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Insect herbivory and plant adaptation in an early successional community

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To address the role of insect herbivores in adaptation of plant populations and the persistence of selection through succession, we manipulated herbivory in a long-term field experiment. We suppressed insects in half of 16 plots over nine years and examined the genotypic structure and chemical defense of common dandelion (*Taraxacum officinale*), a naturally colonizing perennial apomictic plant. Insect suppression doubled dandelion abundance in the first few years, but had negligible effects thereafter. Using microsatellite DNA markers, we genotyped >2500 plants and demonstrate that insect suppression altered the genotypic composition of plots in both sampling years. Phenotypic and genotypic estimates of defensive terpenes and phenolics from the field plots allowed us to infer phenotypic plasticity and the response of dandelion populations to insect-mediated natural selection. The effects of insect suppression on plant chemistry were, indeed, driven both by plasticity and plant genotypic identity. In particular, di-phenolic inositol esters were more abundant in plots exposed to herbivory (due to the genotypic composition of the plots) and were also induced in response to herbivory. This field experiment thus demonstrates evolutionary sorting of plant genotypes in response to insect herbivores that was in same direction as the plastic defensive response within genotypes.

KEY WORDS: **Dandelion *Taraxacum officinale*, experimental evolution, induced defense, microsatellite, phenolic inositol esters, plant defense against herbivory, plant-insect interactions, sesquiterpene lactone.**

The evolution of plant defense against herbivory is a classic area of experimental evolutionary studies, with major advances beginning in the 1980s using quantitative genetics (reviewed in Fritz and Simms 1992; Agrawal 2011; Franks et al. 2012). Conceptually, genetic variation in defense is thought to be maintained by spatio-temporal variation in herbivores and competitors, with natural selection responding to costs and benefits of defense. At the same time, ecological studies on the long-term effects of insect herbivore suppression, especially in a successional context, demonstrated the keystone role herbivores can play in plant community dynamics (Brown and Gange 1989; Müller-Schärer and Brown 1995; Root 1996; Carson and Root 2000). Although some theoretical and empirical work attempted to link these approaches (Uriarte et al. 2002; Hakes and Cronin 2012), multigenerational empirical studies of ecological and evolutionary dynamics imposed by herbivores did not surface until the new millennium.

In 2012, several studies emerged that improved our understanding of the evolutionary impacts of insects on plants, especially in the context of community dynamics. Each of these studies was characterized by a long-term ecological perspective and used a mechanistic approach to understand the evolution of specific plant defense chemicals. Züst et al. (2012) conducted a laboratory selection experiment with a diversity of aphids on *Arabidopsis thaliana*, demonstrating rapid evolution of glucosinolate defenses and a matched natural geographic pattern of defenses to aphid distributions. Using field transplants in multiple populations, Prasad et al. (2012) showed the adaptive genetic differentiation of glucosinolates in native populations of *Boechera stricta*. Studies of tall goldenrod, *Solidago altissima*, showed that the long-term suppression of herbivores favored plot dominance by plant genotypes that were less resistant and more competitive, the latter via the production of allelopathic compounds (Bode

and Kessler 2012; Uesugi and Kessler 2013). Finally, our own work employed experimental evolution in the field with common evening primrose, *Oenothera biennis*, and we demonstrated that herbivore-suppression resulted in increased frequencies of competitive genotypes but decreased frequencies of genotypes chemically defended by ellagitannins (Agrawal et al. 2012). In sum, plant evolution in response to herbivory can be rapid, and often coincides with independent selection and evolution of reduced competitive ability (Uesugi et al. 2017).

Because both plant competitive dynamics and the impact of insect herbivores are predicted to change over successional time (Cates and Orians 1975; Brown 1984; Tilman 1990; Carson and Root 2000; Rasmann et al. 2011), we hypothesized that although plant evolution in response to herbivory may be rapid, the dominant evolutionary drivers may change as natural processes proceed in the field (Hakes and Cronin 2012). For example, herbivory may be critically important during the establishment of seedlings early in the successional process, while plant competition may be the relatively stronger selective agent later (Hanley 1998). Alternatively, the impact of herbivory and competition may both increase from early to midsuccession, causing selection for plant strategies that maintain both defense and competitive ability (Rasmann et al. 2011). Finally, plant phenotypic responses to herbivory (i.e., induced defense) may be most pervasive early in colonization and may play a lesser role later, as herbivory may be less predictable in early succession (Rasmann et al. 2011, Hakes and Cronin 2012). To address changing natural selection and the evolution of defense, here we evaluate the impact of nine years of insect suppression on the abundance, genotypic structure, and chemical defense of a plant that naturally colonizes disturbed sites, the common dandelion (*Taraxacum officinale*).

Dandelion, a dominant, perennial, early successional competitor, naturally recruited into 16 randomly assigned experimental plots we established a decade ago, and early analyses revealed that dandelion populations were dramatically suppressed by insects (Agrawal et al. 2012) (Fig. 1). Dandelion reproduces clonally through seeds (via apomixis), allowing us to track genotype frequencies using a small number of genetic markers and link genotypic change to phenotypic evolution at the plot level. Here, we address the impacts of long-term insect suppression on relative genotype frequencies and genetic diversity at two time points over a decade. Because dandelions naturally colonized our replicate plots, changes in genetic structure and diversity between treatments may be due to selective establishment, mortality of established clones, or differential reproduction in control versus insect-suppression plots. Therefore, observed differences between the plot types represent combined natural processes strong enough to overcome stochastic differences in colonization.

The apomictic nature of dandelion facilitated the phenotypic characterization of defense genotypes in the field. By genotyping and phenotyping plants from experimental plots, we partitioned phenotypic variation due to genotypic identity and plasticity due to the environment (insect suppression treatment). We focused on two classes of plant defense, sesquiterpene lactones and phenolic inositol esters, which were recently shown to be important in the defense of dandelion against herbivory (Huber et al. 2015, 2016a,b). Thus, the current study investigates the roles of herbivory and succession in shaping the genetic structure of dandelion populations and the evolutionary ecology of their defense chemistry.

Materials and Methods

NATURAL HISTORY

Common dandelion (*T. officinale* agg., Asteraceae) was introduced to North America during settlement by Europeans over 200 years ago, and is now well-established in lawns, waste places, and agricultural settings on six continents (Stewart-Wade et al. 2002). It is a perennial lactiferous plant that typically reproduces apomictically (by producing seeds that are clones of the maternal parent), especially in North America where nearly all populations are triploid (Lyman and Ellstrand 1984; Van Dijk 2003; McLeod et al. 2012). Several recent studies have investigated genetic variation, natural selection, epigenetics, phenotypic plasticity, and evolution of dandelion in response to various ecological factors (Vellend et al. 2009; McLeod et al. 2012; Verhoeven and van Gurp 2012; Molina-Montenegro et al. 2013; Verhoeven and Biere 2013; Oplaat and Verhoeven 2015; Huber et al. 2016b).

