Costs of resistance and correlational selection in the multiple-herbivore community of Solanum carolinense

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Although a central assumption of most plant-defense theories is that resistance is costly, fitness costs have proven difficult to detect in the field. One useful, though labor-intensive, method to detect costs is to quantify stabilizing selection acting on resistance in field populations. Here, we report on an experimental field study of Solanum carolinense in which we employed a quadratic phenotypic-selection analysis on 12 types of resistance (defined operationally as one minus the proportion of tissue damaged) involving nine species of herbivores. For seven types of resistance, we found significant stabilizing selection and intermediate optimum levels, indicating the existence of fitness costs. These costs were greatest for resistance against frugivores, moderate for florivores, and lowest for folivores. In addition, significant correlational selection gradients were found for 10 pairs of resistance measures, which we interpret as evidence that the fitness impacts of these pairs of herbivores combined nonadditively. When the herbivores fed on the same type of tissue, their impact was synergistic (greater than additive), and when they fed on different tissues, their impact was antagonistic (less than additive). We suggest this may be a general pattern for correlational selection on resistance to different herbivores.

Plants suffer attacks by a wide range of natural enemies, despite the fact that most plant populations apparently maintain genetic variation for the ability to resist these attacks (Maddox and Root 1987; Karban 1992; Kliebenstein 2014). Thus, a fundamental goal in the field of plant-herbivore evolutionary ecology is to elucidate factors that constrain plants from evolving higher levels of resistance when higher levels would appear to be within reach (Rausher 1996; Pilson 2000). One common explanation is that resistance comes at a cost, and plants are only likely to evolve resistance to the point at which the benefits of reduced damage do not exceed the costs of that resistance (Simms and Rausher 1987; Simms 1992; Bergelson and Purrington 1996; Koricheva 2002). The past few decades have seen extensive work identifying types of resistance costs and devising methods to detect these costs in plants (Rausher 1992b; Vila-Aiub et al. 2011; Cipollini et al. 2014). However, detecting costs of resistance in plant populations has often proved difficult (Bergelson and Purrington 1996; Agrawal 2011). It has been especially challenging to determine costs in a way that is ecologically and evolutionary relevant in natural populations (Cipollini et al. 2014).

One method of detecting costs that is especially relevant to evolutionary ecologists is investigating the pattern of natural selection for resistance in field populations (Agrawal 2011; Cipollini et al. 2014). This method involves the measurement of damage levels (i.e., operational resistance) or candidate resistance traits, estimating plant fitness, and applying regression analysis of fitness on the resistance measurements (Lande and Arnold 1983). When such a selection analysis is run, a negative quadratic regression coefficient for a resistance measure suggests that stabilizing selection is acting on resistance. If stabilizing selection is detected, then it is likely that there is an intermediate optimum level of resistance, above which resistance is too costly and is thus selected against (Simms and Rausher 1987; Rausher 1992b). Although this selection analysis approach can provide powerful evidence of evolutionarily relevant costs of resistance, it is quite labor-intensive (Agrawal 2011). Thus, it has only been employed in a handful of plant-herbivore systems to date (e.g., Rausher and...

Most species of plants are simultaneously or sequentially attacked by multiple species of herbivores, and a plant’s evolution of resistance against them is often not independent (Juenger and Bergelson 1998; Agrawal 1999; Lankau and Strauss 2008). For instance, the evolution of resistance against one herbivore can be slowed by a negative genetic correlation with resistance traits against other herbivores, or by competitive interactions among species of herbivores (Rausher 1992b; Wise 2009; Wise and Rausher 2013; Poelman and Dicke 2014). In addition, the strength or pattern of selection for resistance to one herbivore may depend on the presence of a second herbivore—not because the first herbivore affects the amount of damage by the second herbivore, but because each herbivore affects the plant’s ability to tolerate the damage caused by the other herbivore (Gould 1988; Pilson 1996; Rausher 1996; Stinchcombe and Rausher 2001). On one hand, if damage by a second herbivore increases the fitness impact of damage by the first herbivore, then selection for resistance to the first herbivore will be intensified, which would facilitate the host plant’s evolution of resistance. On the other hand, if damage by a second herbivore lessens the fitness impact of damage by the first herbivore, then selection for resistance to the first herbivore would be weakened, slowing the evolution of resistance. In other words, correlational selection can occur when the fitness impacts of two species of herbivores combine in a nonadditive fashion—that is, when the combined impact of two species is either greater than (synergy) or less than (antagonism) the sum of the fitness impact that the same amount of damage by each herbivore would have if it were feeding alone (Gould 1988).

