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Experimental insect suppression causes loss of induced, but not constitutive, resistance in *Solanum carolinense*

Tyler C. Coverdale[1,2] 🆔 | Anurag A. Agrawal[1,3] 🆔

[1]Department of Ecology and Evolutionary Biology, Cornell University, Ithaca, New York, USA

[2]Department of Organismic and Evolutionary Biology, Harvard University, Cambridge, Massachusetts, USA

[3]Department of Entomology, Cornell University, Ithaca, New York, USA

Correspondence
Tyler C. Coverdale
Email: tc684@cornell.edu

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Abstract

Spatiotemporal variation in herbivory is a major driver of intraspecific variation in plant defense. Comparatively little is known, however, about how changes in herbivory regime affect the balance of constitutive and induced resistance, which are often considered alternative defensive strategies. Here, we investigated how nearly a decade of insect herbivore suppression affected constitutive and induced resistance in horsenettle (*Solanum carolinense*), a widespread herbaceous perennial. We allowed replicated horsenettle populations to respond to the presence or absence of herbivores by applying insecticide to all plants in half of 16 field plots. Horsenettle density rapidly increased in response to insecticide treatment, and this effect persisted for at least 4 years after the cessation of herbivore suppression. We subsequently grew half-sibling families from seeds collected during and shortly after insecticide treatment in a common garden and found strong effects of insect suppression on induced resistance. Feeding trials in field mesocosms with false Colorado potato beetles (*Leptinotarsa juncta*), a common specialist herbivore, revealed that multiyear herbivore suppression drove rapid attenuation of induced resistance: offspring of plants from insect-suppression plots exhibited a near-complete loss of induced resistance to beetles, whereas those from control plots incurred ~70% less damage after experimental induction. Plants from insect-suppression plots also had ~40% greater constitutive resistance compared with those from control plots, although this difference was not statistically significant. We nonetheless detected a strong trade-off between constitutive and induced resistance across families. In contrast, the constitutive expression of trypsin inhibitors (TI), an important chemical defense trait in horsenettle, was reduced by 20% in the offspring of plants from insect-suppression plots relative to those from control plots. However, TIs were induced to an equal extent whether or not insect herbivores had been historically suppressed. Although several defense and performance traits (prickle density, TI concentration, resistance against false Colorado potato beetles and flea beetles, biomass, and seed mass) varied markedly across families, no traits exhibited significant pairwise correlations. Overall, our results indicate that, whereas the divergent responses of multiple defense traits to insect

suppression led to comparatively small changes in overall constitutive resistance, they significantly reduced induced resistance against false Colorado potato beetle.

KEYWORDS

chemical ecology, community ecology, herbivory, jasmonic acid, plant defenses, Solanaceae

INTRODUCTION

Plants have evolved a staggering diversity of defenses against herbivores, and efforts to understand and characterize this diversity have been a cornerstone of ecological and evolutionary research for decades (Burkepile & Parker, 2017; Stamp, 2003). Despite the diversity of both plant defenses and consumers globally, several broadly consistent patterns have emerged from this work that suggest general principles associated with costs, benefits, and trade-offs of different defense strategies (Agrawal, 2020; Agrawal & Hastings, 2019; Moreira et al., 2014). For example, assuming that defenses are both costly to produce and beneficial in the presence of herbivores, spatiotemporal variation in natural selection arising from heterogeneous herbivory should be a major driver of variation in defense (Agrawal et al., 2018). In support of this notion, investigations spanning macroevolutionary (Becerra et al., 2009; Charles-Dominique et al., 2016) and microevolutionary (Bode & Kessler, 2012; Kalske & Kessler, 2020) timescales have repeatedly found tight correspondence between plant investment in resistance traits and the intensity of herbivory. The same is true regarding spatial variation in herbivory across landscapes (Coverdale et al., 2018, 2019).

Even over relatively short timescales, spatiotemporal variation in herbivory is common (Karban, 2011; Karban & Baldwin, 1997) and can affect plant defense phenotype in at least two ways. First, variation in herbivory over successive generations can select for genotypes with greater or lesser constitutive investment in defenses. For example, insect suppression led to a rapid reduction in the constitutive expression of chemical deterrents in evening primrose (*Oenothera biennis*; Agrawal et al., 2012). Similarly, natural variation in the density of specialist herbivores led to parallel patterns in polymorphic defense traits in *Arabidopsis thaliana*, with the genotypic composition of regions with greater herbivore abundance having higher representation of well defended genotypes (Züst et al., 2012). Even in the absence of classic evolutionary mechanisms, herbivory can cause shifts in defense phenotype over time through selective filtering (i.e., mortality) of genotypes that vary in defense traits. Second, variation in herbivory within a single generation can affect defense through phenotypic plasticity, specifically induced

resistance (Karban & Myers, 1989). Simulated browsing on herbaceous understory plants in a Kenyan savanna, for example, caused a 16%–38% increase in defensive spine density within 1 month (Coverdale et al., 2018).

Induced plant responses are widely considered to be a cost-saving alternative to the constitutive expression of the same traits (Agrawal, 2007; Kalske & Kessler, 2020). From an ecological perspective, constitutive and induced resistance have long been considered as opposing ends of a spectrum of plant defense strategies (Agrawal, 2007). The utility of this view is underscored by the fact that across diverse plant lineages (Agrawal & Hastings, 2019; Kalske & Kessler, 2020; Moreira et al., 2014), constitutive and induced resistance often trade-off within species such that individuals with high constitutive resistance typically have low inducibility (and vice versa). Despite the existence of a substantial theoretical literature concerning the conditions under which constitutive and induced resistance should arise (Adler & Karban, 1994; Agrawal, 2005, 2007; Agrawal & Karban, 1999; Hahn & Maron, 2016; Heil, 2002; Karban, 2011; Karban & Myers, 1989; Moreira et al., 2014; Strauss et al., 1999), few studies have addressed the potentially complex responses of constitutive and induced resistance to variation in herbivory. Verbal models of plant defense strategy (e.g., Agrawal & Karban, 1999; Karban & Baldwin, 1997) provide predictions for how constitutive and induced resistance should respond to short-term changes in herbivory pressure, although there remains significant uncertainty concerning the effect of herbivore suppression on the balance of resistance types and the mechanisms by which changes in resistance might occur. For example, given that constitutive resistance is considered more costly than induced resistance, theory predicts that herbivore suppression should cause a reduction in the constitutive expression of defensive traits to reduce energetic costs. As a result, if a genetic trade-off exists between constitutive and induced resistance, a reduction in constitutive resistance may be accompanied by a concordant increase in induced resistance. Over time, however, herbivore suppression may further drive reductions in both constitutive and induced resistance as evolutionary mechanisms act to overcome these trade-offs, thereby reducing the magnitude of both realized (i.e., constitutive) and potential (i.e., induced) defense costs.