Several insect herbivores consume aboveground dandelion tissue at the field site for our long-term experiment. In particular, we have studied a specialized seed predator weevil (*Glocianus punctiger*), which was incidentally introduced to the United States (Fig. 1B) (McAvoy et al. 1983), the cutworm caterpillar of the generalist invasive moth *Noctua pronuba*, and the generalist four-lined plant bug (*Poecilocapsus lineatus*, Hemiptera, Miridae) that is native to the United States. Among dandelion's defenses against herbivory, a dominant sesquiterpene lactone, taraxinic acid beta-D-glucopyranosyl ester (TA-G), has been well-studied and may function both as a deterrent and toxin (Picman 1986; Huber et al. 2016b). Additionally, dandelion produces diverse phenolic inositol esters and these compounds likely breakdown to highly oxidative semi-quinones in insect consumers (Santos-Buelga et al. 2011).

EXPERIMENTAL DESIGN

In 2007 we established 16 replicate experimental field plots near Ithaca, NY. The field site is comprised of a glacial lake bottom (or outwash), with very rocky soil, and had formerly been agricultural land. Dominant vegetation included horse nettle (*Solanum*

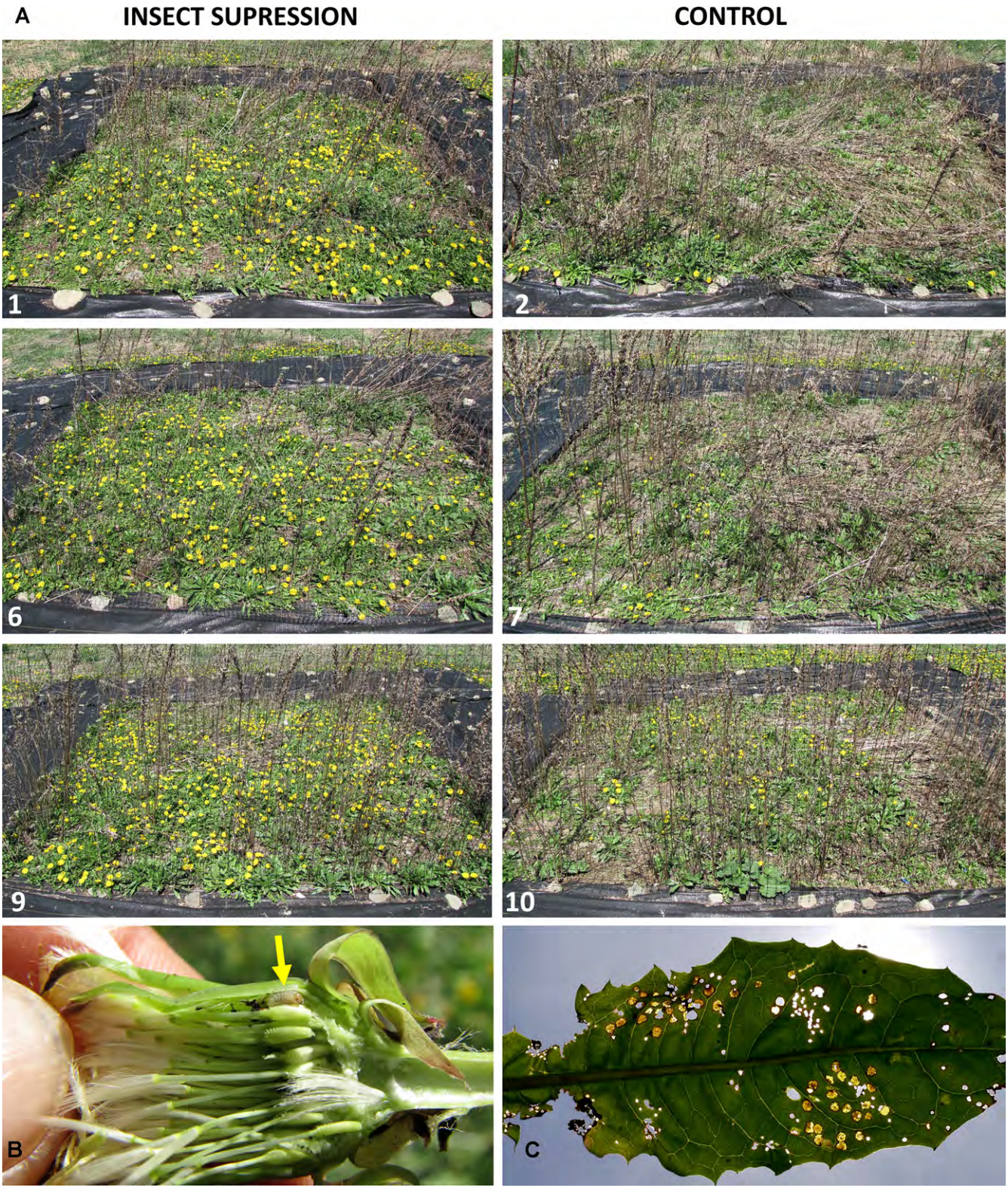

Figure 1. Dandelion plots and herbivores. (A) Photographs of six of the 16 total experimental evolution plots (April 29, 2010). Three representative plots with insect suppression (treated with insecticide, left) and their closest control plot (right); plot identification numbers are shown in each panel. (B) Larva of the specialist seed predator weevil (*Glocianus punctiger*) and (C) mirid leaf damage by the four-lined plant bug (*Poecilocapsus lineatus*).

carolinense), tall goldenrod (*Solidago altissima*), dandelion, and several exotic grasses. Each plot was 13.5 m^2 and plots were spaced a minimum of 10 m apart. Each plot was tilled and sprayed twice with the herbicide glyphosate (Roundup, Monsanto, St. Louis, MO) prior to initiation of the experiment. All plots were planted with 60 individuals of common evening primrose (*O. biennis*) in the center square meter, and results on *O. biennis* ecology and evolution have been reported elsewhere (Agrawal et al. 2012; Agrawal et al. 2013).

Dandelion naturally colonized the plots and was not otherwise manipulated. Although we did not have equal starting conditions in each plot and we assume that there was not a bias in the dispersal into the two plot types, we did replicate and randomize the spatial arrangement of treatments. Furthermore, dispersal should not have been limited given the large population of dandelions in the surrounding field and the capacity of dandelion to disperse >100 m due to their feathery pappus (Tackenberg et al. 2003).

Eight of the 16 plots were randomly assigned to an insect herbivore suppression treatment and were sprayed biweekly every year 2007–2015 during the growing season (April through October) with the insecticide esfenvalerate (Asana XL, Dupont, Wilmington, DE). 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 7.63 mL per liter of water to the vegetation in each of the insect suppression plots. The remaining plots were sprayed on the same schedule with water. Esfenvalerate is a nonsystemic broad-spectrum insecticide that has been shown to have no effects on dandelion germination (Agrawal et al. 2012). Several other plant species have been tested and show no impact of esfenvalerate on plant growth or performance traits (Mitchell 2003; Siemann et al. 2004; Agrawal et al. 2012). Plots were not weeded or otherwise manipulated after experimental plots were established, allowing for natural recruitment of plants. Thus, although differences between plot types can safely be assigned to the insecticide treatment, as discussed later, aspects of demography and indirect ecological interactions likely contributed to the overall effects of insect suppression.

Beginning in 2009 (year 3 of the experiment) through 2016, *T. officinale* were censused annually in early May (during the peak of dandelion flowering) by counting all reproductive stems in each plot from photographs. Flowering stems in each plot were counted independently by two researchers and the average count was used. To verify that this census method accurately captured the abundance of plants in each plot, in 2014 we conducted a complete field survey of all plants (including those not in flower) in each plot at the same time as the photographic census; the two methods were correlated ($n = 16$, $r = 0.78$, $P < 0.001$), although the total number of plants was estimated an average of four times the number of flowering stems ($n = 16$, $\pm$ 0.78 SE; this estimate did not differ between treatments $F_{1,14} = 1.44$, $P = 0.250$).

We have studied three insect herbivores in our experimental plots. First, the relatively specialized seed predator weevil *G. punctiger* was abundant in the developing seed heads of *T. officinale* in our plots in spring (May–June); we previously reported on the effects of our insect suppression treatment on this species (Agrawal et al. 2012). Second, the generalist cutworm caterpillar *N. pronuba* preferentially consumed dandelion over the other dominant plant in our plots, *O. biennis* (Agrawal et al. 2012). Finally, observations of extensive herbivory by the mirid *P. lineatus* in 2014 prompted us to census leaf damage in June. In each of four quadrants in each plot, we randomly tossed a meter stick and assessed every dandelion rosette touching the meter stick (20–40 plants censused per plot, mean 25). If less than 20 plants were touching the meter stick (this occurred in two plots), the closest plants to the stick were sampled so that at least 20 plants were assessed. *P. lineatus* is a "windowing" cell content feeder that leaves scars of approximately 2 $\times$ 2 mm. Dandelion rosettes with >10 scars were counted as "damaged plants." Such damaged plants had, on average, 5% leaf tissue loss. We also searched for root feeding herbivores, which have been shown to be important in the evolution of dandelion defense in Europe (Huber et al. 2016a, b), but did not find any.

MICROSATELLITE MARKERS AND GENOTYPING

To estimate the number of dandelion genotypes and relative frequencies in each plot, we genotyped a subset of plants from each plot using four polymorphic trimeric microsatellite DNA markers (see Appendix 1 for marker development methods and Table S1 for the markers and their primers). Based on our screen, a list of several thousand additional potential markers and corresponding primers are presented in Table S2. While past work identified a maximum of 13 genotypes within natural populations (Falque et al. 1998; Vašut et al. 2004; Vellend et al. 2009; McLeod et al. 2012), our panel of four markers was able to distinguish over 90 genotypes in our experimental plots (which were surrounded by a least a hectare of old-field vegetation with abundant dandelions). Although cryptic genotypes are certainly possible, we believe that this limited number of markers showed sufficient power to provide a strong estimate of the actual number of genotypes in our population, while allowing us to sample a large number of plants. Our confidence in this estimate is further bolstered by the finding that phenotypic measures of secondary metabolites were highly consistent within the genotypes identified using this microsatellite panel (see Results).

We genotyped 95 individuals from each plot in 2011, however in 2014 our sampling was limited by dandelion abundance (mean sample number per plot in 2014 was 75, with the smallest plot having 35 plants sampled). Plants for genotypic analysis

were sampled randomly from within the plots. In total, we sampled 2758 plants. Briefly, tissue was collected in the field on dry ice, stored at –80C, then freeze-dried and ground to a fine powder (Retsch mixer mill). Genomic DNA was obtained by incubating samples at 60°C in a cetyltrimethylammonium bromide (CTAB) buffer containing beta-mercaptoethanol for 20 minutes, and extracted with chloroform. DNA was precipitated from the aqueous phase with isopropanol, washed with ethanol, dried and resuspended in Tris-EDTA buffer (pH8). Samples were diluted with water to yield approximate DNA concentrations of 5–20 ng/μL (Nanodrop ND-1000; Thermo Fisher Scientific, Waltham, MA) to facilitate subsequent PCR and genotype analysis.

For each sample, four microsatellite loci were amplified in a single multiplex PCR reaction (Type-It Microsatellite PCR kit; Qiagen, Valencia, CA). PCR reactions contained 1 μL of diluted genomic DNA as per the manufacturer's protocol (with Q-solution), but scaled to a 10 μL total volume. The forward primer for each locus was labeled with a distinct fluorescent tag (6-FAM, PET, NED, VIC; Applied Biosystems, Life Technologies Corp., Carlsbad, CA). Touchdown PCRs were performed using an initial denaturation step at 95°C for 5 minutes, followed by 35 cycles of 95°C for 30 seconds, 59–50°C for 90 seconds (the annealing temperature decreased by 1°C each cycle for the first 10 cycles) and 72°C for 30 seconds, with a final one-time extension at 60°C for 30 minutes. Multiplex PCR products were visualized for a subset of samples in each plate using agarose gel electrophoresis. Products were then diluted 1:3 with water, and mixed with Hi-Di formamide and Genescan LIZ-500 size standard (Applied Biosystems, Foster City, California). Samples were analyzed on a 3730 × l capillary sequencer (Applied Biosystems) at the CLC. Allele sizes were determined using Genemarker version 2.4.0 software (Soft Genetics, State College, PA), with all calls checked by eye, and a trisomy report was printed, in order to assign triploid genotypes, as described below.

We used this microsatellite data to assign genotypes in two different ways: (1) by using the microsatellites as dominant markers (using only the presence/absence of alleles), and (2) by using peak area ratios to estimate allelic dosage and thereby assign triploid genotypes. This second method of genotype assignment is described in detail below. There was very little difference between the two methods in terms of total genotype number (88 vs 91 genotypes) or assignment of individuals to genotype (five samples were affected). However, analyzing peak area ratios allowed us to identify and exclude a subset of the data affected by amplification issues (e.g., null alleles). Thus we believe the peak area method yields the most robust dataset, which we used for our final analyses.

While use of microsatellites as dominant markers is straightforward, assigning genotypes to triploid organisms can be problematic. Here, a maximum of three alleles are expected at any given locus. Thus, while samples with either one or three peaks at a particular locus are easy to call, assignment of samples with two peaks is less clear. In this case, it is difficult to determine whether a plant individual has two copies of the first allele and one of the second, or vice versa (allelic dosage; i.e., for Locus A, two peaks at 130 and 133 can represent a single-locus genotype of 130/130/133 or 130/133/133). Due to these difficulties, we developed a method for calling triploid genotypes, based on statistical cutoffs. To do this, we used peak area ratios (PARs, based on MAC-PR method: Esselink et al. 2004).

Peak areas were output by Genemarker (in trisomy report), and PARs were calculated between each pair of peaks, with the peak area of the shorter allele being in the numerator. To account for the bias in PCR to preferentially amplify smaller alleles, we created a PAR distribution from all of the data in which three peaks were present, within each locus and each year. Without PCR bias, such a distribution would be centered perfectly around 1, as peak size would be identical for each of the three alleles. In reality, this distribution was shifted to the right, such that the mean PAR was greater than 1, and variable across loci and years. Due to this variability, we used a distinct PAR distribution for each locus and each of our two sampling years (Figs. S1 and S2). While these PAR distributions were well resolved for three of the loci, microsatellite locus tri12 yielded a poorly resolved PAR distribution, with extensive overlap between the samples with three alleles and those with two alleles (Fig. S3). This distribution likely results from locus-specific amplification difficulties, perhaps related to null alleles. Upon close inspection, the inclusion of locus tri12 in our analysis had negligible effects on genotype resolution (out of 91genotypes, only six rare genotypes, affecting 12 individuals, were lost). Due to the problems with reliable amplification as well as its lack of ability to resolve genotypes, we ultimately excluded tri12 from our final analysis.