The same selection analyses used to detect stabilizing selection can be used to detect correlational selection acting on different resistance traits (Brodie et al. 1995; Blows and Brooks 2003). Such approaches have been employed in too few studies for consistent patterns to emerge (Carmona and Fornoni 2013). Therefore, we do not know how frequently nonadditivity of herbivore impacts results in nonindependence of selection for resistance to herbivory, how often correlational selection facilitates or constrains the evolution of resistance, or what causes impacts of different herbivores to combine either synergistically or antagonistically (Strauss and Irwin 2004; Strauss et al. 2005).

Here, we address these issues in a continuation of our investigations into the evolutionary ecology of resistance to herbivores in an experimental field population of the native plant Solanum carolinense (Wise 2007a, 2009; Wise and Rausher 2013). Wise (2007b) measured the damage or abundance of more than two dozen species of herbivores on 960 ramets across 40 genets of S. carolinense. In this report, we employ quadratic selection analysis to examine patterns of selection acting on 12 resistance measures involving nine of the most common species of herbivores (including leaf, flower, and fruit feeders) to address the following questions: (1) How widespread is stabilizing selection for resistance to herbivory in this multiple-herbivore community? (2) Does the type of herbivory affect the likelihood of stabilizing selection? (3) How widespread is correlational selection? and (4) Does the type of herbivory influence whether the correlational selection is positive (facilitative) or negative (constraining)?

Materials and Methods

STUDY SYSTEM

Solanum carolinense L. (Solanaceae), or horsenettle, is a native herb of the southeastern United States, a noxious weed throughout much of the United States, an invasive species in Europe and Asia, and a natural host for several serious agricultural pests (Innicki et al. 1962; Gorrell et al. 1981; Nichols et al. 1992; Mena-Covarrubias et al. 1996; Imura 2003; Wise 2007b). Thus, horsenettle and its herbivores have become a popular study system for evolutionary ecologists with both basic and applied interests (Nichols et al. 1991; Wise 2007b). Wise (2007b) overviewed the herbivore community of horsenettle in northern Virginia, where the current study took place. This community of herbivores includes mainly specialist insects that are able to overcome horsenettle’s impressive arsenal of chemical and physical resistance traits. In a recent study, Wise and Rausher (2013) focused on horsenettle’s resistance to 11 of these herbivores and quantified the genetic variance–covariance matrix for 14 types of resistance in an experimental population. (There were 14 resistance measures for 11 species because one of the species fed on two types of tissue, and another fed on three types of tissues.) The population was found to contain significant genetic variation for all 14 types of resistance, and statistically significant directional selection acted to increase the level of eight of these types of resistance. The current study includes nine of these 11 herbivores, which feed on leaves, flowers, and/or fruits (Table 1). (Damage by the two herbivores that were left out of the current article was measured discretely—i.e., present or absent—rather than continuously, and thus these resistance values were not amenable to quadratic selection analysis.)

In Virginia, horsenettle ramets emerge from perennial roots in late spring. A typical ramet flowers for roughly one month in mid-to-late summer (Wise 2007b). The self-incompatible, bumblebee-pollinated flowers are born on racemes that average about seven flowers each (Wise et al. 2008). Horsenettle is andromonoecious, with a majority of hermaphrodite flowers and a smaller, variable number of female-sterile flowers (Wise and Cummins 2007). An individual flower remains open and receptive to pollination for about four days. Mature fruits are round, yellow-orange berries averaging ~1.5 cm in diameter (Wise and Sacchi 1996).
EXPERIMENTAL DESIGN

The field experiment took place in an Oldfield population of horsenettle at Blandy Experimental Farm in Clarke County, Virginia (39°N, 78°W). Procedures relating to the treatment of plants and the experimental design have been detailed previously (Wise 2007a,b; Wise and Rausher 2013), and thus they are summarized only briefly here. Roots from 120 horsenettle genets were collected from local fields in the spring of 1997 and were grown in a semiprotected outdoor area in 18.9 L plastic pots in commercial growing media (Wesco Growing Media III, Wetsel Seed Company, Harrisonburg, VA). New root growth for each genet was replanted into 18.9 L pots each spring from 1998 to 2000 to help minimize potential maternal, or carryover, effects that might influence plant resistance or reproduction (Roach and Wulff 1987).

In the spring of 2001, 38 equal-sized (2 mL) root cuttings from each of 40 genets were planted individually into 3.8 L plastic pots as a source of plants for the current study. From June 28 to July 2, 2001, 960 horsenettle ramets (24 from each of 40 genets) were transplanted using a randomized block design into an existing oldfield population of horsenettle. Three blocks, with 10 rows each, were separated by 2 m between rows. Each row contained 32 planting positions that were 1.5 m apart. There were roughly 30 times as many resident horsenettle ramets within the blocks as there were transplanted experimental ramets (Wise 2007a). Therefore, there was already a well-established community of horsenettle’s herbivores in the field.

Rather than measuring any particular resistance trait, we used the conventional approach of quantifying resistance operationally, such that resistance to a species of herbivore was calculated as one minus the proportion of tissue damaged by that herbivore (Simms and Rausher 1987; Stinchcombe 2005; Carmona and Fornoni 2013). Details on the methods of damage assessment for the different species of herbivores can be found in Wise (2007b). Importantly, feeding by each herbivore left behind characteristic evidence such that the damage could unambiguously be attributed to a particular species. Data collection on floral herbivory began immediately after transplanting and lasted through plant senescence in early October, when the plants were harvested. Data on leaf damage were the simplest to collect because leaves generally remain on the plant until senescence, and thus measurement for each species of folivore only had to be assessed once (Wise 2007b). Leaf damage by *Epitrix*, *Leptinotarsa*, and *Tildenia* was measured in August, and leaf damage by *Gratiana* and *Prodiplosis* was measured in September. Because reproductive organs are more ephemeral than leaves, each plant had to be visited approximately every three days during flowering to record the fate of all the flowers and fruits. Horsenettle florivores and frugivores also leave characteristic, species-specific evidence of their feeding, such that their damage is identifiable even after the herbivores have left the plant (Wise 2007b). Racemes were collected as their fruits ripened (prior to dispersal), and each fruit was dissected to look for evidence of internal feeding. The diameter (d) of each

| **Table 1. Herbivores included in the study and how they feed.** |
| --- | --- | --- |
| **Order, species (family)** | **Common name** | **Feeding mode and site** |
| **Coleoptera**<br> *Epitrix fuscula* Crotch (Chrysomelidae) | Eggplant flea beetle | Adults chew leaves, causing “shotgun” pattern of small holes |
| *Leptinotarsa juncta* (Say) (Chrysomelidae) | False potato beetle | Adults and larvae chew leaves from margins, as well as flowers and fruits |
| *Gratiana pallidula* (Boheman) (Chrysomelidae) | Eggplant tortoise beetle | Adults and larvae chew less-veiny sections of leaves, causing medium-sized ovals of damage |
| *Anthonomus nigrinus* Boheman (Curculionidae) | Potato bud weevil | Larvae feed inside flower buds |
| **Diptera**<br> *Prodiplosis longifila* Gagné (Cecidomyiidae) | Citrus gall midge | Larvae feed on rolled leaves |
| *Zonosemata electa* (Say) (Tephritidae) | Pepper maggot | Larvae feed on fruit pulp |
| **Lepidoptera**<br> *Frumenta nundinella* (Zeller) (Gelechiidae) | N/A | Larvae eat ovules |
| *Tildenia inconspicuella* (Murtfeldt) (Gelechiidae) | Eggplant leafminer | Larvae mine leaves |
| **Rodentia**<br> *Microtus pennsylvanicus* (Cricetidae) | Meadow vole | Chews flowers and fruits |
fruit was used to estimate seed production using the following prediction equation (Wise and Cummins 2007):

\[
\text{seeds} = 70.1 - 23.0d + 2.18d^2 - 0.0415d^3
\]

**SELECTION ANALYSIS**

Directional selection for resistance was examined using linear phenotypic-selection analysis in a previous paper (Wise and Rausher 2013). In the current article, stabilizing, disruptive, and correlational selection are investigated using the quadratic form of the phenotypic-selection analysis. This analysis involved a regression of relative plant fitness (based on seed production) on 12 damage measurements, the squares of the 12 damage measurements, and the 66 cross-products of each pairwise combination of damage measurements. For each of the 12 types of damage, the damage measurements were standardized to a mean of zero and expressed in units of SD (by subtracting the mean then dividing by the SD) prior to running the analysis. The individual regression coefficients (i.e., the selection gradients) were considered statistically significant if \( P < 0.05 \).

Although the experiment consisted of 960 ramets, measurements for at least one of the 12 types of damage were not available for 123 ramets; therefore, the selection analysis included only the 837 ramets for which all 12 damage types were measured. Each quadratic coefficient (i.e., the regression coefficient for a squared damage term) equals one-half the stabilizing/disruptive selection gradient for resistance to a herbivore (Lande and Arnold 1983; Stinchcombe et al. 2008). If negative, then selection is stabilizing, and if positive, then selection is disruptive. For each damage type for which a significant negative quadratic coefficient was found, we further addressed whether there was an intermediate peak in fitness (rather than just curvature in the fitness–resistance relationship). To do so, we performed separate univariate regressions— including damage from one species of herbivore at a time—and took the first derivative of the regression equation to quantify the damage level in which fitness was maximal. (We first doubled the quadratic coefficient to accurately model the selection gradients, as emphasized by Stinchcombe et al. 2008).