Conversely, if herbivore suppression drives shifts in resistance strategy primarily by reducing the variability or increasing the predictability of herbivory, induced resistance may decrease before changes in constitutive resistance are observed. The only studies that we are aware of that explicitly investigated shifts in constitutive and induced resistance find mixed responses to variation in herbivory regime (e.g., Coverdale et al., 2018; Kalske & Kessler, 2020; Uesugi & Kessler, 2016). Accordingly, it is currently unclear whether the effects of herbivore declines on plant defenses are caused by changes in the *magnitude* or *predictability* of herbivore damage. Given the ubiquity of shifts in mammalian and insect herbivore regimes globally (Ripple et al., 2015; Wagner et al., 2021; Welti et al., 2020) and the multitude of interacting biotic and abiotic factors that also influence defense strategy (e.g., ecological costs of defense, co-occurring abiotic stressors, physiological constraints on defense production), additional studies are needed to characterize rapid responses to changing herbivory pressure and disentangle the role of multiple ecological and evolutionary mechanisms.

Here, we used a replicated, 9-year insect-suppression experiment to assess whether and how the absence of herbivores affects defense traits in horsenettle (*Solanum carolinense*), a common agricultural weed native to the eastern USA with a global invasive range (Follak & Strauss, 2010). Using half-sibling families from insect-suppression and control plots, we examined how insect suppression affected the balance of constitutive and induced resistance against two specialist insect herbivores, the false Colorado potato beetle (*Leptinotarsa juncta*) and flea beetles (*Epitrix* sp.). We also quantified the constitutive and induced expression of two primary physical and chemical defense traits (prickle density and trypsin inhibitor [TI] concentration) to determine whether observed patterns of overall resistance to specialist herbivores were reflected in the responses of individual traits that confer resistance. Within this context, our study had two main conceptual goals: to evaluate (1) the extent to which intraspecific variation in defense strategy can be explained by spatiotemporal variability in herbivory, and (2) how trade-offs among defensive traits constrain resistance against herbivores.

METHODS

Study species and insect-suppression experiment

Horsenettle is a perennial, herbaceous, primarily outcrossing plant native to the southeastern USA (Kariyat et al., 2013). Horsenettle exhibits rapid vegetative reproduction via lateral roots, with sexual reproduction typically restricted to disturbed habitats due to poor competitive ability in seedlings (Wise, 2007a). The stems and leaves of horsenettle are defended by recurved prickles (sharp epidermal outgrowths) and stellate trichomes, and all aboveground parts contain toxic secondary compounds typical of the Solanaceae (Cipollini & Levey, 1997; Kariyat et al., 2013; Walls et al., 2005). In the eastern USA, horsenettle is a common weed in agricultural settings and is consumed by several insects that specialize on species in the family Solanaceae, including Colorado potato beetle (*Leptinotarsa decemlineata*), false Colorado potato beetle (*L. juncta*), and several species of flea beetle (*Epitrix* species; Wise, 2007a). Due to its insensitivity to mechanical and chemical control and proclivity for asexual reproduction, horsenettle is difficult to eradicate once established. Globally, horsenettle causes significant economic losses as a result of competition with crops, degradation of pastures, toxicity to livestock, and spillover of pests and disease to congeneric crops (e.g., potato, *S. tuberosum*; tomato, *S. lycopersicum*; eggplant, *S. melongena*; Follak & Strauss, 2010).

In 2007, we established 16 experimental plots near Ithaca, NY (42.43° N, 76.38° W) to investigate the effect of insect suppression on plant defense strategy (Agrawal et al., 2012). The experiment was initially designed to assess rapid evolutionary responses in the monocarpic forb common evening primrose (*Oenothera biennis*; Agrawal et al., 2012, 2018), but is used here to investigate the effects of insect suppression on defense traits in horsenettle (a perennial). The study site is underlain by rocky, glacial lake bottom sediments and is adjacent to agricultural fields where potatoes and other crops have been grown consistently for more than a decade. Dominant vegetation at the site includes horsenettle, tall goldenrod (*Solidago altissima*), dandelion (*Taraxacum officinale*), and several non-native grasses. In June 2006 (prior to the initiation of the experiment), each 13.5 m² square plot was sprayed with glyphosate herbicide (Roundup, Monsanto, St. Louis, MO) and tilled 2 weeks later. Plots were sprayed with herbicide again in August 2006 and May 2007. Initial tilling and herbicide treatment completely eliminated aboveground plant biomass and plots were not weeded after the initiation of the experiment. Tilling and repeated herbicide application effectively removed aboveground vegetation prior to the onset of the experiment, but by the end of the growing season in 2007 all plots were densely vegetated. We assume that horsenettle, which was among the species that rapidly recolonized the recently cleared plots, did so by a combination of resprouting root fragments and germinating seeds. All plots were fenced with flexible plastic mesh ~1.5 m high to prevent herbivory by deer

(*Odocoileus virginianus*) and were surrounded by a 1 m (horizontal) border of impermeable gardening cloth pinned at ground level to prevent vegetation from growing immediately adjacent to the fences. All plots were at least 10 m from one another.

