The soybean aphid (*Aphis glycines* Matsumura) is currently considered the most important insect pest of soybeans in North America (Hill et al. 2010, Ragsdale et al. 2011). Rapid spread and establishment of the soybean aphid across North American soybean-growing regions has resulted in immense economic damage, an increased dependence on pesticides, and contributed to the success of additional invasive species (Heimpel et al. 2010, Ragsdale et al. 2011, Tilmon et al. 2011). Soybean aphid feeding inflicts damage on the host plant by reducing photosynthetic rates, stunting growth (Ragsdale et al. 2007, Beckendorf et al. 2008, Rhainds 2010), and transmission of soybean viruses (Hill et al. 2001, Wang and Ghabrial 2002). Although chemical control continues to be the most common form of soybean aphid management, additional biological control and plant resistance methods have been proposed (Chandrasena et al. 2011, Ragsdale et al. 2011). In particular, plant resistance has emerged as a cost effective and environmentally safe alternative to insecticides (Michel et al. 2011, Wiarda et al. 2012). However, the future of plant resistance methods is questionable given the existence of naturally occurring aphid biotypes able to overcome several of the most promising aphid-resistant soybean varieties (Kim et al. 2008; Hill et al. 2010, 2012). Although considerable effort has been made to identify aphid-resistant soybean varieties, less emphasis has been placed on characterizing aphid response to soybean plant defenses. As a result, much remains unknown about the interaction between the soybean aphid and soybean defensive mechanisms.

Plants have evolved a variety of defense mechanisms to counteract attack by arthropods (Agrawal 2011, Smith and Clement 2012). An arsenal of morphological and chemical mechanisms exists that impose stress on insect herbivores by decreasing survival and inhibiting growth and reproduction (Chen 2008, Howe and Jander 2008, Smith et al. 2009). In the soybean, antibiotic and antixenotic forms of resistance have been identified that inflict stress on the soybean aphid in several ways (Hill et al. 2004b, Li et al. 2004, Diaz-Montano et al. 2006, Hesler and Dashiell 2007, 2011). In particular, plant resistance has emerged as a cost effective and environmentally safe alternative to insecticides (Michel et al. 2011, Wiarda et al. 2012). However, the future of plant resistance methods is questionable given the existence of naturally occurring aphid biotypes able to overcome several of the most promising aphid-resistant soybean varieties (Kim et al. 2008; Hill et al. 2010, 2012). Although considerable effort has been made to identify aphid-resistant soybean varieties, less emphasis has been placed on characterizing aphid response to soybean plant defenses. As a result, much remains unknown about the interaction between the soybean aphid and soybean defensive mechanisms.

Plants have evolved a variety of defense mechanisms to counteract attack by arthropods (Agrawal 2011, Smith and Clement 2012). An arsenal of morphological and chemical mechanisms exists that impose stress on insect herbivores by decreasing survival and inhibiting growth and reproduction (Chen 2008, Howe and Jander 2008, Smith et al. 2009). In the soybean, antibiotic and antixenotic forms of resistance have been identified that inflict stress on the soybean aphid in several ways (Hill et al. 2004b, Li et al. 2004, Diaz-Montano et al. 2006, Hesler and Dashiell 2007, 2011). In particular, plant resistance has emerged as a cost effective and environmentally safe alternative to insecticides (Michel et al. 2011, Wiarda et al. 2012). However, the future of plant resistance methods is questionable given the existence of naturally occurring aphid biotypes able to overcome several of the most promising aphid-resistant soybean varieties (Kim et al. 2008; Hill et al. 2010, 2012). Although considerable effort has been made to identify aphid-resistant soybean varieties, less emphasis has been placed on characterizing aphid response to soybean plant defenses. As a result, much remains unknown about the interaction between the soybean aphid and soybean defensive mechanisms.

Plants have evolved a variety of defense mechanisms to counteract attack by arthropods (Agrawal 2011, Smith and Clement 2012). An arsenal of morphological and chemical mechanisms exists that impose stress on insect herbivores by decreasing survival and inhibiting growth and reproduction (Chen 2008, Howe and Jander 2008, Smith et al. 2009). In the soybean, antibiotic and antixenotic forms of resistance have been identified that inflict stress on the soybean aphid in several ways (Hill et al. 2004b, Li et al. 2004, Diaz-Montano et al. 2006, Hesler and Dashiell 2007, 2011). In particular, plant resistance has emerged as a cost effective and environmentally safe alternative to insecticides (Michel et al. 2011, Wiarda et al. 2012). However, the future of plant resistance methods is questionable given the existence of naturally occurring aphid biotypes able to overcome several of the most promising aphid-resistant soybean varieties (Kim et al. 2008; Hill et al. 2010, 2012). Although considerable effort has been made to identify aphid-resistant soybean varieties, less emphasis has been placed on characterizing aphid response to soybean plant defenses. As a result, much remains unknown about the interaction between the soybean aphid and soybean defensive mechanisms.

Plants have evolved a variety of defense mechanisms to counteract attack by arthropods (Agrawal 2011, Smith and Clement 2012). An arsenal of morphological and chemical mechanisms exists that impose stress on insect herbivores by decreasing survival and inhibiting growth and reproduction (Chen 2008, Howe and Jander 2008, Smith et al. 2009). In the soybean, antibiotic and antixenotic forms of resistance have been identified that inflict stress on the soybean aphid in several ways (Hill et al. 2004b, Li et al. 2004, Diaz-Montano et al. 2006, Hesler and Dashiell 2007, 2011). In particular, plant resistance has emerged as a cost effective and environmentally safe alternative to insecticides (Michel et al. 2011, Wiarda et al. 2012). However, the future of plant resistance methods is questionable given the existence of naturally occurring aphid biotypes able to overcome several of the most promising aphid-resistant soybean varieties (Kim et al. 2008; Hill et al. 2010, 2012). Although considerable effort has been made to identify aphid-resistant soybean varieties, less emphasis has been placed on characterizing aphid response to soybean plant defenses. As a result, much remains unknown about the interaction between the soybean aphid and soybean defensive mechanisms.
Hesler et al. 2007). Antibiotic resistance functions primarily to reduce aphid survival and fecundity, whereas antixenotic resistance alters aphid feeding and/or behavioral preference for a particular host plant (Smith 2005, Van Emden 2007). Currently six major aphid resistance genes (i.e., resistance to *Aphis glycines* or *Rag* genes) have been identified and mapped to chromosomes in soybean (see review by Hill et al. 2012). These resistance genes have proven to be highly stressful to the aphid, as evidenced by reduced adult survival and reproduction by as much as 90% and complete inhibition of nymph development (Hill et al. 2004a,b). Currently, few studies have measured the effects of plant defensive stress across multiple aphid life history stages (although see Li et al. 2004), and it is unknown whether aphids can recover from acute exposure to plant defenses or if resistance genes cause persistent stressful effects. The induction of insect stress responses (e.g., heat shock proteins, cytochrome P450s) may counteract damage inflicted by plant defenses and promote recovery (Schuler 2011, Zhao and Jones 2012). Alternatively, damage inflicted by ingestion of plant toxins and/or changes in phloem content may be irreversible; thus, continuing to reduce insect fitness despite removal from the source of stress. Evaluating whether persistent detrimental effects exist after brief exposure to *Rag* genes will help determine the effectiveness of plant resistance under field conditions, where aphid migration and dispersal could reduce contact with resistant plants.

Within just a few years of emerging as an agricultural pest in North America, virulent soybean aphid biotypes were identified based on response to the various *Rag* genes. Biotype 2 aphids are able to colonize *Rag1* soybean (Kim et al. 2008), whereas biotype 3 aphids are able to colonize *Rag2* soybean (Hill et al. 2010). The existence of virulent aphid biotypes poses problems for the development and effectiveness of soybean plant varieties harboring aphid resistance genes. