Insect Herbivores Drive Real-Time Ecological and Evolutionary Change in Plant Populations

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Insect herbivores are hypothesized to be major factors affecting the ecology and evolution of plants. We tested this prediction by suppressing insects in replicated field populations of a native plant, Oenothera biennis, which reduced seed predation, altered interspecific competitive dynamics, and resulted in rapid evolutionary divergence. Comparative genotyping and phenotyping of nearly 12,000 O. biennis individuals revealed that in plots protected from insects, resistance to herbivores declined through time owing to changes in flowering time and lower defensive elaiotropins in fruits, whereas plant competitive ability increased. This independent real-time evolution of plant resistance and competitive ability in the field resulted from the relaxation of direct selective effects of insects on plant defense and through indirect effects due to reduced herbivory on plant competitors.

The ubiquitous consumption of plants by insect herbivores represents one of the dominant species interactions on Earth and has been hypothesized to play a strong role in the diversification of plant species and their traits (1–3). The evolution of plant defense has been studied primarily with either a prospective or retrospective approach. Single-generation prospective approaches measure contemporary natural selection on resistance traits and make predictions about how those traits should evolve given estimates of their heritability (4–6). By contrast, retrospective studies compare populations or species that have diverged over time (7–9) and make inferences about the processes driving evolutionary change. More generally, temporal studies of natural species interactions that influence evolutionary dynamics remain rare, and few have been experimental (9–12). Thus, we lack experimental field studies that quantify how species interactions influence selection on traits and the evolutionary response to this selection. As such, it is not well established how rapidly plants adapt to selection by herbivores, which traits are most important in the evolution of defense, and whether herbivory drives predictable parallel changes due to selection across populations.

The ecology and evolution of plant-herbivore relationships is, in part, governed by the reciprocal nature of their interaction and the complexity of communities. For example, plant traits and plant community structure are critical for determining insect occurrence and attack rates (1–3). Conversely, insects can directly reduce focal plant abundance (13) or indirectly affect plant abundance through changes in the density of co-occurring competitors (14). Thus, the evolutionary dynamics of a focal plant species might be influenced through direct selection on resistance traits and/or by indirect selection on traits influencing competitive ability (15, 16). Despite this likely scenario, our understanding of how species interactions influence the evolution of constituent community members is surprisingly limited (17).

We conducted a field study of the selective impact of insects and the evolutionary response

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Fig. 1. Fruit loss to seed predator moths (dominated by M. brevivittella) in 18 genotypes of O. biennis in ambient and insect-suppressed plots (data are from the first generation, 2007–2008, mixed-model analysis of variance, genotype-by-insecticide interaction: χ² = 57.55, P < 0.001). (Left) an opened fruit with a M. brevivittella larvae consuming immature seeds; (right) a flowering O. biennis.
to this selection in the native forb common evening primrose, Oenothera biennis (Onagraceae) (18). We established 16 replicate plots planted with 60 individual seedlings of O. biennis, containing equivalent frequencies of 18 uniquely identified genotypes (19). Half of the plots were treated biweekly during each growing season with esfenvalerate, a nonsystemic insecticide (insect suppression treatment, supplementary text 2a); the remaining plots received an equivalent amount of water as a control (ambient insect treatment). Because O. biennis is annual or biennial, primarily selfing, and has a genetic system that suppresses recombination and segregation of alleles, we could track changes in frequencies of each planted genotype within each replicate population over multiple generations (19). Plots were not further weeded or otherwise manipulated, allowing the natural assembly and early succession of plant species with and without insects (supplementary text 2b). We previously summarized our sampling methods, the effects of flowering phenology on insect attack, and plant life-history evolution in the ambient insect plots (19). Here, we address the effects of experimental insect suppression on ecological and evolutionary change in O. biennis, including changes in genotypic composition, defensive chemistry, competitive ability, and flowering time.

Although there were three native species of specialist seed predator moths at our site, fruit loss in O. biennis was dominated by Mompha brevivittella, which was >5 times as abundant as M. stellella and Schinia florida combined, the other two common specialists. The 18 O. biennis genotypes varied substantially in their resistance to these seed predators (Fig. 1), and insect suppression created an environment with potentially large differences in natural selection. Despite increases in O. biennis abundance in the first 2 years of the experiment (reaching up to 4000 individuals per plot in 2009), recruitment was substantially lower in the insect suppression plots compared to ambient controls (Fig. 2). This reduction in O. biennis density coincided with a rise in the abundance of common dandelion, Taraxacum officinale (Asteraceae), an early successional weed that dominated our plots and was twice as abundant in plots where insects were suppressed compared to controls (Fig. 2 and fig. S1).