Half of the plots (*N* = 8 plots) were randomly assigned to an insect-suppression treatment that involved spraying all plants in the plot with esfenvalerate fortnightly during the growing season (April–October) every year from 2007 to 2015 (2007–2009: Bug-B-Gon, Ortho, EPA Reg. No. 1021-1645-239; 2010–2015: Asana XL, Dupont, EPA Reg. No. 352-515). Esfenvalerate is a non-systemic broad-spectrum insecticide that does not affect plant growth or performance traits (Agrawal et al., 2018). Insect-suppression plots were sprayed at a rate of 7.63 ml insecticide per liter of water. Control plots were watered on the same schedule without insecticide.

To evaluate the effects of insect herbivore suppression on horsenettle abundance, we censused all experimental plots during the growing season in 2011. We estimated the abundance of horsenettle in each plot by counting the total number of individual stems. We note that a single horsenettle genet can produce multiple ramets, however, so our estimate of abundance reflects ramet (not genet) density. We estimated horsenettle ramet density in each plot again in 2017 and 2020 to evaluate the legacy of insect suppression 2 and 5 years after the cessation of insecticide treatment.

In July 2020, we surveyed eight horsenettle ramets in each plot to quantify variation in performance and defense traits. For four plots with fewer than eight horsenettle ramets, we surveyed all ramets in the plot (*N* = 2, 5, 7, and 7 ramets). One plot did not have any horsenettle in 2020. We noted whether each ramet was reproductive (i.e., was flowering or had unopened flower buds) and measured the height from the base to the tip of the tallest branch, the total number of leaves, and the average number of prickles across three fully expanded, haphazardly selected leaves from the main stem. We also surveyed the proportion of leaves on each ramet with flea beetle and false Colorado potato beetle damage. These two insects were the most abundant horsenettle herbivores at our study site and leave distinct browsing scars that are readily distinguishable: flea beetles aggregate in large numbers on horsenettle and chew numerous small (~1–3 mm) holes through the interior of the leaf surface, whereas false Colorado potato beetles chew the leaf margin and typically remove only one or two larger sections of leaf (Appendix S1: Figure S1).

We evaluated changes in horsenettle abundance (number of ramets/plot) over time with repeated measures analysis of variance (ANOVA) using the *aov*

function in R (v. 4.0.3; R Core Development Team, 2015). Data from immediately after tilling in 2007 (when horsenettle and other species had yet to recolonize cleared plots) are included in Figure 1 for illustrative purposes but are excluded from the analysis. Data on individual horsenettle traits (height, number of leaves, number of prickles, flea beetle damage, false Colorado potato beetle damage, and proportion of ramets with flowers or buds) were analyzed with separate one-factor ANOVA, with insecticide treatment (insect suppression vs. control) as the factor. Data from individual ramets were averaged within plots prior to all analyses; unless otherwise noted, all subsequent comparisons were performed using the *lm* function in R.

Induction experiment

At the end of the growing season in 2011 (during insecticide treatment) and 2017 (2 years after insecticide treatment stopped), we collected fruits from 1–5 horsenettle ramets in each plot. Each fruit contained >40 seeds, which we subsequently used to grow replicated families. We from this point forwards refer to these as "half-sibling families," although we note that it is possible that some fraction of seeds from a single fruit could be full siblings (i.e., pollen from a single genet could have fertilized multiple seeds per fruit). Although up to five fruits were collected from individual ramets, we only grew half-sibling families from one fruit per ramet. While it is possible that some of the half-sibling families grown from seeds collected in 2011 and 2017 had the same maternal genotype, we consider this unlikely given that we grew at most two
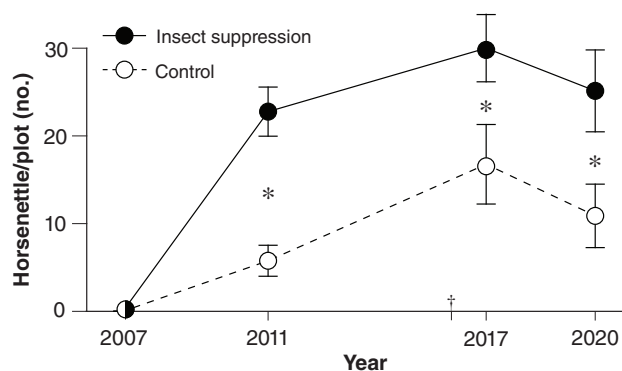
FIGURE 1 Abundance of horsenettle (*Solanum carolinense*) in tilled plots sprayed with insecticide (black dots) and unmanipulated control plots (white dots; *N* = 8 plots/treatment). Insecticide was applied fortnightly during the growing season from 2007 to 2015. †Cessation of insecticide treatment (2016). *Significant pairwise difference between treatments within years. Data are means ± SEM.

half-sibling families from a single plot at each time point, and each plot contained a large number of horsenettle ramets during this phase of the experiment (2011: mean = 14.3 ramets/plot, range 0–34 ramets/plot; 2017: mean = 23.4 ramets/plot, range 3–42 ramets/plot); marked differences in both defensive and non-defensive traits between half-sibling families grown from the same plots further supported our assumption that even physically proximal parent plants were likely to be different genets. In 2011, we also collected seeds from 10 ramets from the non-manipulated habitat between plots to increase replication for family-level comparisons of trait trade-offs (please refer to *Chemical defense assays* and *Performance and physical defense assay*; Morris et al., 2006). Because each of these ramets were at least 10 m apart, we similarly considered it likely that they represented unique genets.

