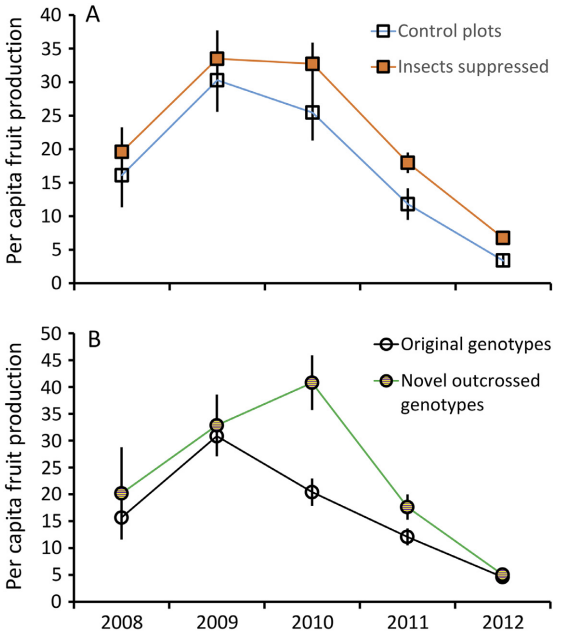

FIG. 1. Effects of (A) insect suppression and (B) original vs. novel genotypes on lifetime per capita fruit production of *O. biennis* across 16 experimental populations (n = 8 experimental populations per treatment). Values are back-transformed LS means (±SE).

original genotypes (28% increase; treatment × reproductive type $F_{1,\ 2675} = 4.43$, $P = 0.035$; Fig. 4A). Again, excluding the most abundant novel genotype (new60) did not qualitatively alter this result ($F_{1,\ 2590} = 4.56$, $P = 0.033$). Despite this fecundity advantage of novel genotypes in insect-suppressed populations, the abundance differed less between novel and original genotypes in insect-suppressed vs. herbivore-present populations towards the end of the experiment (analysis from 2011 before the populations declined, treatment × reproductive type: $F_{1,14} = 5.66$, $P = 0.032$, Fig. 4B). Indeed, although the frequency of original and novel genotypes converged to 50% of plants across all populations in 2012, novel genotypes were significantly less frequent in insect-suppressed populations than in herbivore-present populations during both 2010 and 2011 (Fig. 5A).

Oenothera biennis density was halved in the insect suppression populations due to release of an interspecific competitor (Agrawal et al. 2012). Nonetheless, per capita fecundity of original or novel genotypes did not vary with population density (density $F_{1,79.38} = 0.294$, $P = 0.589$; density × reproductive type $F_{1,74.91} = 0.390$, $P = 0.534$). However, fecundity differed significantly between reproductive types in this analysis, indicating that independent of a population's plant density, novel genotypes had higher fecundity (across years) than original genotypes (reproductive type $F_{1,74.91} = 34.62$, $P < 0.001$).

The density of flowering plants was the best predictor of the frequency of novel genotypes created by

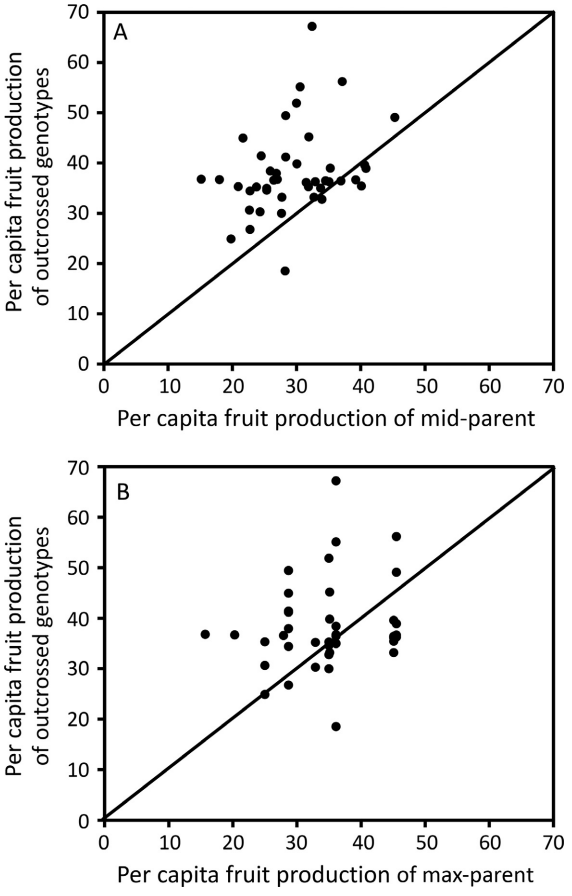

FIG. 2. Among 43 newly generated novel genotypes of *O. biennis,* lifetime per capita fruit production as predicted by the (A) mean of the two parents (mid-parent) and (B) the maximum of their two parents (max-parent). Data points are LS means from a mixed-model analysis (see *Methods* for details).