Note that while we describe these analyses in terms of selection on resistance, we used the damage measurements directly as the predictor variables (rather than converting them first into operational-resistance values). The inferences would be identical using either damage or resistance values, but the results of the analysis of additivity of impact are more intuitive when thinking in terms of damage measurements rather than resistance measurements.

The cross-product coefficients from the quadratic regression analysis represent correlational selection gradients. A significant coefficient means that the strength of selection for resistance to one herbivore depends on the amount of damage the plant incurs from a second herbivore. A negative value for the correlational gradient means that the combined damage causes an additional decrease in fitness beyond the additive components, and thus the strength of selection for resistance to one herbivore is intensified by damage caused by a second herbivore. In contrast, a positive correlational gradient means that the selection for resistance to one herbivore is lessened by the presence of damage caused by a second herbivore. In other words, if the correlational gradient is negative, then the combined fitness impact of the two herbivores is greater than additive (i.e., synergistic), whereas if the correlational gradient is positive, then the combined fitness impact is less than additive (i.e., antagonistic).

We performed an additional series of standardized quadratic selection analyses to examine more closely the selection surfaces acting on resistance to pairs of herbivores. With these analyses, we were able to plot how plant fitness varied across a range of damage levels by a pair of herbivore species while holding damage levels by all other species to their standardized mean value (i.e., zero).

Finally, we performed a quadratic selection analysis in which the standardized damage levels were summed within the type of tissue fed upon (viz., leaves, flowers, and fruits). Before analysis, the damage levels within each of the three feeding “guilds” were standardized to a mean of zero and expressed in terms of SD from the mean.

It should be noted that a genotypic-selection analysis (in which the regression is performed on breeding values of family-mean values of fitness and resistance) is generally preferred because of the potential for gradients from a phenotypic-selection analysis to be influenced by unmeasured environmental correlations between phenotypes and fitness (Rausher 1992a; Mauricio and Mojonnier 1997; Stinchcombe et al. 2002). However, a quadratic genotypic analysis including the 12 types of resistance cannot be performed on our dataset because the number of genets (40) is less than the number of regression coefficients that would have to be measured for the full analysis (90, including linear, squared, and cross-products). Thus, the interpretation of the selection gradients calculated in this study must be evaluated with the caveat that they may contain some influence of unmeasured environmental variables because they were estimated from a phenotypic-selection analysis.

**Results**

**HERBIVORE DAMAGE LEVELS**

This study included five insect species that caused appreciable damage to horsenettle leaves. Epitrix adults are highly mobile and caused “shotgun” hole damage to every ramet, and to almost every leaf. Larvae and adults of Leptinotarsa damaged ~90% of the ramets, with a mean of 5.6 ± 7.3% (SD) of leaf area removed per ramet. Damage by Gratiana and Tildena occurred on ~40%
Comparison of damage levels (solid bars) predicted by the univariate selection analyses to lead to maximal plant fitness, with actual damage levels (hatched bars, means ± 1 SE) measured in the field. The graph includes the seven resistance types on which significant stabilizing selection was found to be acting in the full selection analysis. Types of tissue damaged are abbreviated as follows: lv., leaves (green bars); flw., flowers (purple bars); and frt., fruits (orange bars).

Figure 1. Comparison of damage levels (solid bars) predicted by the univariate selection analyses to lead to maximal plant fitness, with actual damage levels (hatched bars, means ± 1 SE) measured in the field. The graph includes the seven resistance types on which significant stabilizing selection was found to be acting in the full selection analysis. Types of tissue damaged are abbreviated as follows: lv., leaves (green bars); flw., flowers (purple bars); and frt., fruits (orange bars).

of the ramets, while Prodiplosis larvae were found on ~6% of the ramets.

Roughly, half of the flower buds produced in this horsetail population were destroyed by herbivory, with Anthonomus, Leptinotarsa, and Microtus being the most damaging florivores (Fig. 1). In addition, Frumenta destroyed a mean of 2.7 ± 8.3% of the flower buds. Flower damage by Anthonomus, Leptinotarsa, Microtus, and Frumenta occurred on 93%, 64%, 26%, and 26% of the ramets, respectively. Three-fourths of the fruits set by horsetail were destroyed prior to seed dispersal (Fig. 1). The frugivore species responsible were Zonosemata, Leptinotarsa, and Microtus, with damage by these species occurring on 76%, 57%, and 39% of the ramets, respectively.

SELECTION ANALYSIS

The full quadratic selection analysis revealed significant curvature in the relationship between relative fitness and seven resistance measures across five herbivore species (Table 2A). Specifically, the quadratic selection gradients were significantly negative for resistance to one of the five folivores, three of the four florivores, and all three frugivores. Similarly, in the guild analysis, the quadratic selection gradients for resistance to folivory and frugivory were highly significantly negative (Table 2C). The seven negative quadratic gradients from the full selection model suggest that stabilizing selection acted on these types of resistance.

For all seven of these resistance types, the univariate analyses showed that there was a nonzero level of damage at which fitness was maximized (Fig. 1). For one herbivore (Epitrix), the quadratic coefficient in the full selection model was nearly significantly positive (P = 0.05),7 suggesting at least weakly disruptive selection acting on resistance to adult flea beetles. The guild-level analysis suggested that stabilizing selection was twice as strong for resistance against frugivores than florivores, and negligible for folivores (Table 2C).

Ten of the 66 cross-product terms (i.e., correlational selection gradients) in the full quadratic selection analysis were significantly different from zero (Table 2B). Correlational selection was strongest for resistance to frugivores and weakest for resistance to folivores, as only one folivore (Gratiana) was involved in a significant cross-product. The significant correlational selection gradients were negative for resistance to all five pairs of herbivores that fed on the same plant-organ type (e.g., frugivore vs. frugivore), and the significant gradients were positive for resistance to all five pairs that fed on different plant-organ types (e.g., florivore vs. frugivore). The guild-level selection analysis echoed this last point, as the correlational selection was significantly positive only for combined flower and fruit damage (Table 2C).

Figure 2A shows the selection surface as damage levels range from two SDs below to two SDs above the mean for frugivory by Zonosemata and by Microtus (with damage by all other herbivores held at their mean values). Contour lines indicate damage combinations of equal fitness, and different colors designate areas with different fitness. The “+” inside the red oval indicates the combination with the highest fitness. This peak occurs above the lowest damage levels of both types of damage, consistent with the pattern of stabilizing selection found for both resistance measures. Rather than being symmetrical (circles), the contour lines of equal fitness are stretched into ovals that create a ridge in the direction from upper left to lower right. This pattern indicates that the damage caused by these two herbivores combines in a greater than additive (synergistic) fashion, which results in selection to decrease the covariance between resistances to these herbivores.

The selection surface for florivory by Leptinotarsa and frugivory by Microtus is displayed in Figure 2B. As in Figure 2A, the peak of fitness occurs at an intermediate level of resistance to both types of herbivory, consistent with stabilizing selection. In contrast with the pattern in Figure 2A, however, the contour lines are stretched from the lower left to the upper right, creating a selective ridge in the direction of positive covariance between resistances to these two herbivores. This pattern indicates that damage caused by these two herbivores combines in a less than additive (antagonistic) fashion.

The selection surface for folivory by Epitrix and frugivory by Leptinotarsa shows a third pattern—a saddle (Fig. 2C). The peak
Table 2. Results of the quadratic selection analyses on resistance.