For the remaining three loci, in each case in which a sample showed two peaks at a given locus, we used its PAR to determine whether it was statistically unlikely to fit the null expectation of a 1:1 ratio, and thus much more likely to fall in either the 1:2 or 2:1 category. We used a 95% cutoff, such that a 2-allele sample with a PAR below the 2.5 percentile, or above the 97.5 percentile of the PAR distribution, was categorized as 1:2, or 2:1, respectively. Samples showing two alleles with PARs falling within 95% of the PAR distribution curve were categorized as "unresolved" and were excluded from final analyses. In total, we recovered good microsatellite data for 95% (2610) of the 2758 samples, after removing samples for which PCR failed at 1 or more loci, as well as those for which we suspected PCR contamination due to either the presence of additional peaks or highly skewed PARs. Using our three reliable microsatellite loci, we were able to completely resolve 95% of this final dataset (2480 plants) to a multilocus genotype, leaving 130 samples unresolved. As discussed above,

these samples were excluded from the analysis due to problems with PCR amplification. Although it is possible that a subset of these samples could potentially be diploids, our estimates show this to be unlikely

CHEMICAL ANALYSIS

In 2014, we collected additional samples of leaf tissue to analyze secondary metabolites. In particular, we assessed the concentrations of the dominant and defensive sesquiterpene lactone taraxinic acid beta-D-glucopyranosyl ester (TA-G) and five different phenolic inositol esters (PIEs, with either two (di-) or three (tri-) phenylacetic acid moieties). Here we report TA-G, the total concentration of di-PIEs, and the total concentration of tri-PIEs. Tissue was collected from the same set of plants sampled for genotyping (up to 95 per plot). The youngest fully expanded leaf was collected from each plant on dry ice and stored at –80C. Tissue was then freeze-dried and stored until genotype analysis on corresponding leaf tissue was complete.

To test for the genotypic contribution to expression of these secondary metabolites, we ground leaf tissue collected from a subset of 10 individuals of each of the 15 most common dandelion genotypes from the field experiment. Together, these 15 genotypes comprised >70% of individuals in the experiment and were also the most abundant genotypes in 2011. Because secondary metabolite expression may have been impacted by the presence of insects (i.e., induced responses to herbivory) and plot to plot variation, we analyzed five leaf samples for each genotype from each treatment and from 3–5 plots within each treatment (total $n = 147$ leaf samples for chemical analysis).

To determine the concentrations of TA-G and PIEs in the leaves, 20 mg freeze-dried tissues was ground and extracted with 1 mL methanol containing 20 μg/mL loganin and 200 μg/mL salicin as internal standards for TA-G and PIEs, respectively. Samples were vortexed for 5 minutes, centrifuged at room temperature at 17,000 *g* for 15 min and supernatant analyzed on an Agilent Technologies 1100 series HPLC, coupled to a photodiode array detector (G1315A DAD, Agilent Technologies, Santa Clara, CA). Metabolite separation was accomplished with a Nucleodur Sphinx RP column (250 × 4.6 mm, 5 μm particle size, Macherey-Nagel, Duren, Germany). Injection volume was 5 μL. The mobile phase consisted of 0.1% acetic acid (A) and acetonitrile (B) utilizing a flow of 1 mL/min with the following gradient: 0 min: 5% B, 18 min: 43% B, followed by column reconditioning. Peak area was integrated at 245 for TA-G and at 275 for PIEs. TA-G and PIEs were quantified based on their respective internal standards.

STATISTICAL ANALYSES

ANOVA was used to compare plot means for numbers of reproductive stems and proportion of plants with mirid herbivory. Similarly, analyses of single genotype frequencies, Simpson's (genotypic) Diversity Index, genotypic richness, and genotypic evenness were assessed with one-way ANOVA. To analyze the effect of insect suppression on the multivariate genetic structure of dandelion populations, we employed canonical correspondence analysis (CCA) and a permutation test for each of the two years (implemented in Canoco V4.5, Braak and Šmilauer 2002). For the subset of dominant genotypes (six that each were at least 5% of the plants), we analyzed the effect of treatment using one-way MANOVA. Finally, to specifically address genetic change at the plot level between 2011 and 2014, we again conducted a one-way MANOVA, but here used the difference in frequency of each of the dominant genotypes as the response variables. In other words, in this analysis we asked if the magnitude and direction of change in genotype frequencies was dependent on the insect suppression treatment.

For analyses of plant chemical defense phenotypes from field-collected leaf tissue (in 2014), we employed mixed-model ANOVAs including insect suppression treatment (fixed effect), genotype (random effect), and plot nested with treatment (random effect). Limited sampling precluded an analysis of interaction terms. We then calculated the genotype-based plot level chemical phenotypes by multiplying the frequencies of genotypes by their genotypic (least squares mean) value for each defense trait; differences between insect suppression and control plots were compared with one-way ANOVA. These calculations were conducted for 2011 and 2014; nonetheless, the two sampling years are not strictly comparable as the environmental component of quantitative phenotypes may have differed across years.

Because in 2008–2010 the plots were dominated by evening primrose (102 plants/m^2 in 2010, Fig. 2), we used the abundance of *O. biennis* in each plot in 2010 as covariate for all analyses of data from 2011; neither this nor other plant density covariates were significant for 2014 and therefore were not used. All ANOVAs were conducted using JMP PRO V12; mixed models were implemented with restricted maximum likelihood, and fixed effects were tested using type III sums of squares. Assumptions of normality of the residuals and homoscedasticity were checked and no transformations were employed.

Results

ECOLOGICAL EFFECTS OF INSECT SUPPRESSION

Two years after initiating the experiment, in 2009, we observed substantial dandelion recruitment to the plots and nearly twice the number of flowering stems in insect-suppressed plots compared to ambient controls (Figs. 1 and 2). This statistically significant twofold treatment effect was persistent for the next two years, but dwindled to about a 40% effect of insect suppression in 2012–2016, and was not significantly different from controls in these latter five years. In the peak dandelion year, 2012, there were just