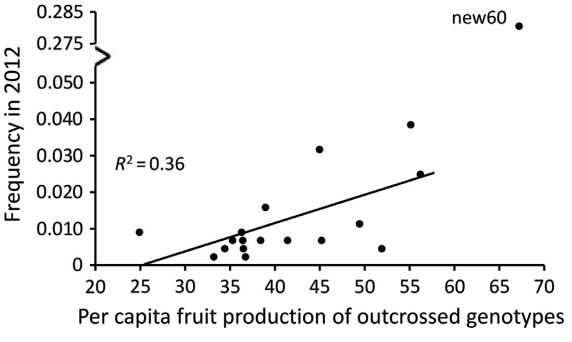

FIG. 3. Novel genotypes of *O. biennis* with higher per capita fruit production (LS means, estimated from across all years) are proportionally at higher frequency at the end of the experiment (2012). Note that the regression line (and R^2) is drawn excluding the outlying new60 genotype. The relationships are also statistically significant with this genotype ($R^2 = 0.45$, analyses were also significant with a nonparametric Spearman's test: $\rho = 0.562$, $P = 0.015$). Not all novel genotypes are represented because many did not persist through 2012.

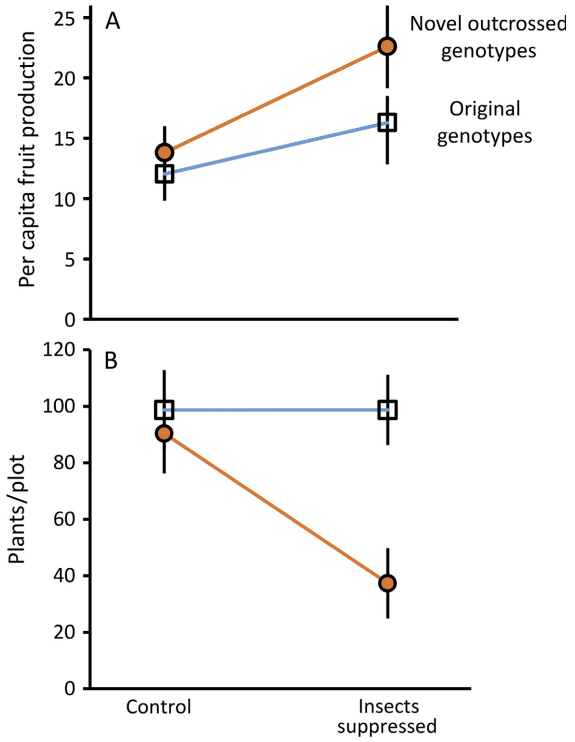

FIG. 4. The impact of insect suppression on back-transformed LS mean (±SE) (A) per capita lifetime fruit production and (B) plant abundance per experimental population for original and novel genotypes of *O. biennis* (n = 8 experimental populations per treatment).