A specialist seed-feeding beetle of T. officinale, Glossina punctiger, was significantly reduced by insect suppression, and a highly abundant generalist moth caterpillar at the site, Noctua pronuba, showed a preference for T. officinale over O. biennis (supplementary text 2c). Thus, we hypothesized that the release of T. officinale from its herbivores led to competitive suppression of O. biennis. Indeed, populations of O. biennis and T. officinale showed a negative correlation at the population level (simple correlation, N = 16, r = −0.523, P = 0.038), and an independent field experiment at the same site confirmed that T. officinale inhibits early establishment of O. biennis (supplementary text 2d). Because O. biennis requires light for germination, we hypothesize that T. officinale likely affects germination as well as early seedling survival. Therefore, insect suppression substantially affected plant competitors in addition to our focal species.

The net evolutionary consequence of insect suppression was a change in the genetic structure of O. biennis populations (Fig. 3, repeated measures canonical correspondence analysis, permutation test, trace = 0.275, F = 8.511, P = 0.020), with the strongest effect observed in the final year of the experiment (fig. S2). This parallel genetic differentiation among our replicated insect suppression plots in comparison to control plots (Fig. 3) implies that selection by insects, and not genetic drift, drove the observed

**Fig. 2.** Effects of insect suppression on the densities of focal O. biennis (univariate analysis of variance, F = 8.97, P = 0.010) and a major colonizing competitor (T. officinale, univariate analysis of variance, F = 12.96, P = 0.003) in 2009 (means ± SEM); also shown are representative ambient and insect-suppressed plots when T. officinale was in peak bloom.
Genotypic evolution. At the end of the experiment, 1 of the original 18 genotypes was entirely extirpated, and two additional genotypes were represented only in insect suppression plots (Fig. 3). Only six genotypes consistently maintained a frequency of 2% or higher within the populations. Three novel genotypes, derived from outcrossing events between known parents, each also reached >2% of the populations in 2011 (supplementary text 2e). Thus, across both treatments, nine genotypes dominated all plots (>2% each) at the end of the experiment. Genotypic evenness was >50% higher in insect suppression plots compared to controls (Smith and Wilson’s evenness index, $E_{\text{var}}$, univariate analysis of variance, $F_{1,14} = 15.037, P = 0.002$), suggesting an erosion of genotypic variance in the presence of insect herbivores. Nonetheless, insect suppression affected neither genotypic richness (univariate analysis of variance, $F_{1,14} = 0.287, P = 0.600$) nor diversity (Simpson’s index, univariate analysis of variance, $F_{1,14} = 0.377, P = 0.549$).

On the basis of annual estimates of genotypic frequencies in each plot and genotype-specific means of $M. \text{breivittella}$ attack measured in control plots during 2007, we found that experimental suppression of insects resulted in evolution of relaxed plant defenses (Fig. 4). We next determined the evolutionary response of traits responsible for this divergence in resistance. We had previously shown that the extent of early-season flowering (number of open or senesced flowers after 50% of the plants had at least a single open flower) positively correlated with $M. \text{breivittella}$ attack in 2 years (19) (i.e., later-flowering plants suffered less damage). There was no difference between treatments in the first year of our study (2007), as early season flowering was not different among the two treatments (univariate analysis of variance, $F_{1,14} = 0.320, P = 0.581$); by 2010, there was a significant shift toward earlier flowering in plots with suppressed insects (univariate analysis of variance, $F_{1,14} = 6.105, P = 0.027$). This effect was even stronger in 2011 (Fig. 4, univariate analysis of variance, $F_{1,14} = 7.025, P = 0.019$) and was predictable on the basis of genotype frequencies and genotype-specific means for early flowering (Fig. 4). Thus, a predicted response to selection was evident in only three to four generations. We found no divergence in annual versus biennial phenotypes of $O. \text{biennis}$ (repeated measures multivariate analysis of variance, $F_{1,14} = 0.935, P = 0.350$).

$O. \text{biennis}$ produces diverse hydroxylated tannins, including the largest ellagitannins known from any plant species (20), and these compounds have very high oxidative capacity, negatively affecting insect herbivores (21). One trimer, oenothein A (fig. S3), was the dominant ellagittannin in $O. \text{biennis}$ fruits (up to 7.8% dry mass). We previously showed that oenothein A is favored by natural selection in $O. \text{biennis}$ leaves (6), and its production in fruits was negatively genetically correlated with $M. \text{breivittella}$ attack in the current study (simple correlation, $N = 17, r = -0.491, P = 0.045$) (supplementary text 2f), implicating a role of this compound in defense. Despite a genetic correlation between foliar and fruit ellagittannin chemistry (simple correlation, $N = 18, r = 0.848, P < 0.001$), only fruit chemistry significantly diverged in our experimental plots (Fig. 4), suggesting that selection was strongest for defense against flower- and fruit-feeding herbivores like $M. \text{breivittella}$. A negative genetic correlation between early-season flowering and the production of oenothein A in fruits (simple correlation, $N = 23, r = -0.67, P < 0.001$) is consistent with the rapid and joint evolution of these traits (i.e., early flowering with low oenothein A in insect-suppressed plots) (Fig. 4).

Given that insect suppression not only affected herbivory on $O. \text{biennis}$, but also resulted in stronger plant competition through larger $T. \text{officinale}$ populations, we hypothesized that $O. \text{biennis}$ would evolve greater competitive ability in the absence of insects. We measured the relative competition index (RCI) in total above- and belowground biomass of different genotypes in an independent experiment (fig. S4). We then used these genotype-specific values of competitive ability to determine if the competitive phenotypes diverged between control and insect-suppressed plots. Insect suppression resulted in the evolution of enhanced competitive ability relative to ambient insect plots (Fig. 4), and because this effect was not genetically correlated with early-season flowering, resistance to insects increased without a corresponding decrease in competitive ability.

Fig. 4. Phenotypic evolution in $O. \text{biennis}$ plots. (A) In the presence of seed predator insects, higher plant resistance evolved [estimated by multiplying genotype-specific means measured in control (ambient insect) plots by genotype frequencies in each plot] (repeated measures multivariate analysis of variance, $F_{1,14} = 6.322, P = 0.025$), and this effect did not vary over time (time-by-treatment interaction, repeated measures multivariate analysis of variance, $F_{1,14} = 1.459, P = 0.275$). (B) Earlier flowering was observed in the insect suppression plots (univariate analysis of variance, $F_{1,14} = 7.025, P = 0.019$). We confirmed that this was an evolutionary response by multiplying genotype-specific means by genotype frequencies (univariate analysis of variance, $F_{1,14} = 4.530, P = 0.050$); the former field assessment includes the possible effects of phenotypic plasticity, whereas the latter is based on genotypic values. (C) Leaf defensive chemistry did not diverge (univariate analysis of variance, $F_{1,14} = 1.716, P = 0.221$) but fruit chemistry changed (univariate analysis of variance, $F_{1,14} = 8.917, P = 0.010$) as predicted by genotypic means and genotype frequencies. (D) Competitive ability of $O. \text{biennis}$ when grown with $T. \text{officinale}$; plants in the insect-suppressed plots evolved a greater ability to maintain above- and belowground biomasses, and competitive ability increased with genotype frequency. (E) Competitive ability of $O. \text{biennis}$ when grown with $T. \text{officinale}$; plants in the insect-suppressed plots evolved a greater ability to maintain above- and belowground biomasses, and competitive ability increased with genotype frequency.
Natural Enemies Drive Geographic Variation in Plant Defenses

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Plants defend themselves against attack by natural enemies, and these defenses vary widely across populations. However, whether communities of natural enemies are a sufficiently potent force to maintain polymorphisms in defensive traits is largely unknown. Here, we exploit the genetic resources of Arabidopsis thaliana, coupled with 39 years of field data on aphid abundance, to (i) demonstrate that geographic patterns in a polymorphic defense locus (GS-ELONG) are strongly correlated with changes in the relative abundance of two specialist aphids; and (ii) demonstrate differential selection by the two aphids on GS-ELONG, using a multigeneration selection experiment. We thereby show a causal link between variation in abundance of the two specialist aphids and the geographic pattern at GS-ELONG, which highlights the potency of natural enemies as selective forces.

Intraspecific genetic variation is essential in enabling species to respond rapidly to evolutionary challenges such as changing environmental conditions (1) or the emergence of novel pests and pathogens (2). This diversity often reflects the balance between the strength of local selection and the current and historical levels of population substructure and gene flow (3, 4). Geographic analyses of genetic variation in several plant species have revealed clear genetic signals of local adaptation (5), caused by differences in the selective regime among locations. These analyses are further supported by reciprocal transplant experiments, in which home genotypes generally outperform those transplanted from other populations (6, 7). Although the drivers of local adaptation often remain unidentified, there

References and Notes
18. Materials and methods are available as supplementary materials on Science Online.

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Supplementary Materials
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ing financial responsibility for their national malaria control programs. The international financial crisis and redirection of political and financial attention to other pressing global health problems such as malnutrition and family planning are potential threats to the investment needed to sustain and expand malaria control and research. Past experience shows the disastrous consequences of letting up on effective malaria control. It’s time to reap the benefits of the mosquito and parasite genomes to aggressively tackle this disease. Otherwise, this highly adaptable parasite and its vector will continue to outwit us and continue to kill millions.

References

How Insect Herbivores Drive the Evolution of Plants
J. Daniel Hare

The most common biological interaction among species on Earth is that between plants and the insects that feed on them (1). Insect herbivores are thought to impose natural selection, which favors resistant plant genotypes and drives the evolutionary diversification of plant species. Two reports in this issue—by Züst et al. on page 116 (2) and Agrawal et al. on page 113 (3)—independently provide strong empirical evidence for the rapid evolution of plant traits that confer resistance to herbivores when herbivores are present but for the evolution of traits that confer increased competitive ability when herbivores are absent.

If resistance to insects benefits plants, then why are not all plants now resistant? Several answers to this long-standing question have been proposed. One is based on the assumptions that plant defenses are costly, that resistant genotypes are favored when the probability of insect damage is high, but that these genotypes pay a cost for resistance and are disfavored when the probability of herbivore attack is low. In its simplest form, this hypothesis states that chemical resources obtained by plants can be allocated maximally either to growth and reproduction or to defense, but allocation to both processes cannot be maximized simultaneously (4). A second hypothesis is that defense polymorphisms are the result of variation in selection regimes due to variation in the size and membership of herbivore communities at different locations (5). The two studies in this issue find strong evidence for variation in plant defense traits in response to particular herbivore species, but only partial support for the allocation hypothesis.

Züst et al. studied natural populations of Arabidopsis thaliana in Europe. They compared the geographic variation in the profiles of glucosinolates [a class of defensive chemical compounds in the plant family Brassicaceae (6)] with the distribution of two aphid species that feed only on brassicaceous plants. The frequency of the GS-ELONG gene, which determines the length of the carbon side chain on glucosinolate molecules, varied both with latitude and longitude. The aphid Brevicoryne brassicae predominated in areas where the plants produced glucosinolates with four-carbon side chains, whereas the aphid Lipaphis erysimi predominated in areas where plants produced glucosinolates with three-carbon side chains.

The authors next used a synthetic plant population consisting of genotypes that produce several different combinations of glucosinolates. After only five generations of selection, feeding by each aphid species selected for plant genotypes with glucosinolate profiles identical to those in the field locations where each aphid species predominated. By contrast, the plant genotype that predominated after five generations in a “no aphid” treatment produced relatively low levels of glucosinolates. This genotype was, however, eliminated from populations exposed to all aphid treatments (see the figure).

In a related but independent study, Agrawal et al. conducted a four-generation
DEVELOPMENTAL BIOLOGY

Intestinal Wound Healing Requires a Wnt Balancing Act

Terrence A. Barrett

Inflammatory bowel disease (IBD) encompasses a group of disorders of the colon and small intestine including Crohn’s disease and ulcerative colitis. It affects roughly 396 per 100,000 persons worldwide (1) and in the United States is responsible for more than $1.7 billion in overall health care costs. The chronic or recurrent inflammation associated with this disease causes severe damage to the epithelial lining of the intestine. On page 108 of this issue, Miyoshi et al. (2) present a model for wound healing in the intestinal tract that may have clinical relevance to mucosal repair in disorders of intestinal ulceration.

Within the epithelial lining of the small intestine and colon is a gland composed of subunits called intestinal crypts or crypts of Lieberkühn. After mucosal wounding, channels of epithelial cells move under exposed surfaces to begin to recreate normal crypt architecture. Epithelial proliferation at wound edges is driven by the Wnt signaling pathway, particularly the canonical Wnts, which signal through β-catenin.

Miyoshi et al. have found that creation of new crypts requires release of noncanonical (β-catenin–independent) Wnt5a. Mesenchymal Wnt5a-secreting cells are derived from serosal mesothelial (WT1+) stem cells and appear to migrate into areas to participate in tissue repair. Wnt5a lowers epithelial proliferation rates and induces epithelial channel invaginations or clefting under the wounded surface. Miyoshi et al. suggest that Wnt5a potentiates transforming growth factor–β (TGF-β) signaling (via Serpin1 and Smad3) to reduce epithelial proliferation and cause clefting of epithelial channels. Clefting alters the polarization of highly proliferative crypt structures at wound margins, allowing them to branch into new crypt units. The authors did not detect Wnt5a-mediated inhibition of canonical Wnt signaling as reported by others (3). This inhibition, when observed, is context dependent (3), suggesting that noncanonical Wnt5a may affect canonical Wnt signaling in other mucosal healing processes in the intestine.

Wnt5a is needed for wound healing. Image of transmural ulcer from a Crohn’s disease resection specimen showing an area of hyperproliferative branching crypts producing an epithelial monolayer (EM). An area of epithelial channel clefting is highlighted (dark arrow).
Supplementary Materials for

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1) Materials and Methods

a. Experimental design

In May 2007, we established 16 13.5 m$^2$ plots in an old field in Tompkins County, Ithaca, NY. Plots were spaced at least 10 m apart. The field encompassing the plots had homogeneous vegetation and shared an identical land use history (abandoned agriculture). Plots were plowed and sprayed twice with herbicide prior to commencing the experiment, and were protected from deer herbivory by mesh fencing. In the center of each plot, we established a 1x1 m focal area that was planted with experimental $O. biennis$ populations composed of 60 equally spaced individual $O. biennis$ seedlings at the 1$^{st}$ true leaf stage. There was no further weeding or manipulation of the plots, and seedling recruitment in subsequent years occurred throughout the 13.5 m$^2$ plot area. The nearest natural populations of $O. biennis$ were over 1 km from our plots and therefore the potential for outside contamination was low.

We initially planted three individuals from each of 16 genotypes and 6 individuals from each of two additional genotypes ($3 \times 16 + 6 \times 2 = 60$ total individuals per plot) into each plot, with the location of each genotype randomly assigned. The latter two genotypes were originally thought to be four distinct genotypes, but extensive additional analyses indicate that these were in fact indistinguishable (see details in ref. 19). The 18 genotypes were selected to span the range of phenotypic characteristics from a total of 40 genotypes, which were all grown in a common garden in 2006 (23). The 40 original collections were all from early successional habitats (one per site, separated by at least 0.5 km; average and maximum distance between sites was 12 km and 30 km, respectively) within Tompkins County, New York, USA. $O. biennis$ grows in recently disturbed successional habitats, where populations are typically small (<100 individuals) and discretely dotted across the landscape; our experimental populations attempted to mimic such natural populations. Although our field experiment was also located in Tompkins County, the closest collection site was $\approx 2$ km away. To reduce the impact of maternal environment effects, we grew all genotypes for a generation in a common garden at the same site and used their offspring in the current experiment.

Half of the 16 plots were randomly assigned to the insect suppression treatment. We applied 0.425 % esfenvalerate (Bug-B-Gon, Ortho, EPA Reg. No. 1021-1645-239; 2007-2009, or Asana XL, Dupont, EPA Reg. No. 352-515; 2010-2011) at a rate of 1 U.S. fluid oz. per gallon of water to the vegetation in each of the insect suppression plots every two weeks (April through October) during each of the five growing seasons. Control plots were sprayed with an equal amount of water.

In each year we assessed seed production and seedling recruitment. We counted fruits for all original 60 plants in each focal area (2007 and 2008). However, due to the large episode of recruitment beginning in 2008, we sub-sampled populations in each year between 2008 and 2011 by counting fruits on at least 50 individuals from each plot (and identifying their genotypes as well, see below). We estimated seed production based on genotypically variable mean fruit size (analysis of variance, $F_{17,44}=3.61, P<0.001$) and the regression slope of fruit size (volume) vs. seed number ($Seed\ number=2.33(fruit\ volume) -102.47, r^2=0.60, F_{1,26}=38.47, P<0.001$) (further details are provided in ref. 19).

Our experimental populations were reflective of natural populations. $O. biennis$ typically has discrete patchy populations, representing meta-populations ranging in size
from a few individuals to thousands of plants. The genetic diversity of *O. biennis* populations has been estimated at up to 14 genotypes (26), although this estimate is likely conservative given the nature of the genetic tools employed in 1977.

b. Population trends for *Oenothera biennis* and *Taraxacum officinale*

*T. officinale* was the dominant competitor of *O. biennis* in the spring and early summer throughout the five year experiment (see images in Fig. 2). We estimated the size of each of the 16 *O. biennis* populations in July of each year (2008-2011) by counting the number of rosettes and flowering plants within each of nine quadrats (625 cm²), and then multiplying the mean abundance per m² by plot area. Seedlings with fewer than four true leaves were not included in our demography estimates. *T. officinale* was censused annually in May by counting all reproductive stems in all plots.

c. Competition between *Oenothera biennis* and *Taraxacum officinale* and impacts of herbivores on *T. officinale*

During October and November 2009, we established 28 small plots (930 cm²) at the same field site as our larger experimental plots in order to estimate competitive impacts of *T. officinale* on germination and establishment of *O. biennis*. All plots had naturally occurring *T. officinale* at a minimum density of three large rosettes per plot, and plots were weeded of all other existing vegetation. In half the plots, we carefully weeded *T. officinale* (dandelion exclusion plots), cutting stems at soil level to avoid soil disturbance. In late January 2010, we added 160 *O. biennis* seeds to each plot. Seeds were added to 2 mL of dried sand, mixed, and sprinkled with a salt shaker to allow for even application of seeds within each plot. Beginning in early April 2010, we maintained the dandelion exclusion plots by weeding, as above, once every 1-2 weeks and conducted regular seedling counts through mid-June.

A specialist seed predator weevil *Glocianus punctiger* was abundant in the developing seed heads of *T. officinale* in spring (May-June). In 2012, we surveyed 10 randomly selected nearly mature seed heads of *T. officinale* from each of the 16 experimental plots and opened each to assess the number of *G. punctiger* larvae.

A choice test was conducted with nearly mature larvae of the abundant generalist *Noctua pronuba* in May 2010. Larvae were collected from the old field habitat surrounding field plots. Pairs of leaf discs (one each of *O. biennis* and *T. officinale*, 2.5 cm²) were offered to individual larvae on a bed of soil in small ventilated containers (600 mL) and damage was assessed after 12 hours (N=24 pairs).

d. Genotyping

Approximately 190 plants were genotyped per plot per year by randomly sampling rosettes and flowering plants according to their relative proportion of the total population of recruits within the plot in that year. Plot level genotype frequencies were used in the canonical correspondence analysis. Genotyping methods are given in ref. 19. Briefly, genomic DNA was obtained from freeze-dried leaf tissue using a standard cetyltrimethylammonium bromide (CTAB) extraction protocol (total N=11,731 samples). For each sample, four microsatellite loci (Oenbi2tri2, Oenbi2tri3, Oenbi2tri6 (27), Oenbi102 (19)) were amplified in a single multiplex PCR reaction (Type-It Microsatellite PCR kit; Qiagen, Valencia, CA). Samples were analyzed on a 3730xl DNA Analyzer (Applied
Biosystems, Foster City, CA) at the Cornell University Life Sciences Core Laboratories Center. Allele sizes were determined using Genemapper v3.5 (Applied Biosystems), with all calls checked by eye. Plants were assigned to a particular genotype only if their alleles at all four loci were representative of that genotype.