In June 2020, we germinated 10–24 seeds from each of 44 half-sibling families (N = 904 total plants). Of the 44 families, 27 were collected in 2011 (10 families from insect-suppression plots, seven families from control plots, and 10 families from the unmanipulated habitat between plots), and 17 were collected in 2017 (10 families from insect-suppression plots and seven families from control plots). Seeds were initially sown ~5 mm beneath the surface of 72-cell seedling trays in a mixture of Lamberts 111 (75%; Lambert, Quebec, Canada) and perlite (25%). Seedlings were watered *ad libitum* with a dilute fertilizer mix for the duration of the experiment and transferred to 0.5 L pots after 5 weeks. Plants were randomized within a single greenhouse and grown without supplemental light with a constant daytime temperature of 27°C and nighttime temperature of 22°C. Approximately 7 weeks after the initiation of the experiment, half of the plants from each family were randomly assigned to a jasmonic acid (JA) treatment to trigger defense induction (from this point forwards "induced" plants). Jasmonic acid (84 mg) was dissolved in acetone (3 ml) and added to 747 ml of deionized water for a final concentration of 0.53 mM (following Thaler et al., 2014). Each plant was removed from the greenhouse and sprayed with ~3 ml of JA solution using an atomizer. The remaining (from this point forwards "control" plants) were sprayed with an identical acetone–water mixture without JA. The leaf immediately below the terminal bud was marked with a wire loop on all plants immediately after spraying to differentiate between leaves developed before and after manipulation. Plants were returned to the greenhouse and grown under identical conditions until they were randomly assigned to three separate assays of plant performance and defense (please refer to *Chemical defense assays*, *Performance and physical defense assay*, and *Beetle bioassays*).

Chemical defense assays

At 6 days after JA application, we collected all (i.e., two or three leaves/plant) newly developed leaves from three induced and three control plants per family; for three families with low germination rates, we collected leaves from two plants per treatment per family. After removing stems and midribs, we pooled, freeze dried, and ground all tissue to a fine powder. We then quantified TI concentration using a radial diffusion assay (modified from Jongsma et al., 1993, 1994; please refer to Appendix S1 for detailed methods). We report the average TI concentration of each family as μg TI/mg of freeze-dried leaf tissue (Cipollini et al., 2002; Cipollini & Bergelson, 2000; Kessler et al., 2006).

We assessed the independent and interactive effects of insect suppression and defense induction (JA vs. control) on TI concentrations with a two-factor ANOVA; replicate samples from each family (N = 34 families) were pooled prior to analysis and families from between experimental plots were excluded from this analysis.

Performance and physical defense assay

Approximately 2 weeks after JA application, we selected an additional 2–5 plants per treatment per family to quantify aboveground biomass and prickle density. Five families with <2 replicates per treatment were excluded from this assay (N = 38 families). For each plant, we measured the total number of prickles on the midrib and petiole of the youngest fully expanded leaf, all of which emerged after JA application. We then harvested all aboveground biomass, dried tissue to a constant mass at 60°C, and weighed each sample. We assessed the independent and interactive effects of insect suppression and defense induction on prickle density and aboveground biomass with separate two-factor ANOVA.

Beetle bioassays

At 9 days after JA application, we selected four plants per family per treatment to assess horsenettle resistance to a specialist insect herbivore (false Colorado potato beetle). Previous work (e.g., Thaler et al., 1996, 2014) has shown that experimental induction with comparable concentrations of JA causes significant differences in individual defense traits and resistance to herbivores for at least 14 days after application. Although it is likely that peak defense induction occurs more quickly, we elected to allow for a longer period between JA application and feeding assays to allow for potential changes in physical defense investment. For this assay, we included 30 families from

experimental plots (insect-suppression plots: 18 families; control plots: 12 families) and 10 families from between plots. From this point forwards, we differentiate between "resistance" (the vulnerability of plants to herbivores) and "defense traits" (the individual components of plant defense that, collectively, confer resistance). Plants were randomly assigned to one of four mesocosms at a local field site, each of which measured 2 m × 3 m × 2 m (W × L × H; Appendix S1: Figure S2) and was constructed of fine (~1.5 mm) mesh. Each mesocosm contained one induced and one control plant per family ($N = 80$ plants/mesocosm) and the location of each plant within each mesocosm was haphazardly assigned prior to the initiation of the bioassay. Plants were allowed to acclimate to field conditions for 36 h, at which time 16 adult false Colorado potato beetles collected nearby were released into each mesocosm and allowed to disperse freely for the duration of the experiment. Two additional beetles were released into each mesocosm on the second day of the experiment. Plants were watered *ad libitum* for the duration of the trial. After 11 days, we removed and photographed all leaves with signs of beetle herbivory and measured the total area of leaf tissue consumed by beetles during the trial for each plant using LeafByte (Getman-Pickering et al., 2020).

We assessed the independent and interactive effects of insect suppression and defense induction on resistance against false Colorado potato beetles with a two-factor ANOVA. For simplicity—and because scaling the total area of leaf tissue consumed by family-level average biomass did not qualitatively affect the results—we elected to compare the absolute area of tissue consumed. Data were pooled at the level of half-sibling families prior to analysis and square-root transformed to meet assumptions of normality; only plants from insect-suppression and control plots were included in this analysis (Figure 2). We initially included collection year (2011 vs. 2017) but, because the effect of collection year was not significant in this or any other analysis, we removed it from all models for simplicity. Five (out of 240) plants were severely wilted at the conclusion of the experiment and were excluded from the analysis because we were not able to estimate leaf area loss. To test whether a genetic trade-off exists between constitutive and induced resistance in horsenettle, we compared resistance in the control treatment to the magnitude of induction (resistance in induced plants minus resistance in control plants) at the level of half-sibling families. For this comparison, we included all 40 half-sibling families (Figure 3). Data were analyzed with a permutation test in MATLAB following the modified Monte Carlo procedure developed by Morris et al. (2006) to account for spurious negative correlation between constitutive and induced resistance.

In a separate bioassay, we germinated a second set of horsenettle plants from the original insect-suppression experiment that comprised a subset of the families germinated in the first trial: 21 families collected in 2011 (four from control plots, seven from insect-suppression plots, and 10 from the unmanipulated habitat between plots) and seven families collected in 2017 (four from control plots and three from insect-suppression plots). Plants were started in the greenhouse as in the first induction experiment before being transplanted directly from 72-cell seedling trays to the field site. Prior to planting, the field site was sprayed with a broad-spectrum herbicide, mowed, and tilled to remove all aboveground plant biomass. Approximately 1 week after transplant, half of the replicates from each family ($N = 4$ plants/family/treatment) were sprayed with JA following the same protocol as above. To prevent overspray, plants were enclosed in an impermeable box prior to spraying and for 60 s afterward. After 1 month, we photographed each plant and estimated resistance to flea beetles by counting the total number of holes caused by flea beetle damage. Data were analyzed using a two-factor ANOVA, with historical insect suppression and induction treatments as factors.

Family-level trait correlations

Finally, to test for correlations among performance and defense traits across families, we compiled five traits from the experiments and bioassays described above: (1) aboveground biomass, (2) TI concentration, (3) prickle density, (4) resistance to false Colorado potato beetles, and (5) resistance to flea beetles (*Epitrix* sp.). We also measured (6) the mass of 10 haphazardly selected seeds from each half-sibling family collected from the same fruit as those used in performance and defense assays.

In all cases, trait values were averaged at the level of half-sibling families. Data from control and experimentally induced plants were compared separately using Pearson's *r* (using the function *rcorr.adjust*); for the comparison of induced plants, we did not include seed mass because all seeds were collected from unmanipulated field-grown plants in 2011 and 2017. Here, 10 families from between experimental plots were included in all comparisons. We corrected all pairwise *p*-values using the Holm–Bonferroni method to account for spurious significance for multiple comparisons (Appendix S1: Figure S3). Although we consider it unlikely that plants collected from the same plot (whether within or across years) are from the same genet, we note that the results of all analyses described here and in the preceding three sections) are qualitatively similar whether or not we treated each half-sibling family as a unique genet or pool

all data (including across years) at the level of experimental plot (i.e., assuming that each plot contained only a single genet, and that this genet was the same in 2011 and 2017).

RESULTS

Long-term insect suppression

Horsenettle recolonized all experimentally cleared plots (Figure 1) and was more than four-fold more abundant in insect-suppression plots compared with control plots after 4 years of insecticide treatment. This effect persisted for at least 5 years after the cessation of insecticide application, at which point horsenettle remained more than twice as abundant in insect-suppression plots (treatment × time interaction: $F_{1,14} = 3.24$, $p = 0.03$). Horsenettle plants in insect-suppression plots were also nearly twice as likely to be reproductive when censused in July 2020 (insect suppression = $37.7 \pm 4.6\%$; control = $19.6 \pm 6.0\%$; $F_{1,14} = 5.79$, $p = 0.03$), although they did not differ from horsenettle in control plots in any other trait measured (height, number of leaves, number of prickles, proportion of leaves with flea beetle damage, or proportion of leaves with false Colorado potato beetle damage; all $F_{1,14} < 1.56$, $p > 0.23$).

Induction experiment

Chemical defense assays

Insect suppression and defense induction had opposing effects on TI concentration across families (Figure 4): horsenettle families from control plots had, on average, ~30% more TI/mg of leaf tissue compared with families from control plots ($F_{1,64} = 13.99$, $p = 0.0004$), whereas treatment with JA increased TI concentration by >50% relative to control plants ($F_{1,64} = 55.13$, $p < 0.0001$). Notably, defense induction had a comparable effect on the TI concentration of plants from insect-suppression and control plots (insect suppression × defense induction interaction: $F_{1,64} = 0.003$, $p = 0.95$). In other words, historic insect-suppression treatment was associated with constitutively reduced plant TIs, but this response did not impact the magnitude of inducibility.

Performance and physical defense assay

Neither insect suppression nor defense induction affected plant biomass or prickle density (all $F_{1,56} < 3.43$,

$p > 0.05$) in greenhouse-grown plants. Nonetheless, the number of prickles per leaf was greatest in plant families from control plots (average constitutive prickle density $\pm$ SE = 17.4 ± 0.7), which had, on average, ~10% more prickles compared with families from insect-suppression plots (average constitutive prickle density $\pm$ SE = 15.9 ± 0.4), although this effect was not statistically significant ($F_{1,56} = 3.44$, $p = 0.069$). Differences in prickle density across insect suppression treatments were therefore comparable in direction and magnitude with those observed for TI concentration.
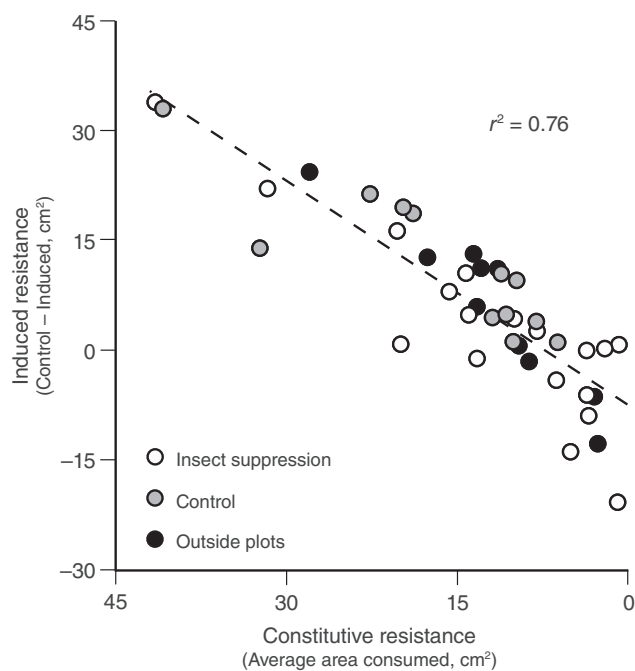
Beetle bioassays