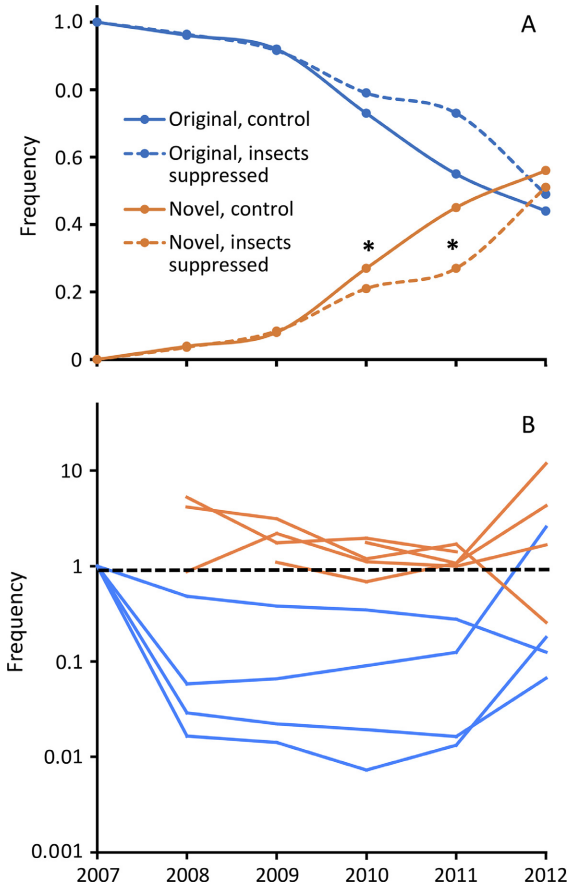

FIG. 5. The frequency of original and novel genotypes of *O. biennis* over six years (three to five plant generations). (A) Points are the mean of eight experimental populations for each treatment. Novel genotypes were naturally generated and recruited into populations. Original and novel genotypes are a mirror image because they sum to a frequency of 1. Asterisks indicate significant differences ($P \leq 0.05$) in frequency between insect suppressed and control populations for each reproductive type. (B) Mean relative frequency (herbivore-present/insect-herbivore-suppressed populations) over time of each genotype that represented 1% or greater frequency at the end of the experiment in 2012 (blue are original genotypes and orange are novel genotypes). Means are derived from the eight herbivore-present and eight insect-suppressed populations. The dashed black line indicates the null hypothesis of no divergence in genotype frequencies between treatments. The y-axis is on a log scale to facilitate equal visualization of under- and over-represented genotype frequencies depending on treatment.

outcrossing. Since our experiment started in 2007 with near equal densities of clones across all populations, and there was relatively little flowering in 2008, 2009 was the first year in which there was substantial variation in the density of flowering *O. biennis* among replicate populations. The density of flowering *O. biennis* in herbivore-present populations in 2009 predicted the frequency of novel genotypes observed in 2010 (n = 8, r = 0.873, P = 0.028, Fig. 6). Density of flowering *O. biennis* was a substantially better predictor of novel genotype frequency than total plant density, that latter of which included thousands of non-flowering rosettes and did not correlate with frequency of novel genotypes in 2010 (n = 8, r = 0.709, P = 0.205). Within insect-suppressed populations, which were one-half as dense as herbivore-present populations, there was no relationship between density in one year and the frequency of novel genotypes in the next (n = 8, r = 0.438, P = 0.646; Fig. 6).

Genotype frequencies at the end of the experiment

The frequency of novel genotypes created by outcrossing increased dramatically during the experiment. By the end of the experiment in 2012, *O. biennis* densities had dropped from a high of >1,500 plants/plot during 2008–2010 to an average (±SE) of only 19.13 ± 4.98 and 38.25 ± 15.42 plants/plot in herbivore-present and herbivore-suppressed populations, respectively. One-half of these plants were novel genotypes (Fig. 5A). Although most novel genotypes were rare (<1% frequency), by 2012, five novel genotypes occurred in experimental populations at or above 1% overall frequency (Four of which are shown; Fig. 5B). One of these common genotypes, (new60) represented, on average, 20% and 32% of all plants in herbivore-present and herbivore-suppressed populations, respectively. Similarly, by 2012, only four of the 18 original genotypes occurred at or

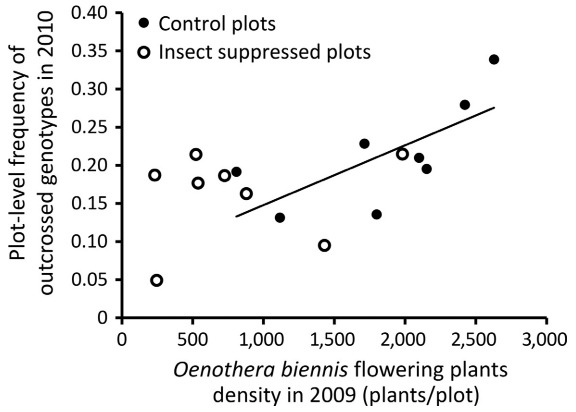

FIG. 6. The frequency of novel genotypes of *O. biennis* as a function of the density of flowering *O. biennis* in the previous year. The significant correlation is shown for herbivore-present populations, while the relationship is not significant for insect-suppressed plots (see *Results* for details).

above 1% frequency, with one genotype composing an average of 47% and 18% of all plants in herbivore-present and herbivore-suppressed populations, respectively.

DISCUSSION

In a 5-yr experiment initiated by establishing replicate experimental populations of *O. biennis* with the same 18 original clonal genotypes, striking population dynamic and evolutionary change occurred, as evidenced by altered genotypic frequencies within and among populations (Agrawal et al. 2012, 2013). These changes in existing genotypes were supplemented by the creation of novel genotypes created by a low frequency of outcrossing. Several of the novel genotypes substantially increased in frequency, and by the end of experiment, nearly 50% of plants were of outcrossed origin, suggesting a major role of rare outcrossing events in creating genetic novelty that affects the ecological and evolutionary dynamics of *O. biennis* populations.