To evaluate the drivers of trait evolution we conducted several genetic correlations (based on genotype means) between phenotypic traits measured (flowering phenology, leaf and fruit chemistry, competitive ability, resistance to attack, relative competition index). These analyses were conducted with simple Pearson product moment pairwise correlations, with sample sizes that ranged from 16-23 genotypes. Sample size was variable based on available data; late in the experiment, no phenotypic data was available for rare or extinct genotypes. In some cases, we evaluated phenotypes not only on the 18 original genotypes, but the additional 8 outcrossed genotypes that became abundant in the plots. In these analyses, as in others involving analysis of variance, the residuals were checked for normality and homoscedasticity.

e. Phenolic fruit chemistry of Oenothera biennis

For 900 samples, taken from the leaves and fruits of the 16 field plots in 2010, we collected samples on dry ice, lyophilized tissues, and ground them to a fine powder. Samples were analyzed by ultra-performance liquid chromatography/diode-array detection coupled to a triple quadrupole mass spectrometer with an electrospray ionization interface (Waters Acquity Xevo TQ) (21, 23). Individual polyphenols were separated into hydrolyzable tannins (HTs) and flavonoids.

f. Genotypic variation in competitive ability in Oenothera biennis

In 2011, we conducted a competition experiment between 26 genotypes of Oenothera biennis (18 original genotypes used in the experimental evolution plots plus the eight most common outcrossed genotypes) and T. officinale. All plants were germinated from seed in plug trays containing potting soil, then transplanted at the cotyledon stage into field soil (500 mL plastic pots) either alone or with a single T. officinale competitor (competing plants were planted 0.8 cm apart). Plants were grown in the absence of herbivores on an open air roof-top patio in full sun (watered and weeded as needed). After 60 days of growth plants were harvested, roots were washed and separated from shoots by species (26 genotypes x 2 treatments x 5 replicates = total n=260), dried at 40-50 °C until reaching a constant weight and then weighed. For each genotype, we calculated competitive ability (RCI) (28) as [(Bo - Bi)/ Bo] X 100, where Bo was the total biomass of the plant at the end of the experiment growing alone and Bi was the total biomass of the same genotype growing with T. officinale.

g. Evidence for divergent natural selection among treatments

For each year (2007 and 2008 are combined for the first generation of plants, some of which did not reproduce until the second year), we calculated total genotypic selection separately for early flowering, oenothein A, and competitive ability using analysis of covariance. Genotype means of traits were regressed against mean genotypic fitness (lifetime seed production) (N=18 genotypes for each analysis). In particular, an interaction between traits and our experimental manipulation of insects would indicate divergent selection caused by insect suppression.
2. Supporting online text

a. Effects of insecticide on *Oenothera biennis* and *Taraxacum officinale*

To test for any direct effects of the insecticide (esfenvalerate) on plant germination or growth, we conducted four trials, outlined below, using the same methods for insecticide application as in the main experiment.

Trial #1: Effects on *O. biennis* shoot and root growth. Forty-two plants were grown in pots on an open air roof-top patio free of insect herbivores. A randomly chosen set of 21 plants were sprayed with insecticide (esfenvalerate, trade name Bug-B-Gon) every two weeks for a total of three applications. All plants were harvested one week after the final application. There was no effect of the insecticide on shoot (mean ± SEM grams dry mass, control 1.20 ± 0.03, insecticide 1.15 ± 0.03, univariate analysis of variance, $F_{1,40}=1.18$, $P=0.28$) or root biomass (mean ± SEM grams dry mass, control 0.73 ± 0.04, insecticide 0.71 ± 0.04, univariate analysis of variance, $F_{1,40}=0.26$, $P=0.61$).

Trial #2: Effects on *O. biennis* shoot and root growth controlling for plant genotype. 60 plants from the 18 genotypes from our experiment were randomly assigned to be sprayed with insecticide (esfenvalerate, trade name Asana XL) or water controls every two weeks exactly as in the experimental evolution plots. Plants were 2-3 months old, grown in 500 mL pots with field soil, and were maintained on an open air roof-top patio. After three applications, above and below-ground tissue were harvested and there were no effects of insecticide on shoot (mean ± SEM grams dry mass, control 1.13 ± 0.04, insecticide 1.15 ± 0.04, mixed model analysis of variance $F_{1,40}=1.18$, $P=0.67$) or root biomass (mean ± SEM grams dry mass, control 0.88 ± 0.06, insecticide 0.92 ± 0.06, mixed model analysis of variance, $F_{1,40}=0.33$, $P=0.57$). Plant genotypes varied substantially in shoot (mixed model analysis of variance, $P=0.001$) but not root (mixed model analysis of variance, $P=0.13$) biomass. There was no genotype-by-treatment interaction.