Historic insect suppression did not affect the area of leaf tissue consumed by false Colorado potato beetles in the offspring of plants from the insect-suppression experiment (insect suppression effect: $F_{1,56} = 0.01$, $p = 0.90$; Figure 2). By contrast, defense induction by JA reduced beetle feeding damage by nearly 50% overall (defense induction effect: $F_{1,56} = 7.95$, $p = 0.007$), and this effect varied substantially as a function of historic insect suppression treatments (defense induction × insect
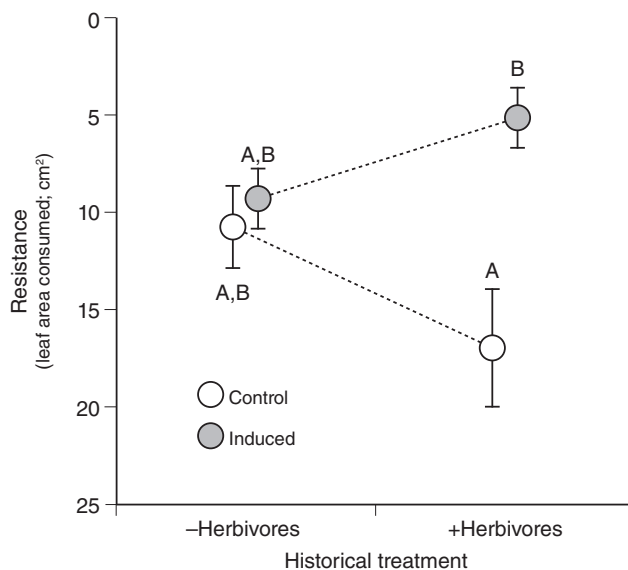
FIGURE 2 Effects of insect suppression and experimental defense induction on horsenettle resistance to false Colorado potato beetles (*Leptinotarsa juncta*). Note that, because resistance was measured by quantifying the area of leaf tissue consumed per plant, the *y*-axis is reversed (i.e., larger areas consumed [bottom] correspond to lower resistance). Please refer to Appendix S1: Figure S3 for representative photographs of beetle herbivory damage. Data are means $\pm$ SEM; letters denote pairwise differences (Tukey's HSD test) in post hoc comparisons. Note that half-sibling families from between the experimental plots are not included in this analysis.

suppression effect: $F_{1,56} = 7.96$, $p = 0.007$; Figure 2). Specifically, induction decreased beetle feeding damage by ~70% in plants from control plots, but had a negligible (<15%) effect on herbivore damage in plants from insect-suppression plots. Although there was a large - difference in constitutive resistance between insect-suppression and control plots, with the former having ~40% greater constitutive resistance against beetles, this pairwise difference was not statistically significant (Tukey's honestly significant difference (HSD): $p = 0.17$; compare white points in Figure 2). There was a strong trade-off between constitutive and induced resistance across families, with plants investing more in constitutive defenses producing more muted induced responses to JA compared with families with lower constitutive resistance (after correction for spurious correlations: observed $r = -0.87$, lower 5th percentile $r = -0.49$, $p < 0.0001$; Figure 3). By contrast, neither historical insect suppression treatment ($F_{1,56} = 0.21$, $p = 0.65$)

nor experimental induction ($F_{1,56} = 0.06$, $p = 0.81$) affected resistance against flea beetles.

Family-level trait correlations

There was marked variation among families across all traits, with several traits exhibiting moderately strong pairwise correlations (|Pearson's correlation coefficients| > 0.25). Notably, the correlation between control prickle density and resistance to false Colorado potato beetles was strong (but not statistically significant following correction; Appendix S1: Figure S3), with plants that produced more prickles incurring less damage during feeding trials.

DISCUSSION

Understanding how variation in defense phenotype arises is a central goal in the study of plant–herbivore interactions (Burkepile & Parker, 2017; Stamp, 2003). In recent years, several studies have investigated how marked changes in herbivory regime affect intraspecific patterns of plant defense (e.g., Agrawal et al., 2012, 2018; Bode & Kessler, 2012; Coverdale et al., 2018; Hahn & Maron, 2016). Consistent with the notion that herbivores can drive rapid shifts in plant defense strategy, these studies showed that herbivore suppression affects constitutive defense phenotype through both ecological and evolutionary mechanisms, and that heterogeneity in herbivore abundance and activity is a major contributor

FIGURE 3 Trade-off between constitutive and induced resistance across 40 half-sibling families of horsenettle (*S. carolinense*). Note that, because resistance was measured by quantifying the area of leaf tissue consumed per plant, the *x*-axis is reversed (i.e., larger areas consumed [left] correspond to less defended plants). Induced resistance is reported as the difference between control and induced plants; larger numbers (top) correspond to greater induced resistance. Negative values of induced resistance are indicative of induced susceptibility. Data are family-level means and were analyzed following Morris et al. (2006) to account for spurious negative correlations between induced and constitutive defenses.
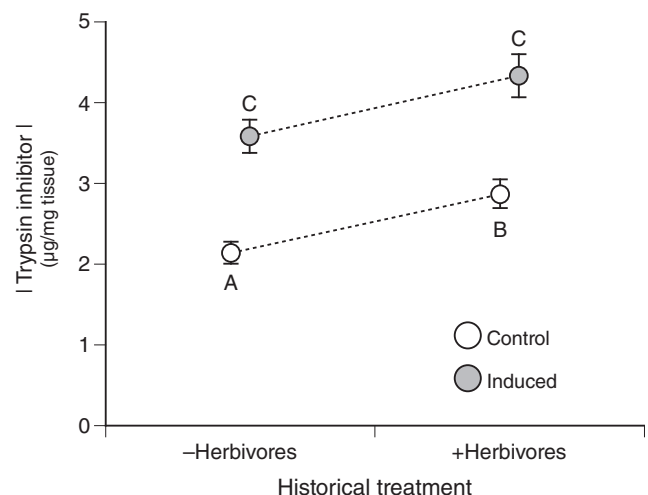
FIGURE 4 Effects of insect suppression and experimental defense induction (jasmonic acid) on trypsin inhibitor (TI) concentration. Data are means ± SEM; letters denote pairwise differences (Tukey's HSD test) in post hoc comparisons.

to the standing variation in defense phenotype observed within populations (Poelman & Kessler, 2016). Although the contributions of herbivores to intraspecific variation in defense investment are becoming increasingly clear, how changes in constitutive defense expression interact with phenotypic plasticity (i.e., induced resistance), another major contributor to individual variation in defense phenotype, is less understood. We sought to address this gap by investigating the effects of multiyear insect suppression on constitutive and induced resistance, and found that herbivore suppression can significantly reduce phenotypic plasticity and drive divergent patterns of herbivore resistance across populations.