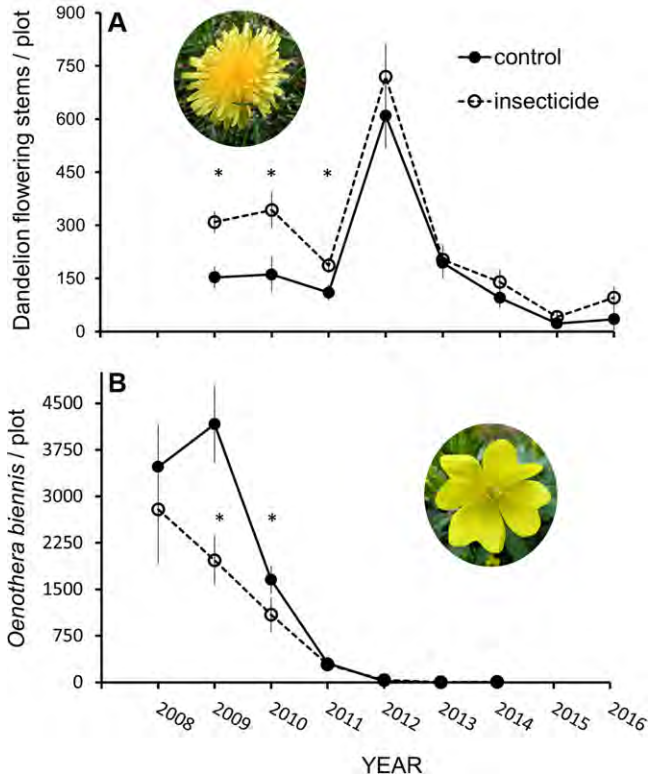

Figure 2. **Impacts of insect suppression (insecticide treatment) on the mean ± SE abundance of (A) reproductive dandelion and (B) evening primrose plants in each plot 2009–2016. Asterisks indicate $P < 0.05$ by one-way ANOVA, $n = 16$ plots.**

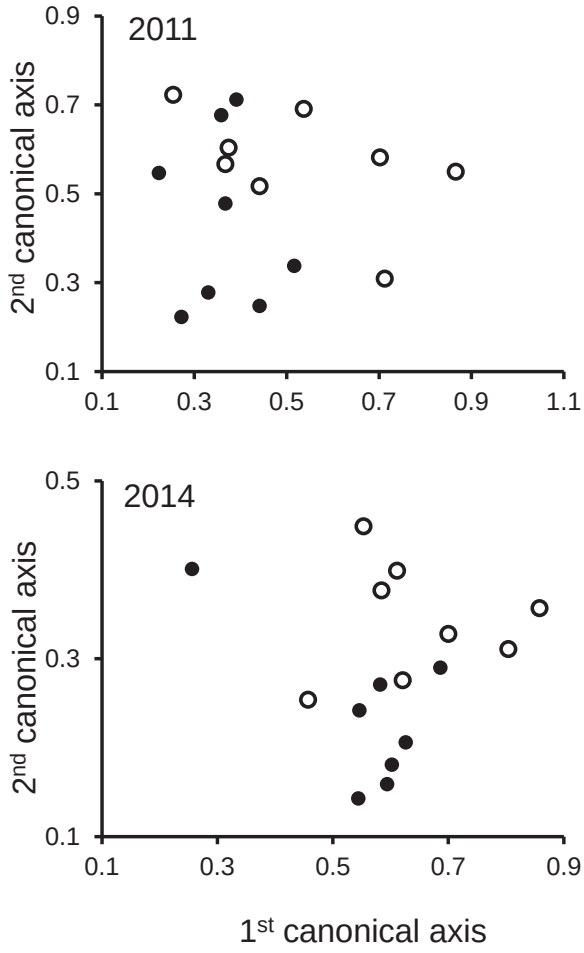

Figure 3. **Results from an unconstrained correspondence analysis on frequencies of dandelion genotypes in 2011and 2014 as a means to visualize the genetic structure of each plot. Each dot represents an experimental plot (total $n = 16$); ambient (open dots) and insect suppressed (black dots) treatments were coded after the analysis. A constrained canonical correspondence analysis revealed substantial genetic differentiation between treatment plots in both years (see Results).**

under 50 flowering stems/m^2 averaged across the plots (≈ 200 total plants/m^2). During 2008–2010, low vegetation in the plots was dominated by *O. biennis*, dandelion, strawberry (*Fragaria* spp.), and horsenettle (*Solanum carolinense*); as an overstory species, tall goldenrod (*Solidago altissima*) began to recruit into the plots during this period. By 2012, *O. biennis* was almost absent from the plots (Fig. 2), as it is an early successional species that relies on light to germinate.

As previously reported (Agrawal et al. 2012), dandelion seed predator weevils were half as abundant in the insect suppression plots compared to controls when censused in 2011 (mean ± SEM number of larvae per 10 seed heads, control plots 9.50 ± 1.30, insect suppression plots 4.63 ± 1.30, $F_{1,14} = 7.01$, $P = 0.019$). Application of insecticides also resulted in a reduction of leaf feeding by the four-lined plant bug (*P. lineatus*). Plots treated with insecticide had half as many plants damaged by these mirids as compared to controls (mean ± SE percent plants with damage, control 27 ± 3, insecticide 14 ± 3, $F_{1,14} = 7.613$, $P = 0.015$).

IMPACTS ON GENOTYPIC COMPOSITION OF PLOTS

Across the 16 plots, our microsatellite screen revealed 71 triploid dandelion genotypes in 2011 and 70 genotypes in 2014 (50 were overlapping in the two years) (raw frequencies are given in Table S3). To test for differences in the genotypic composition of our sprayed versus control plots, we employed a constrained canonical correspondence analysis (CCA). In both 2011 and 2014, we found substantial genotypic differentiation due to insect suppression (Fig. 3, CCA, 2011: Trace = 0.037, $F = 2.094$, $P = 0.039$; 2014: Trace = 0.066, $F = 1.933$, $P = 0.043$). As elaborated below, the effects on genetic structure in these two sample years were distinct.

One means to characterize the genetic structure of each plot, especially since plants were natural colonists, is its genotypic diversity. The diversity of dandelion genotypes (Simpson's Index) was 29% lower in the insect-suppressed treatment compared to controls in 2011 (Fig. 4, mean ± SE control 0.098 ± 0.008,

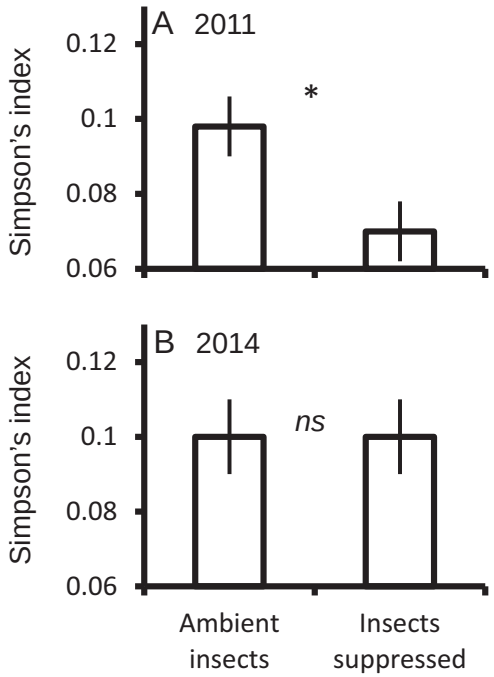

Figure 4. **Impacts of insect suppression on genotypic diversity. Shown are means ± SE for (A) 2011 and (B) 2014. Asterisks indicate $P < 0.05$ by one-way ANOVA, $n = 16$ plots.**

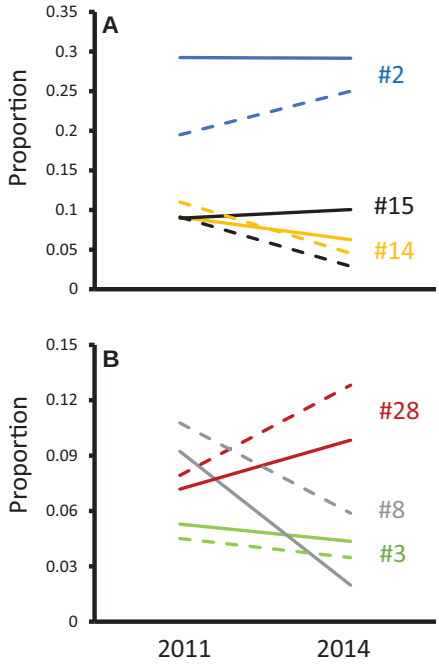

Figure 5. **Reaction norm plots showing the change in frequencies of the dominant genotypes over time. Mean frequency across eight control plots shown in solid lines and mean frequency of eight insect suppression plots in dashed lines. Genotypes are distinguished by line color and identities are shown to the right. Two panels are shown for clarity; note the difference in Y scale in the top and bottom panel.**

insecticide 0.070 ± 0.008, $F_{1,13} = 5.945$, $P = 0.029$), and this effect was associated with a marginal (9%) reduction in genotypic richness ($F_{1,13} = 3.717$, $P = 0.076$). Evenness of genotypes was not affected by insect suppression in 2011 or 2014 (Smith and Wilson's evenness index, E_{var}, 0.62 in 2011 for both treatments, $F_{1,13} = 1.716$, $P = 0.213$; 2014: $F_{1,14} = 0.761$, $P = 0.398$). In 2014, neither of the measures of genetic diversity were significantly different between treatments (Simpson's index: control 0.101 ± 0.013, insecticide 0.101 ± 0.013, $F_{1,14} = 0.001$, $P = 0.981$; Richness: control 23.0 ± 2.04, insecticide 22.3 ± 2.04, $F_{1,14} = 0.067$, $P = 0.799$).

We next assessed the impact of the insect suppression treatment on the relative frequency of the dominant genotypes. A single genotype of *T. officinale* dominated the plots (genotype #2), accounting for >20% of all dandelions across years. Five other genotypes each represented 5–10% of the plots, followed by many less frequent genotypes. We first used MANOVA to determine the effect of insecticide treatment on relative frequencies in each year, and found an effect in 2014 (exact $F_{6,9} = 9.397$, $P = 0.002$) but not in 2011 (exact $F_{6,9} = 1.073$, $P = 0.444$) (Fig. 5). Dominant genotype #2 was 68% more abundant in control plots compared to insect suppression plots in 2011 (Fig. 4, $F_{1,13} = 8.297$, $P = 0.013$). However, by 2014, this difference in the frequency of genotype #2 was no longer apparent (Fig. 4, $F_{1,14} = 0.999$, $P = 0.334$). Among the other abundant genotypes, the frequency of only one of five was impacted by herbivore suppression (in 2014) (genotype 15, control frequency = 0.10 ± 0.01, insecticide frequency = 0.03 ± 0.01, $F_{1,14} = 22.012$, $P < 0.001$).

To specifically address evolutionary change in the plots, we examined differences in genotype frequencies between the two sample years as a function of the insecticide treatment. Using MANOVA with the six most abundant genotypes, we found that the genotypic trajectories were indeed dependent on treatment (exact $F_{6,9} = 3.373$, $P = 0.050$, Fig. 5). In univariate analyses, only one genotype (#15) showed a significant effect of treatment on change in frequency ($F_{1,14} = 18.100$, $P < 0.001$, Fig. 5A).

EVOLUTION OF PLANT DEFENSE CHEMISTRY

To address the genotypic basis of plant defensive chemistry, we conducted HPLC analysis on replicate samples from the 15 most abundant genotypes, each collected from multiple sprayed and control plots (Table S4). We found a strong genotypic basis for the production of taraxinic acid beta-D-glucopyranosyl ester (TA-G), total concentration of di phenolic inositol esters (di-PIEs), and total concentration of tri phenolic inositol esters (tri-PIEs). In pairwise analyses, there was a lack of a significant genetic correlation between the three compound classes ($n = 15$, $P_s > 0.765$,

Table 1. Mixed-model ANOVA for effects of insect suppression treatment, plant genotype, and collection plot on foliar chemistry of common dandelion.

		Taraxinic acid beta-D-glucopyranosyl ester (TA-G)	Total di phenolic inositol esters	Total tri phenolic inositol esters
Treatment	**d.f.**	1, 8.941	**1, 11.57**	1, 5.76
	F	0.107	**15.242**	4.594
	P	0.751	**0.002**	0.077
Genotype	**LR**	**13.6**	**7.7**	**7.2**
	P	**<0.001**	**0.003**	**0.004**
Plot[Treatment]	**LR**	0	0.8	0.8
	P	0.5	0.168	0.168

Treatment was a fixed effect and the degrees of freedom (DF) and *F* ratio are shown. Genotype and plot nested within treatment were random effects and were tested with a Likelihood ratio (LR) test. Significant effects are shown in bold.

Fig. S4). Insect suppression treatment was significant only for di-PIEs (Table 1), with di-PIEs showing 72% higher concentrations when exposed to insects compared with sprayed plots (mean ± SE μg/mg dry tissue, insects suppressed: 0.65 ± 0.12, controls: 1.12 ± 0.13). Because genotypic identity was accounted for in the analysis (and plant sampling was balanced between sprayed and control plots) this effect was due to phenotypic plasticity, most likely insect damage induced increased expression of di-PIEs.

To test for genetically determined differences in chemical profiles between control and treated plots, we calculated the plot level chemical phenotypes by multiplying the frequencies of the most common genotypes by their genotypic value of defense (least squares means, accounting for plasticity). Based on these estimations, in both 2011 and 2014, di-PIEs were genetically differentiated between control and insect suppression plots, with insect exposure raising di-PIEs genotypically by about 10% at the plot level (Fig. 6, Table S5). Neither TA-G nor tri-PIEs showed evidence of genetic differentiation between control and insect suppression plots (Table S5).

The dominant genotype (#2) showed the highest expression of di-PIEs (77% higher than the average of the other 14 genotypes) and the lowest expression of tri-PIEs (33% lower than average) (Fig. S4). Accordingly, this dominant genotype was largely responsible for the plot level differences in chemistry in both years (i.e., the treatment effect is no longer significant if we exclude genotype 2 from the analyses). This was also the only genotype that did not show an increase in di-PIE expression in the presence of insects compared to sprayed plots (the range among the 14 other genotypes was a 10–75% increase, while genotype #2 showed a 6% decrease). Genotype 15, the only other genotype significantly impacted by the treatment on its own, was near the average for both constitutive and induced levels of di-PIEs. TA-G was near the average for these two genotypes compared to the rest of the genotypic pool.