Trial #3: Effects on *O. biennis* germination. During the spring of 2012, six standard 1020 greenhouse trays were filled with field soil and moistened thoroughly. Three hundred primrose seeds (from a homogenous mixture of five genotypes) were sprinkled on top of the soil of each flat. Flats were watered as needed and germination was permitted to occur for 17 days prior to the first insecticide application (very few seedlings emerged during this period). Esfenvalerate (Asana XL) was applied at one-quarter the rate per area applied to our evolution plots, as a conservative estimate for the amount of chemical that actually reaches the soil surface during each application. Insecticide was applied every two weeks (3 applications in total) and did not affect percent germination (William’s corrected G-test = 1.85, $P=0.174$).

Trial #4: Effects on *T. officinale* germination. During the spring of 2012, flats were prepared for dandelion germination, as above (Trial #3) with the exception that 100 seeds were sown in each flat. Again, germination was not affected by treatment (William’s corrected G-test < 0.001, $P=1$).

b. Impacts of insect suppression on other plants

Beginning in 2008, >20 other plant species also recruited into the plots, but most were relatively rare. In 2011 we surveyed the vegetation in each of the plots (species identity and abundance) of those plants that naturally recruited into the plots. We
assessed the impacts of insect suppression treatment on species richness (number of plant species per plot), evenness (Smith and Wilson’s $E_{\text{var}}$) (29), and diversity (Simpson’s index). Species richness was not impacted by our treatments (mean ± SEM plants per plot in 2011, insect suppression 13.3 ± 1.2, ambient insect controls 13.3 ± 1.2, univariate analysis of variance, $F_{1,14}<0.001$, $P=1.0$). In addition to $O. \text{biennis}$ and $T. \text{officinale}$, one other species, $Solanum \text{carolinense}$, was significantly impacted by our insect suppression treatment in 2011. $Solanum \text{carolinense}$ populations were larger in insect suppression plots (mean ± SEM plants per plot, insect suppression 22.8 ± 2.3, ambient insect controls 5.8 ± 2.3, univariate analysis of variance, $F_{1,14}=26.401$, $P<0.001$).

c. Herbivores of $T. \text{officinale}$
Weevils ($Glocianus \text{punctiger}$) were half as abundant in the insect suppression plots (mean ± SEM number of larvae per 10 seed heads, control plots 9.50 ± 1.30, insect suppression plots 4.63 ± 1.30, $F_{1,14}=7.01$, $P=0.019$).

In 18 out of the 24 choice tests (see Methods above under 1.c), larvae of an abundant generalist moth, $Noctua \text{pronuba}$, consumed more of $T. \text{officinale}$ than $O. \text{biennis}$ tissue (Wilcoxon signed rank test, $Z=-2.779$, $P=0.005$).

d. Impacts of removing $T. \text{officinale}$ on $O. \text{biennis}$ By mid-June, establishment of $O. \text{biennis}$ was >3.5 times higher where $T. \text{officinale}$ was removed compared to control plots (mean ± SEM number of established $O. \text{biennis}$, control 2.64 ± 0.97, removal plots 9.64 ± 0.97, univariate analysis of variance, $F_{1,26}=5.09$, $P<0.001$). See 1.d above for methods.

e. Outcrossing in experimental plots of $\text{Oenothera biennis}$
Despite the fact that $O. \text{biennis}$ typically produces seeds that are clonal replicates of the parents (via selfing and a near complete suppression of recombination and free segregation caused by a permanent translocation heterozygote genetic system) (30, 31), a low level of outcrossing can occur. In 2008, 7.3% of the naturally recruited plants in our plots were outcrossed genotypes, and our insecticide treatment did not affect the proportion of outcrossed recruits (analysis of variance, $F_{1,14}=0.593$, $P=0.454$). By 2011, 36% of the plants across plots were outcrossed genotypes, either produced through the repeated outcrossing events or the clonal propagation of outcrossed genotypes in 2008. Four parental genotypes of abundant outcrossed genotypes were rare by 2011 (>1% of the final population), suggesting that the success of outcrossed genotypes was not simply dependent on the abundance of parentals.

In a canonical correspondence analysis (CCA) where we only analyzed genotype frequencies of the original genotypes, ambient insect and insect suppression plots still show a high level of genotypic divergence (CCA on 2011 genotype frequencies, Trace = 0.077, $F = 2.590$, $P=0.007$) and differentiation in insect resistance ($F_{1,14}=22.117$, $P<0.001$). In other words, the outcrossed genotypes were not critical for the evolutionary change observed.

f. Phenolic fruit chemistry of $\text{Oenothera biennis}$
Because the main herbivores of $O. \text{biennis}$ are seed predators and there is a genetic correlation between leaf and fruit chemistry (simple correlation, $N=18$, $r=0.848$, $p<0.001$), here we focus on fruit chemistry. Among the total phenolics in the fruits, 97%
were hydrolysable tannins (HTs), and 75% of HTs were ellagitannins. The ellagitannins were dominated by a trimer (oenothin A, which comprised 56% of all ellagitannins) and a dimer (oenothin B, 33%).