<table>
<thead>
<tr>
<th>Herbivore (feeding site)</th>
<th>Gradient</th>
<th>(SE)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>A. Quadratic selection gradients</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Tildenia</em> (leaves)</td>
<td>−0.03769</td>
<td>(0.03412)</td>
<td>0.27</td>
</tr>
<tr>
<td><em>Gratiana</em> (leaves)</td>
<td>−0.10463</td>
<td>(0.04325)</td>
<td>0.016</td>
</tr>
<tr>
<td><em>Prodiplosis</em> (leaves)</td>
<td>−0.02260</td>
<td>(0.03793)</td>
<td>0.55</td>
</tr>
<tr>
<td><em>Leptinotarsa</em> (leaves)</td>
<td>0.00786</td>
<td>(0.03299)</td>
<td>0.82</td>
</tr>
<tr>
<td><em>Epitrix</em> (leaves)</td>
<td>0.12264</td>
<td>(0.06247)</td>
<td>0.050</td>
</tr>
<tr>
<td><em>Anthonomus</em> (flowers)</td>
<td>−0.30530</td>
<td>(0.06920)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td><em>Leptinotarsa</em> (flowers)</td>
<td>−0.27946</td>
<td>(0.08320)</td>
<td>0.0008</td>
</tr>
<tr>
<td><em>Frumenta</em> (flowers)</td>
<td>−0.03364</td>
<td>(0.06028)</td>
<td>0.58</td>
</tr>
<tr>
<td><em>Microtus</em> (flowers)</td>
<td>−0.19245</td>
<td>(0.07585)</td>
<td>0.011</td>
</tr>
<tr>
<td><em>Leptinotarsa</em> (fruits)</td>
<td>−0.45945</td>
<td>(0.11163)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td><em>Zonosemata</em> (fruits)</td>
<td>−0.57844</td>
<td>(0.09945)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td><em>Microtus</em> (fruits)</td>
<td>−0.61246</td>
<td>(0.13374)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td><strong>B. Significant correlational selection gradients</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Gratiana</em> (leaves) × <em>Leptinotarsa</em> (fruits)</td>
<td>0.09517</td>
<td>(0.04451)</td>
<td>0.033</td>
</tr>
<tr>
<td><em>Anthonomus</em> (flowers) × <em>Leptinotarsa</em> (fruits)</td>
<td>−0.15094</td>
<td>(0.06420)</td>
<td>0.019</td>
</tr>
<tr>
<td><em>Anthonomus</em> (flowers) × <em>Microtus</em> (flowers)</td>
<td>−0.20269</td>
<td>(0.07806)</td>
<td>0.0096</td>
</tr>
<tr>
<td><em>Leptinotarsa</em> (flowers) × <em>Leptinotarsa</em> (fruits)</td>
<td>0.09212</td>
<td>(0.04491)</td>
<td>0.041</td>
</tr>
<tr>
<td><em>Leptinotarsa</em> (flowers) × <em>Zonosemata</em> (fruits)</td>
<td>0.09782</td>
<td>(0.04855)</td>
<td>0.044</td>
</tr>
<tr>
<td><em>Leptinotarsa</em> (flowers) × <em>Microtus</em> (fruits)</td>
<td>0.12410</td>
<td>(0.05522)</td>
<td>0.025</td>
</tr>
<tr>
<td><em>Frumenta</em> (flowers) × <em>Zonosemata</em> (fruits)</td>
<td>0.12416</td>
<td>(0.05440)</td>
<td>0.023</td>
</tr>
<tr>
<td><em>Leptinotarsa</em> (fruits) × <em>Zonosemata</em> (fruits)</td>
<td>−0.36873</td>
<td>(0.08829)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td><em>Leptinotarsa</em> (fruits) × <em>Microtus</em> (fruits)</td>
<td>−0.43588</td>
<td>(0.11416)</td>
<td>0.0001</td>
</tr>
<tr>
<td><em>Zonosemata</em> (fruits) × <em>Microtus</em> (fruits)</td>
<td>−0.51745</td>
<td>(0.11699)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td><strong>C. Quadratic and correlational selection gradients for herbivore-guild analysis</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Folivores (quadratic)</td>
<td>−0.02294</td>
<td>(0.03225)</td>
<td>0.48</td>
</tr>
<tr>
<td>Florivores (quadratic)</td>
<td>−0.22443</td>
<td>(0.05637)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Frugivores (quadratic)</td>
<td>−0.45815</td>
<td>(0.06033)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Folivores × florivores</td>
<td>0.00383</td>
<td>(0.04125)</td>
<td>0.93</td>
</tr>
<tr>
<td>Folivores × frugivores</td>
<td>−0.00550</td>
<td>(0.03583)</td>
<td>0.88</td>
</tr>
<tr>
<td>Florivores × frugivores</td>
<td>0.12421</td>
<td>(0.03370)</td>
<td>0.0002</td>
</tr>
</tbody>
</table>

SEs of the gradients are shown in parentheses. (A) The quadratic gradients and SEs equal twice the values of the partial regression coefficients for the squared terms. Negative quadratic gradients indicate concave-downward curvature in the fitness–damage relationship, and thus suggest an intermediate optimum for resistance (i.e., stabilizing selection). A significant positive quadratic gradient would indicate concave-upward curvature, and thus suggest disruptive selection for resistance. Quadratic selection gradients in boldface are significant at $P < 0.05$. (B) Only the statistically significant ($P < 0.05$) correlational selection gradients are shown. For the other 56 cross-product coefficients, $P > 0.05$. (C) Quadratic and correlational selection gradients from the guild-level selection analysis. Selection gradients in boldface are significant at $P < 0.05$.

fitness for resistance to *Leptinotarsa* florivory is at an intermediate value, consistent with the negative quadratic selection gradient for this damage (Table 2). Resistance to *Epitrix*, in contrast, has two local peaks—a pattern illustrating disruptive selection, which was indicated by the marginally significant ($P = 0.05$) positive quadratic gradient for this damage (Table 2). The slight rotation of the saddle in this figure is in the direction of selection for positive covariance between the resistance traits, which is consistent with the positive (but not statistically significant) correlational selection for these two resistance measures, and with the fact that the two types of damage combined in a slightly antagonistic fashion to impact the plants’ fitness.

**Discussion**

Previous work has shown that horsenettle populations maintain genetic variation for resistance to herbivores even in the face of high levels of damage and directional selection to increase resistance (Wise 2007a; Wise and Rausher 2013). The current study reveals that the evolution of increased resistance to some
Figure 2. Quadratic selection surfaces for resistance to damage by three pairs of herbivores. Damage levels were standardized to a mean of zero, and are expressed in terms of SDs. The graphs display the predicted relative fitness of plants as damage levels range from two SDs below to two SDs above the means for a pair of herbivores, with the damage levels by all other herbivores set to their mean value. The contour lines indicate combinations of damage that result in equal fitness. The areas in red indicate the highest relative fitness, and those in purple indicate the lowest relative fitness. In panels A and B, the “+” sign indicates the position of the peak relative fitness.