Previous research on dandelion and evening primrose conducted in the same experimental plots suggests that insecticide reduced overall phytophagous insect damage by 50%–90% across a diverse suite of herbivores (Agrawal et al., 2012, 2018). Our results similarly showed that insects exert a strong top-down control on horsenettle: the prevalence of insect damage was consistently high across all plots in 2020 (after the cessation of insecticide treatment), and experimental insect suppression led to rapid and lasting increases in horsenettle density that persisted for at least 4 years after insecticide application ceased (c.f. Kim et al., 2015). Although it is possible that these differences were driven entirely by top-down control by insect herbivores, it is also possible that the composition or density of neighboring plant communities initially diverged in response to insect suppression, and that this may have indirectly contributed to changes in horsenettle abundance by decreasing the strength of interspecific competition. This initial divergence could have been further amplified by differences in the rate of asexual reproduction by horsenettle of different sizes or as a function of differences in the intensity of competition. By 2014, tall goldenrod (Solidago altissima) was dominant in all plots, suggesting that long-term differences in horsenettle abundance were most likely to be driven, at least in part, by changes occurring early in the experiment (Agrawal et al., 2018). Regardless of the exact mechanism by which insect suppression altered horsenettle densities, the persistently lower density of horsenettle in control plots provides strong evidence that insects have the potential to drive rapid shifts in defense traits through top-down effects on horsenettle.

Antiherbivore defenses in horsenettle comprise a diverse suite of physical traits (e.g., prickles and trichomes) and chemical traits (e.g., glycoalkaloids, protease inhibitors, polyphenols) that collectively confer resistance against the >30 species of insect herbivores in its native range (Wise, 2007a). Although less is known about the impact of vertebrate herbivores, numerous bird and mammal species also consume horsenettle fruits and

seeds (Williams & Ward, 2006). We measured a subset of defense traits on plants grown under different herbivory regimes and found divergent responses to insect suppression. Although physical defenses are widely considered to be primarily deterrent against vertebrate herbivores (Cooper & Owen-Smith, 1986), previous reports have suggested that horsenettle prickles confer some resistance against tobacco hornworms (Manduca sexta) by impeding caterpillar movement along stems and petioles (Kariyat et al., 2017). Although not statistically significant following correction for multiple comparisons, we found that prickle density was negatively correlated with feeding damage by false Colorado potato beetles (Appendix S1: Figure S3). Although there were nearly two-fold differences in prickle density among families in our study, however, we found no effect of insect suppression or experimental induction on prickle density. Notably, previous work has suggested that horsenettle prickles can be induced by insect damage, and that this response is variable across genotypes and assumed to be mediated by the jasmonate pathway (Kariyat et al., 2013).

With regard to chemical defense traits, we found that insect suppression substantially reduced the constitutive expression of TIs but had no effect on their inducibility: experimental induction strongly increased TI concentration irrespective of insecticide treatment. We note that because we used JA to induce plants, we may have potentially overestimated inducibility across insect suppression treatments, as there may be differences in endogenous plant production of JA following natural herbivory. Although TI concentration was not correlated with feeding damage by adult false Colorado potato beetles, previous studies have indicated that beetle growth at the larval stage is reduced by TIs in horsenettle (McNutt et al., 2017). Therefore, although TIs are only one aspect of defense in horsenettle, their constitutive expression shifted in response to insect suppression and is likely to have contributed to the observed variation in resistance to false Colorado potato beetles in our feeding trial. In this particular case, the cost of constitutive TI expression in the absence of relevant herbivores appears to be quite high, given that constitutive (but not inducible) TI concentration varied as a function of herbivore suppression treatment (Cipollini et al., 2014; Zavala et al., 2004). For plants with multifaceted defense strategies, herbivores may impact different traits independently, with differential impacts on co-existing herbivore species or life stages (Lankau, 2007). The traits we measured in this study, for example, appear to have responded independently to insect suppression because they did not show correlated responses (and we did not detect genetic correlations between any defense traits). Bode and Kessler (2012) similarly documented compartmentalization of defenses

driven by the differential effects of experimental suppression on rare versus dominant herbivores, suggesting that short-term responses to changes in herbivore regime may commonly be complex.

Because levels of individual defensive traits may not be reflective of either overall impacts on herbivores or costs to the plant, we elected to investigate trade-offs between constitutive and induced resistance with data from our false Colorado potato beetle feeding trial. Consistent with the results of the only other studies of which we are aware that explicitly investigated changes in induced resistance over subdecadal timescales (Coverdale et al., 2018; Kalske & Kessler, 2020), but in opposition to our predictions and those of induced defense theory, we found that insect suppression led to rapid attenuation of induced resistance. Given that levels of constitutive resistance were statistically indistinguishable, our results further suggested that herbivore suppression may act to reduce the energetic costs of plasticity per se (e.g., the maintenance of sensory and defense production machinery; DeWitt et al., 1998). Our results are also consistent with aspects of the "moving target" model of induced defenses (Adler & Karban, 1994), specifically the assumption that the benefits of defense variability are only realized in the presence of herbivores (Karban, 2020). We note, however, that although we did not detect a statistically significant difference between families from insect-suppression and control plots in terms of constitutive resistance, the former experienced ~40% less beetle damage when not treated with JA. This result suggested that, counterintuitively, insect suppression triggers appreciably higher investment in constitutive resistance (compare white dots in Figure 2). These opposing patterns of induced and constitutive resistance are consistent with the significant trade-off we documented between these two types of resistance across a larger pool of half-sibling families (Figure 3; please refer also to McNutt & Underwood, 2016), but are inconsistent with the theoretical prediction that putatively more costly constitutive defenses should be lost before their induced counterparts in the absence of herbivores.