*O. biennis* produces ellagitannins that are dimers, trimers, tetramers, pentamers, hexamers, and heptamers. In our dataset, there were 15 possible pairwise genetic correlations among these classes, and all but one were highly statistically significant. Remarkably, dimers were negatively correlated with each of the larger classes (4 of the 5 genetic correlations had \( r \)-values between -0.71 and -0.38, all \( P \)-values < 0.02), while all larger classes (trimers to heptamers) were significantly positively genetically correlated (\( r \)-values ranged from 0.62 to 0.95, all \( P \)-values < 0.001). We conclude that in environments with ambient insects, natural selection has favored larger ellagitannin oligomers, while the relaxation of selection by herbivores favors the basic dimer structure.

There was no difference among our treatment plots in total phenolics (univariate analysis of variance, \( F_{1,14}=0.023, P=0.882 \)), total hydrolyzable tannins (univariate analysis of variance, \( F_{1,14}<0.001, P=0.977 \)), or total ellagitannins (univariate analysis of variance, \( F_{1,14}=0.383, P=0.546 \)). Rather, the suppression of insects appears to have driven evolution of specific fruit ellagitannin oligomers. For example, in the main text we report the evolution of lower oenothin A in fruits in response to insect suppression. This effect appears to come at the expense of oenothin B (which is increased in insect suppressed plots, univariate analysis of variance, \( F_{1,14}=16.384, P=0.001 \)). Hence, the ratio of dimers to trimers substantially increased (85%) in our insect suppression plots (univariate analysis of variance, \( F_{1,14}=6.636, P=0.022 \)). In this and several previous experiments, we see a negative genetic correlation between plant allocation to dimers and trimers (simple correlation, \( N=17, r=-0.693, P<0.001 \)), with the trimers being more strongly genetically correlated with insect resistance (simple correlation of trimers and *Mompha* attack: \( N=17, r=-0.491, P=0.045 \), simple correlation of dimers and *Mompha* attack: \( N=17, r=0.275, P=0.286 \)). Furthermore, total concentrations of hydrolysable tannins in fruits were not genetically correlated with *Mompha* attack (simple correlation, \( N=17, r=-0.259, P=0.316 \)).

It is expected that specific phenolic compounds will show strong negative effects on insect performance even when total phenolic concentrations show little effect. Most previous studies of plant phenolics measured total phenolic concentrations, or the protein binding capacity of phenolics. Yet, it is now recognized that these measures of phenolic chemistry often show little relationship with resistance to insects (32), even though specific phenolic metabolites can have large negative effects on resistance to insects. In particular, ellagitannins produced by *Oenothera* have a demonstrated negative impact on insect herbivores (21).
3. Supporting figures

Figure S1. Population trends for *Oenothera biennis* (A) and *Taraxacum officinale* (B) in experimental plots. Shown are means ± SEM. N=8 plots per treatment.
Figure S2. Correspondence analysis and visualization of genetic differentiation among experimental field plots analyzed each year. Numbers indicate plot identifications, and polygons were drawn to represent treatments (purple=ambient insects, black=insect suppression) after running an unconstrained model. Statistical analysis was conducted by assigning treatments using canonical correspondence analysis and a permutation test reported for each year in the panels. Repeated measures canonical correspondence analysis, trace=0.275, $F=8.511$, $P=0.020$. 
Figure S3. The structure of ellagitannin trimer oenothein A.
Figure S4. Heritable variation in response to plant competition among the 18 genotypes of *Oenothera biennis* from the field experiment. Shown is the combined above and belowground biomass of *O. biennis* when grown alone or with a major competitor (*Taraxacum officinale*). Genotypes showed heritable differences in their competitive ability (genotype-by-competition interaction: likelihood ratio test for random factors in mixed model analysis of variance, $\chi^2=3.99$, $P=0.023$).
**Figure S5.** Divergent natural selection among treatments. Divergent selection was detected for each trait in at least one year and is depicted above: A) selection on early flowering (2009) and B) selection on fruit oenothein A concentrations (2011). In both cases there was a significant interaction term between insect suppression treatment and the trait for predicting lifetime seed production (see table S1).
Table S1. Evidence for divergent natural selection among our treatments for traits observed to evolve in the experimental evolution plots. Shown are analysis of covariance; an interaction between treatment (insect suppression) and the trait for predicting fruit production indicates divergent selection. Shown below are F-values, * indicates \( P<0.05 \), while † indicates \( P<0.1 \).

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References and Notes


18. Materials and methods are available as supplementary materials on *Science* Online.


27. E. L. Larson, S. M. Bogdanowicz, A. A. Agrawal, M. T. J. Johnson, R. G. Harrison, Permanent genetic resources: Isolation and characterization of polymorphic microsatellite loci in common evening primrose (*Oenothera biennis*). *Molecular