Given the manifold costs of sex, sexual reproduction must have clear ecological and evolutionary advantages over exclusively clonal reproduction (Williams 1975, Maynard-Smith 1978, Agrawal 2006, Schwander et al. 2014). For species such as *O. biennis*, which typically reproduce via a functionally asexual genetic system (suppressed recombination and segregation due to permanent translocation heterozygosity) it was unclear what the implications of rare outcrossing might be. Our results suggest that even when outcrossing produces offspring with an exact copy of one chromosome set from each parent, and no independent assortment and recombination, this genotypic novelty is ecologically and evolutionarily important. Indeed, the "mixed" reproductive strategy of functional asexuality with occasional outcrossing has distinct advantages for the individual fecundity of plants and, the longer-term population dynamics of *O. biennis*. Our results are consistent with the more general prediction that even rare outcrossing can lead to rapid increases in genetic diversity within populations (Charlesworth and Charlesworth 1995). Such increased genetic diversity is known to have positive impacts on the growth and stability of populations (Hughes et al. 2008).

Novel genotypes that established and rose in frequency were substantially more fecund than their parents and on average 8% more fecund than their most fecund parent. Given that outcrossing could theoretically generate 306 novel genotypes (18 original genotypes × 17 = 306, given the genetic system of *O. biennis*), perhaps only the highest fecundity genotypes persisted. A few high fecundity genotypes (e.g., new60) were independently produced in all populations, suggesting that a floral (morphological) mechanism, such as greater herkogamy (i.e., stigma–anther separation), may have allowed for greater outcrossing between particular parents. Many other novel genotypes were generated with reduced frequency. Nonetheless, the enhanced fecundity we observed was general across 43 novel genotypes. Similar fitness gains resulting from occasional sexual reproduction have also been quantified in organisms such as clonal freshwater snails (Vergara et al. 2014), planaria (D'Souza and Michiels 2010), and a perennial grass (Kelley et al. 1988), although their genetic systems differ from that of *O. biennis*. In the only other study of the fitness effects of outcrossing in a PTH species, Heiser and Shaw (2006) found evidence for reduced fitness of outcrossed (with pollen from distant populations) *Oenothera serrulata* (=*Calylophus serrulatus*) in a controlled environment compared to clonal parentals, which may have resulted from genetic incompatibilities among genotypes.

One possible advantage to outcrossing is that it may allow purging or masking of deleterious mutations (Muller 1964, Charlesworth 2012). Outcrossing in *O. biennis* (which results in offspring containing one haploid set of chromosomes from each parent) can also facilitate masking the expression of recessive deleterious mutations that were homozygous in a clonal parent. This may be particularly important if heterozygous loci become homozygous due to gene conversion, which may be particularly prevalent in asexual lineages (Flot et al. 2013, Tucker et al. 2013). Accordingly, one interpretation of our results is that outcrossing can alleviate genetic load, which can impose long-term negative fitness effects in asexual lineages (Lynch and Gabriel 1990, Andersson and Hughes 1996, Hollister et al. 2015). Thus, our data suggest that rare outcrossing may provide "the best of both worlds," wherein plants gain the advantages of asexuality with occasional outcrossing generating novel genotypes that potentially mask recessive deleterious alleles present in clonal parents.

Fecundity advantages possessed by novel genotypes resulted in significant increases in their frequencies within experimental populations. This is supported by the fact that mean per capita fruit production for these novel genotypes (across all years) predicted their relative frequency at the end of the experiment. Of the novel

genotypes that were identified, five in particular became abundant, and were represented at >1% frequency across experimental populations in 2012. Similarly, four original clonal genotypes also became relatively abundant, and occurred at >1% frequency across all populations (Fig. 5B). We speculate that differential survival at an early life history stage accounts for the selective advantages accrued to novel genotypes. This interpretation is consistent with results suggesting strong selection during the seed and seedling stage had large effects on the evolutionary trajectory of experimental populations (Agrawal et al. 2013).

Previous analyses of the experiment revealed that selection strongly favored genotypes with a greater propensity for annual (as opposed to biennial) reproduction and that the dominance of just a few original genotypes through time was driven by differential recruitment of those genotypes (Agrawal et al. 2013). These processes likely explain the rapid increase in frequency of a few novel genotypes as well, in that the dominant outcrossed genotype (new60) was also highly annual (*unpublished data*). However, the selective advantage of novel compared to original genotypes was unlikely explained by differences in annuality between these genotypes; novel outcrossed genotype annuality was predicted by the mid-point of their parents' annuality ($F_{1,6} = 10.153$, $P < 0.019$). Additionally, at least one of the five abundant novel genotypes was primarily biennial (<20% probability of bolting in the first year).