Although our combined experimental approach allowed us to characterize the multifaceted defense *response* of horsenettle to insect suppression, we lack the necessary information to conclusively determine that these effects were the result of a specific (micro)evolutionary *mechanism*. It is possible, for example, that the effects we detected are the result of purely epigenetic mechanisms acting to shift gene expression in the absence of gene frequency changes at the plot level (Sobral et al., 2021); intriguingly, this mechanism is also compatible with each plot being colonized and occupied by only a single horsenettle genet for the duration of the experiment. It is also possible that insect suppression facilitated colonization by novel genotypes from the surrounding habitat with greater constitutive (and lower induced) resistance (Agrawal et al., 2012). However, given the experimental design and duration of the experiment, we consider it most likely that the rapid loss of inducibility in insect suppression plots—and the simultaneous increase in constitutive resistance—is the result of non-random filtering of early colonizing genotypes within the experimental plots, coupled with a strong genetic trade-off between constitutive and induced resistance across families (Agrawal, 2011). Although it is beyond the scope of the present study to elucidate these mechanisms, we nonetheless suggest that several aspects of the experimental design and horsenettle life history are consistent with our conclusion. For example, we consider it likely that the initial horsenettle population within each plot arose through a combination of regrowth from root fragments (following tilling) and seeds (which can remain viable for years in the seed bank; Wise, 2007a). Importantly, the establishment of new ramets coincided with the initiation of insecticide treatment, such that there was a strong selective filter during this crucial life history stage. Insects also exerted strong top-down control on horsenettle density (Figure 1). Initial genetic diversity is also a necessary precondition for non-random filtering of genotypes by herbivores, and we consider the alternative (that each plot contained only a single genet) unlikely given the density of ramets in the plots, the marked differences in defensive and performance traits across families (even within the same plot), and the size of our experimental plots (c. 3.5 m × 3.5 m) relative to the typical (c. 1 m; Kiltz, 1930) and longest (c. 5.5 m; Ilnicki & Fertig, 1962; Wise, 2007b) records we could find of lateral root growth in horsenettle. Horsenettle is also obligately outcrossing, so the presence of viable fruits in all of our plots across multiple years suggests at least moderate genetic diversity within the foraging range of the site's dominant buzz pollinators (Kariyat et al. 2013). These assertions notwithstanding, we suggest that disentangling the mechanisms of rapid defense shifts in plants following herbivore suppression is a fruitful area for future research, particularly given the growing evidence of the ubiquity of these types of responses across systems (Coverdale et al., 2018; Kalske & Kessler, 2020; Uesugi & Kessler, 2016).

Synthesis and conclusions

Although considerable effort has been devoted to understanding the conditions under which inducibility evolves (e.g., Heil et al., 2004; Thaler & Karban, 1997), comparatively little is known about how and why induced

resistance may be lost (Kalske & Kessler, 2020). We suggest that this lack of clarity stems, at least in part, from the possibility of conflicting evolutionary pressures associated with marked reductions in herbivory. For example, given that induced resistance is widely considered to be a cost-reducing strategy for minimizing the costs of tissue loss (Agrawal, 2005, Karban, 2011, but please also refer to Zangerl, 2003), herbivore suppression (or, by extension, herbivore extinction), might be predicted to select first for reduced constitutive resistance if selection acts on the cost of defense. Conversely, if the predictability of herbivory (rather than its energetic cost) is the primary selective force driving changes in defense investment, consistently low herbivory pressure may select for loss of inducibility more rapidly than loss of constitutive resistance. To our knowledge, no study has simultaneously manipulated both the magnitude and predictability of herbivory, although this would be a promising approach to disentangle the effects of variable herbivory regimes on plant defense strategy.

Our results suggest that the loss of inducibility in horsenettle under herbivore suppression was rapid (occurring within 4 years of the onset of the experiment) and persistent (lasting at least 2 years after insect suppression ended), whereas changes in constitutive resistance over the same period were in the opposite direction and comparatively muted. Comparable evidence for directional shifts in constitutive resistance in previous studies is mixed, with both marked decreases (e.g., Coverdale et al., 2018) and increases (e.g., Kalske & Kessler, 2020) in constitutive resistance having been observed under reduced herbivory pressure. The latter case potentially represents a striking example of an evolutionary constraint (a trade-off between constitutive and induced resistance) driving costly and maladaptive investment in constitutive resistance in the absence of herbivores. Resolving how herbivore suppression affects the magnitude and direction of induced and constitutive resistance—and why these responses may be constrained—is a promising area for future research.

The evolution of plant defenses is complex, particularly when discrete components of multifaceted defense strategies respond independently to selection by different herbivores (Agrawal et al., 2018; Bode & Kessler, 2012). Inducible defenses further complicate efforts to understand changes in defense traits over time because studies of ecological communities—where defense induction is typically investigated—often assume that the phenotypes of focal species are fixed or, at the very least, that phenotypic evolution is sufficiently slow that it is unlikely to affect the outcome of ecological interactions (Thompson, 1998; Weber et al., 2017). There is now ample evidence to reject this assumption in the context of plant resistance against herbivores (e.g., Agrawal et al., 2012, 2018; Bode & Kessler, 2012; Coverdale et al., 2018;

Kalske & Kessler, 2020; Turley et al., 2013). For plant defenses, it is also clear that the prevalence of different phenotypes can change rapidly in response to shifts in herbivore regime, and that this variation can result from independent shifts across multiple defense traits, trade-offs among traits, and differences in trait plasticity.

AUTHOR CONTRIBUTIONS

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

ORCID

Tyler C. Coverdale https://orcid.org/0000-0003-0910-9187
Anurag A. Agrawal https://orcid.org/0000-0003-0095-1220

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