Specifically, we detected significant stabilizing selection for seven resistance types (out of 12 tested), including for one folivore (*Gratiana*), three florivores, and three frugivores. Instead of the highest fitness being at 0% for damage by three florivores and three frugivores, the calculated highest plant fitness occurred at damage levels ranging from 7.6% to 18.6% of the flowers or fruits damaged per herbivore species.

The significant stabilizing selection gradients suggest that allocation costs may be constraining horsemint from evolving complete resistance to some of its herbivores, particularly the flower- and fruit-feeding species. However, the actual damage levels for several herbivores was well above the levels at which the plants were predicted by the gradients to experience maximal fitness. In particular, the levels of florivory by *Anthonomus* weevils and frugivory by *Leptinotarsa* beetles and *Zonosemata* larvae were roughly twice as high as the levels predicted to maximize plant fitness. There are at least two explanations for the discrepancies between the predicted optimal resistance levels and the actual damage levels—one that relates to the way resistance was quantified and the other that relates to ecological costs of resistance.

In the selection analyses, we used operational measures of resistance, which are based on the amount of damage a plant exhibits. The amount of damage will depend not only on plant-resistance traits that control the relative damage levels among plants, but also on how abundant the herbivores are, which will control the overall mean amount of damage in the plant population. It is possible that the population sizes of some herbivores were unusually high the year of this field study due to the reasons other than plant resistance, such as favorable abiotic factors or low levels of natural enemies. If this were the case, then the mean levels of damage per plant caused by these herbivores would be higher than the predicted levels calculated with the quadratic selection analysis. Nevertheless, the mean level of damage over a longer time period would still be expected to center around the levels at which plant fitness is maximized as population sizes of the herbivores vary from year to year.

The costs of resistance suggested by stabilizing selection gradients can best be seen as allocation (or internal) costs of resistance that affect the pattern of selection and result in an intermediate optimal level of resistance. However, the evolutionary response of a plant population to that selection for resistance to a herbivore can be constrained by ecological (or external) costs that result from the presence of other species of herbivores (Simms 1992; Stowe 1998; Strauss et al. 2002). For example, negative genetic correlations in resistance to different species of herbivores likely play a role in keeping resistance levels below their optima for some species in this system. Specifically, resistance to
frugivory by *Leptinotarsa* and *Zonosemata* are strongly negatively genetically correlated with resistance to frugivory by voles as well as resistance to folivory by *Epitrix* flea beetles (Wise 2007a; Wise and Rausher 2013). Thus, an evolutionary increase in resistance to *Leptinotarsa* or *Zonosemata* would be accompanied by a decrease in resistance to voles and flea beetles, and vice versa.

A second type of ecological cost of resistance likely played a strong role in keeping resistance levels below the selective optima in this system: interspecific competition between herbivores. For example, there was significant evidence of competition (suggested by negative environmental correlations in damage) between *Anthonomus* weevils and two of the other florivores, and even stronger competition among the three species of frugivores (Wise 2009). Therefore, even as selection would tend to push the resistance against a single herbivore toward the optimum, response to that selection would be slowed because, as the damage level for that species went down, damage levels by competing species would go up, thus lessening the fitness gains achieved by increasing the resistance to the first herbivore.

Although only a few other studies have reported attempts to detect stabilizing selection on resistance to herbivory, a comparison of their results with those of the current study are instructive. For example, in a field study of *Ipomoea purpurea*, Rausher and Simms (1989) found stabilizing selection for fruit damage by corn earworms, but not for leaf damage by flea beetles, tortoise beetles, or generalist insects. Similarly, Pilson (2000) found significant stabilizing selection for resistance to seed-pod-feeding weevils but not to leaf-feeding flea beetles in *Brassica rapa*. In another field study of *I. purpurea*, Tiffin and Rausher (1999) did not detect significant stabilizing selection on resistance to apical-meristem damage or folivory.

Combined with the results of the current investigation, these studies suggest that although stabilizing selection for resistance is far from universal, it may nevertheless be relatively common. Moreover, it may be more likely to be detected for flower- and fruit-feeding herbivores than for leaf-feeding herbivores. This finding suggests that resistance to frugivores and florivores may be more costly than resistance to folivores. A possible explanation for this pattern may be related to herbivore foraging or oviposition behavior: Just as pollinators are often attracted to larger floral displays (Schemske 1980; Cruzan et al. 1988; Andersson 1996; Conner and Rush 1996) and seed dispersers can be attracted to plants where fruits are concentrated (Korine et al. 2000), herbivores of flowers and fruits may preferentially feed on plants with relatively large displays of flowers or fruits. If this is the case, then the trait of producing few flowers or fruits would essentially be a mechanism for resistance against florivory or frugivory—albeit a costly mechanism in terms of loss of opportunities for sexual reproduction.

In addition to the seven significant cases of stabilizing selection, there were 10 significant correlational selection gradients for pairs of resistance. Just as with stabilizing selection, the strength of correlational selection varied by type of herbivore damage. Three of the significant correlational gradients involved pairs of frugivores, two involved pairs of florivores, four involved a frugivore and a florivore, and only one involved a folivore. The relative frequency of significant correlational selection gradients among herbivore types roughly reflected how strongly damage by the herbivores affects plant fitness, as directional selection for resistance was previously shown to be greatest for frugivores, followed by florivores, and then folivores (Wise and Rausher 2013). We believe this pattern in turn reflects a gradient in the effects of damage on fitness, with a given amount of damage to fruits reducing fitness more than an equivalent amount of damage to flowers or leaves.

In addition to the type of damage influencing the magnitude of correlational selection gradients for resistance across the herbivore community, the type of damage was also related to the signs of the gradients. Specifically, when two herbivores fed on the same type of organ, the correlational selection gradient for their damage was negative, and when the two herbivores fed on different types of organs, the gradient was positive. This finding indicates, for instance, that selection against damage by a frugivore would be intensified by damage from other species of frugivores. In other words, the impacts of the two frugivores would be synergistic, or greater than additive. In contrast, selection against damage by a frugivore would tend to be weakened in plants with higher levels of damage by florivores or folivores. In other words, the fitness impacts of the different herbivores would combine antagonistically, or in a less than additive fashion.

Numerous studies across a diversity of plant-herbivore systems have observed that impacts of two herbivores can combine in a nonadditive fashion (Strauss 1991; Hufbauer and Root 2002; Strauss and Irwin 2004). Although some of these studies focused on ecological implications of additive versus nonadditive impacts, others have emphasized the role that nonadditivity might play in the coevolutionary dynamics between plants and their herbivores (Hougen-Eitzman and Rausher 1994; Iwao and Rausher 1997; Strauss and Irwin 2004). Specifically, if impacts combine in a greater than additive fashion, then selection for resistance against both herbivores may be facilitated (Gould 1988). In contrast, if the impacts of two herbivores combine in a less than additive fashion, then the evolution of resistance to both herbivores may be slowed.

Because most empirical studies on combined-herbivore impact have focused on only two herbivore species, there has been little exploration of patterns involving different types of nonadditivity. In particular, a question that has not previously been addressed is whether there are particular conditions that cause
impacts to combine synergistically or antagonistically. With 12 separate operational–resistance measurements across nine species in the current study, 66 pairwise comparisons were possible. Although only 15% of the correlational selection gradients were statistically significant, the consistent pattern in those gradients allows us to suggest the following hypothesis: if two herbivores feed on the same type of plant organ, the combined impact is likely to be greater than additive. If, however, two herbivores feed on different plant organs, the combined impact is likely to be less than additive.

The rationale for this hypothesis lies in a consideration of limiting factors on plant reproduction. For instance, a plant may be able to compensate for an amount of leaf damage caused by one herbivore by increasing its rate of photosynthesis on undamaged leaves to partially make up for the loss of carbohydrate production caused by folivory. However, feeding by a second folivore may exacerbate the carbohydrate limitation initiated by the first folivore, thus causing an increasingly negative fitness impact on the host plant. Thus, damage by one leaf-feeder causes the plant to be less tolerant of a second leaf-feeder. In contrast, a second herbivore feeding on flowers instead of leaves might have less impact on the plant because a carbohydrate-limited plant is likely to produce more flowers than the number of fruits it will ultimately be able to fill (Lloyd et al. 1980; Stephenson 1981; Wise and Cummins 2006). In other words, damage by a leaf feeder would cause the plant to be more tolerant of a flower feeder, whereas damage by a flower feeder would cause a plant to be more tolerant of a leaf feeder. Therefore, although two herbivores that affect the same resource are more likely to have a synergistically (i.e., greater than additive) negative impact on the host plant, the fitness impacts of two herbivores that affect different resources are more likely to combine antagonistically (i.e., less than additively).

Because the current study was largely observational (in the sense that insects chose which plants to feed upon), the patterns related to nonadditivity revealed by the correlational selection gradients are merely suggestive. With so many types of damage and other influences on the plants in the field setting, there is a lot of noise that can obscure patterns. Manipulative experiments in which damage levels are independently varied will be necessary to test our hypothesis. Nevertheless, it is a hypothesis that could help to reconcile a large number of otherwise miscellaneous findings related to nonadditivity of herbivore impact.

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