Another possible explanation for the rise in frequency of a few successful novel genotypes could be negative frequency-dependent selection. This might explain why novel genotypes did proportionally better in experimental populations that were exposed vs. protected from insect herbivores. Negative frequency-dependent selection can be important in maintaining genetic variation in populations (Hartl and Clark 1997) and is hypothesized to be important in the evolutionary dynamics of host–enemy interactions (Thompson and Burdon 1992). For example, trematode parasites of a freshwater snail that is polymorphic for sexual vs. asexual reproduction, impose selection in favor of rare snail genotypes (Koskella and Lively 2007). This negative frequency-dependent selection is credited with maintaining genetic variation within populations, and is thought to play a role in the maintenance of sex (Morran et al. 2011). Although we cannot reject a role for frequency dependence driving the dynamics observed in *O. biennis*, such dynamics are most frequently suggested in gene-for-gene models of resistance (Otto and Nuismer 2004), and two observations suggest that this was not the primary driver in our system. First, relatively few of the novel genotypes became abundant, and among those that did become abundant, per capita fecundity predicted their frequency. Second, the winnowing of genotypic diversity seen among novel genotypes mirrored that seen in the original 18 genotypes that were planted at equal frequencies in the first generation.

Selection for outcrossing in the presence of herbivores

Suppression of insect herbivores enhanced overall plant fecundity and the fecundity advantages of novel over original genotypes. The frequency of novel genotypes increased more in populations with ambient herbivory compared to insect-suppressed populations. Although this latter result is consistent with the Red Queen hypothesis (Bell 1982, Becks and Agrawal 2012), why was it that the higher fecundity of novel genotypes within populations experiencing suppressed herbivory did not lead to an increase in the frequency of these genotypes within these populations? Perhaps intense competition with dense common dandelions limited evening primrose recruitment so strongly that fecundity gains by outcrossed genotypes were not manifest at the population level. Dandelions disproportionally increased within herbivore-suppressed populations, due to relaxed damage by their own insect herbivores (Agrawal et al. 2012). This caused precipitous declines in evening primrose density in insect suppressed populations that exceeded the overall experiment-wide decline due to successional changes (Agrawal et al. 2012).

Novel genotypes arose more frequently in control populations with higher density of flowering evening primrose plants. Indeed, during 2010, the frequency of novel genotypes varied positively among control populations with their flowering density during the preceding year, when *O. biennis* was particularly abundant (Fig. 6). Because self-pollination of *O. biennis* typically happens before flowers open and flowers are only open for one night, opportunities for pollination, and therefore outcrossing, are limited. As in self-incompatible plants, outcrossing is likely most frequent in high-density populations (Kunin 1993, Dauber et al. 2010). Thus, the fecundity advantages of outcrossing in insect-suppression populations were apparently counterbalanced by their more intense interspecific competition and lower density of intraspecific mates compared to herbivore-present populations.

Outcrossing frequency compared to natural populations

An important question is whether the observed outcrossing rates reflect those observed in natural populations. Typically, *O. biennis* plants produce seeds that are clonal replicates of their parents; however, 7.3% of the naturally recruited plants in our populations originated from outcrossing in 2007, with no difference between herbivore-present and herbivore-suppressed populations (Agrawal et al. 2012). This estimate of outcrossing frequency is remarkably similar to that of Hoff (1962), who estimated an outcrossing rate of 0.7–7.8% (depending on the time of year) based on heritable phenotypic markers. Substantial outcrossing is expected only for larger populations and those with multiple genotypes, as in our experiment. Certain habitats, such as sandy lake margins, consistently support large *O. biennis* populations (M. T. J. Johnson and A. A. Agrawal, *personal observations*). Populations in more stable habitats are often small and ephemeral, although

infrequent disturbance can trigger germination from large seed banks (Steiner and Levin 1977).

Conclusion and implications

Limited sexual reproduction can be a critically important source for generating increased genetic variation in asexual populations of plants and animals (Charlesworth and Charlesworth 1995, D'Souza and Michiels 2010, Navarro et al. 2013). Given the range of reproductive strategies in plants that span from asexuality to complete outcrossing, the generation and propagation of novelty occurs through many means. Our experimental results illustrate that even limited outcrossing, in the absence of recombination and segregation, can generate novel genetic variation that can then be propagated through clonal reproduction and influence population genetic structure. These results support long-standing theory that even limited sex can provide important advantages to plants (D'Souza and Michiels 2010).

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Supporting Information

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Data Availability

Data associated with this study are available from the Dryad Digital Repository: https://doi.org/10.5061/dryad.bn37p