Discussion

Various approaches have been employed to study the evolution of plant defense against herbivores. Classically, quantitative genetic methods were used in a single generation to estimate costs, benefits, genetic correlations, and natural selection on defensive traits (Rausher 1996; Mauricio and Rausher 1997; Shonle and Bergelson 2000; Franks et al. 2012). Additionally, historical approaches, especially those employing phylogenies, have been used to infer the evolution of defense and their causes (Fine et al. 2004; Becerra et al. 2009; Desurmont et al. 2011). A recent surge of interest in rapid evolution and its consequences has spurred more direct multigeneration and experimental approaches to studying defense evolution, many of which are initiated with known genotypes (Meyer et al. 2006; Agrawal et al. 2012; Züst et al. 2012).

Several previous studies have taken advantage of "natural experiments" to examine the impacts of altered selection regimes by herbivores (Vourc'h et al. 2001; Salgado and Pennings 2005; Zangerl and Berenbaum 2005; Stenberg et al. 2006; stenKato et al. 2008; Woods et al. 2012; Martin et al. 2015), including the large literature on nonnative plants that may escape enemies in their introduced range (Franks et al. 2012; Felker-Quinn et al. 2013). In the present study, and in a few others (Bode and Kessler 2012; Uesugi and Kessler 2013), naturally colonizing plant genotypes recruit into manipulated field plots and evolve through additional selective recruitment or sorting in experimentally manipulated communities. Because of the spatial proximity of the experimental plots and the stark contrast in the selection regimes, differences in the genotypic composition and phenotypes of the plots can be attributed to the impacts of herbivores, although some of these effects may be indirect and due to altered population abundances and competitive dynamics. In our study, plot-level insect suppression shifted the balance of competition between dandelion and

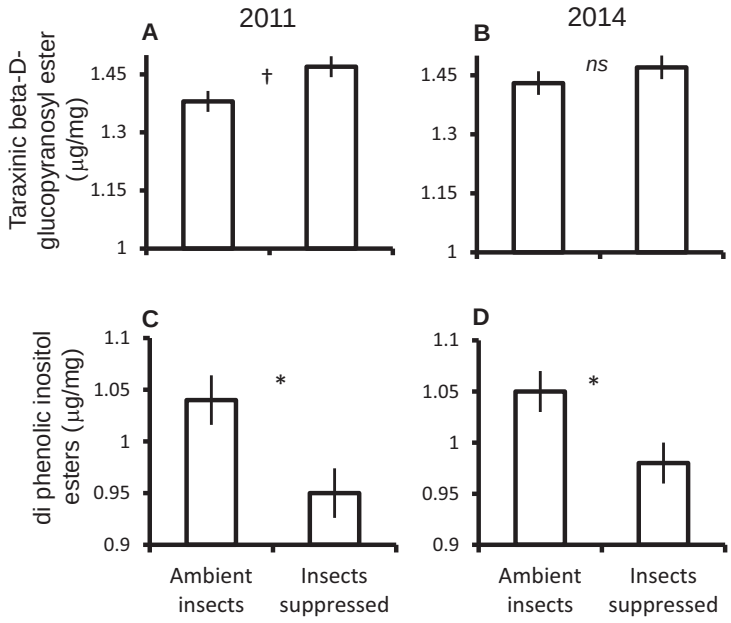

Figure 6. Impacts of insect suppression on plot-level defensive chemistry as determined by genotype frequencies and genotype-specific phenotypic values (see Methods). Shown are means ± SE for 2011 and 2014. Data for tri-PIEs was not different between treatments in either year and data are not shown. Asterisks indicate $P < 0.05$ by one-way ANOVA, $n = 16$ plots. † indicates $P < 0.1$.

evening primrose in favor of dandelion (Agrawal et al. 2012), and these effects persisted for the first five years of the experiment (Fig. 2). There are many reasons why the population and evolutionary effects of herbivores may decline over successional time, including changing competitive dynamics (Bazzaz 1979), the intensity of herbivory (Brown et al. 1987; Carson and Root 1999), and eco-evolutionary feedbacks (Agrawal et al. 2013). In the current study, 2012 was a turning point, where the previously dominant plant in the plots (*O. biennis*) strongly declined due to its lack of competitive ability and need for light to germinate (Fig. 2., Agrawal et al. 2012).

Because dandelion is an apomictic species, we were able to track genotype frequencies in the field using a small number of microsatellite markers. This is a similar system to our past research in the same plots, which employed *O. biennis*, a species which typically reproduces clonally through seeds (Agrawal et al. 2012, 2013). Nonetheless, unlike *O. biennis*, dandelion is perennial and recruited naturally into the plots via seeds, with many more genotypes represented. More than 90 distinct triploid genotypes of dandelion were identified in our screen of the >2500 plants across all plots and years. Our work revealed a much greater within-population diversity of genotypes than past research on dandelion (which reported 1–13 genotypes per population), although previous research employed dimer microsatellites (Falque et al. 1998; Vašut et al. 2004; Vellend et al. 2009; McLeod et al. 2012). Given the replication and spatial randomization of our plots, the effects of insect suppression on plant genotypic structure appear robust, although we neither controlled for the initial genotypic composition, nor the relative extent of effects caused directly by herbivory, versus indirect effects of plant competition.

We found several potent herbivores at the site, including a specialist seed feeding weevil, in addition to caterpillars (a cutworm) and mirid bugs. Suppression of these herbivores had a nearly twofold effect of the abundance of reproductive stems of dandelion over the first five years of this experiment (we only censused dandelions in years 3–5, as they were not apparent in the first two years). A previous study in Europe reported that exclusion of molluscan herbivores also enhanced recruitment of dandelions over one year (Hanley et al. 1995). In our study, as populations of dandelions peaked (2012), the impacts of herbivores on dandelion abundance declined, and this lack of an effect of insect suppression persisted over the subsequent five years as populations of dandelion gave way to competition and further successional suppression. As succession proceeded into the old-field phase beginning in 2014, tall goldenrod, *Solidago altissima*, became dominant.

At the community level, the effects of herbivores on plants (and predators on prey) can increase or decrease diversity, although intermediate levels of grazing typically increase plant species diversity (Lubchenco 1978; Olff and Ritchie 1998; Allan and Crawley 2011). Nonetheless, very few studies have evaluated the role of herbivores on intraspecific genetic structure and diversity. In previous work in the same experimental evolution plots, we found strong impacts of herbivores on the genetic structure (relative abundance of genotypes) but not diversity of *O. biennis*

(Agrawal et al. 2012). Our analysis of the genotypic structure and defensive phenotypes of the dandelions revealed an effect in the fifth year of study (2011), with suppression of insects resulting in a reduced abundance of the dominant genotype, altered relative abundance of genotypes (in the CCA, but not in MANOVA of the dominant genotypes), and reduced genetic diversity overall. It thus appears that ambient herbivory maintained genetic diversity and also favored the most abundant genotype in this system during the first half of the experiment (Fig. 4).

The genotypic structure of our plots was not entirely consistent among the two sample points. In 2014, effects of insect suppression on genotypic structure persisted (in the CCA, and now also in the MANOVA), but effects on the genotypic diversity and the frequency of the dominant genotype were no longer significant. At this stage, herbivores no longer had an impact on dandelion abundance (Fig. 2), and competitive dynamics may have taken hold. Importantly, we found evidence of insect-dependent changes among the dominant genotypes between 2011 and 2014 (Fig. 5). Although these effects were relatively weak, with at least three genotypes showing largely parallel responses in the two treatments across time (Fig. 5B), other genotypes were consistently on different trajectories when insects were suppressed (Fig. 5A); we emphasize that these analyses were conducted on the mean of eight replicate plots of each treatment. Because this analysis directly accounts for starting conditions (2011), we interpret the finding as evidence for insect-dependent evolutionary change (i.e., change in genotype frequency due to differential establishment, survival, or reproduction) as succession proceeded in the latter half of the experiment.

EVOLUTION OF PLANT CHEMICAL DEFENSE

The evolution of plant defensive chemistry can be complex and modified by the type and extent of herbivores attacking plants. For example, in our study, di-PIEs showed an evolutionary response (reduction) to release from herbivorous insects, while the sesquiterpene lactone TA-G showed a marginal increase in the same herbivore-free treatment. These responses appear to be independent, as we did not find a genetic correlation between expressions of these two defenses. For di-PIEs, herbivores may have selected for genotypes with particularly high secondary metabolite levels. Indeed, the most abundant genotype (#2), which was by far the highest producer of di-PIEs, was 68% more abundant in control plots (ambient herbivory) compared to insect suppression plots. Although the mode of action of PIEs as defenses remain largely unclear, these highly reactive compounds may breakdown to produce reactive semi-quinones and thereby induce oxidative stress in insect guts (Santos-Buelga et al. 2011). The fact that dandelions exposed to herbivory also showed a strong induced response in di-PIEs is consistent with the hypothesized defensive role. In-depth studies on the function of PIEs may provide further insights as to whether the observed differentiation in leaf chemistry between sprayed and unsprayed plots was adaptive in response to herbivores.

For TA-G we observed some evidence for an evolutionary decline with herbivory, which is more difficult to explain. One possibility is that the specialized seed weevil, *G. punctiger*, may be attracted to these "defense" compounds, as is the case for several other specialist herbivores (Adler et al. 1995; Giamoustaris and Mithen 1995; Ali and Agrawal 2012). Analyses of TA-G concentrations in the capitula and bioassays of insect oviposition preference could further substantiate this hypothesis. In dandelion roots, TA-G concentration is likely under positive selection by belowground feeding generalist insects (Huber et al. 2016a,b). Dandelion populations exposed to severe belowground herbivory over several decades had higher TA-G concentration in their root latex compared to lightly infested populations in the field; both phenotypic plasticity and genotypic differentiation contributed to this differentiation. The selection pattern for PIEs in dandelion roots is less clear. Although total PIE concentration in root latex was higher in dandelion populations subject to long-term root herbivory compared to controls, this pattern was likely shaped predominantly by phenotypic plasticity (Huber et al. 2016a). These results compared to the current study highlight that the evolution of plant defense chemistry can be distinct above and belowground, and the extent of pleiotropy between these plant compartments awaits further study.

We inferred the evolutionary responses of PIEs and TA-G to above ground herbivore selection by multiplying the genotype frequencies of plants in each plot by the genotypic values of their defense traits. As such, we have gained insight into how the populations evolved both genotypically and phenotypically, in terms of defensive chemistry. Interestingly, we found evidence for induction of di-PIEs as well. Indeed, plants from control (ambient herbivory) plots showed $>70\%$ higher di-PIE values than insect suppression plots. The consistency of the plot-level response to selection and phenotypic response to the presence of insects is highly suggestive of an important role for di-PIEs in plant defense. Nonetheless, the induction of PIEs in the leaves by above ground herbivores contrasts with the reduction of these compounds in root latex upon below ground herbivore attack (Huber et al. 2016a,b). Differences in the genotype composition, herbivore identity, and feeding intensity may account for the divergent responses.

CONCLUSION AND SPECULATION

Asexual (or highly inbreeding) species like dandelion have been the focus of several experimental studies examining the evolution of plant chemical defense, including glucosinolates, diterpenes, and ellagitannins (Agrawal et al. 2012; Bode and Kessler 2012; Züst et al. 2012). These systems have the empirical advantage of being able to track the frequencies of genotypes, but also the

limitation of reduced trait mixing and the potential for overestimating the evolutionary impact of herbivores. In studies of local adaptation, and especially systems where the genes of interest are known, evolution can be studied in outcrossing species, but these are still few and far between (Savolainen et al. 2013). The rapidity of adaptation may be weaker in such outcrossing systems. Interestingly, we have recently shown that even for highly clonal (through seed) species like evening primrose (*O. biennis*), fitness can be enhanced through rare outcrossing events in the face of herbivores (Maron et al. 2018). Thus, an important avenue for further work is unraveling the different evolutionary trajectories imposed by herbivores across the continuum of plant mating systems.

In conclusion, our study demonstrated differentiation of common dandelion genotypes and chemical defense phenotypes in field plots over a decade of insect suppression. This differentiation occurred in the face of continued colonization of our plots from the larger population, likely in each year. The relative abundance of specific dandelion genotypes, genetic structure of the plots, and genetic diversity were all impacted by insect suppression, leading to altered defensive chemistry phenotypes. As early succession proceeded and the effects of herbivores subsided, evolutionary change persisted and differences in plant defense chemistry were maintained. Differentiation among plots in genetic structure persisted, but whether there were additional phenotypic consequences is unclear.

We speculate that as intraspecific competition dominated, followed by the vegetational community moving into the next phase of dominance by tall and dense forbs such as goldenrod, the selection regime changed as did the evolutionary response in the plants. The extent to which evolutionary trajectories in communities change throughout succession is an unresolved question. Furthermore, as such early successional populations may be resurrected decades later by disturbance, the legacy of past demographic and evolutionary change in populations may or may not shape the population biology of plants in the next cycle.

AUTHOR CONTRIBUTIONS

AAA and APH conceived the project. APH led all field and laboratory research. AAA led the statistical analyses and writing the of manuscript. MH led chemical analyses. DMF contributed to genotyping and field work. SB contributed to genotyping. All authors contributed to revising the manuscript.

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DATA ARCHIVING

Raw data are provided in Supporting Information and microsatellites have been archived in GenBank (see Table S1).

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's website:

Appendix 1. Supporting methods: Development of microsatellite markers.
Figure S1. Peak area ratio (PAR, see Esselink et al. 2004) distributions from the 3 microsatellite loci used for 2011 analysis.
Figure S2. PAR distributions from the 3 microsatellite loci used for 2014 analysis.
Figure S3. PAR histograms for microsatellite locus "tri12" – excluded from final analysis due to irregularity in allele frequencies.
Figure S4. Genotype means for concentrations of three foliar defense compounds of dandelion.
Table S1. Microsatellite markers used for genotype determination in *T. officinale*.
Table S2. A list of 5638 potential microsatelite markers and their corresponding primers obtained from *T. officinale* DNA extracted from an individual at our field site.
Table S3. Frequencies of the 15 most abundant dandelion genotypes given for 2011 and 2014 for each experimental plot. Two additional tabs on this file provide the full raw data for abundance and frequency of all genotypes in each year.
Table S4. Raw data for chemistry of dandelions for all samples.
Table S5. Statistical tests for genetic differentiation between control and insect suppression plots in plant chemistry (TA-G, di-PIEs, and tri-PIEs).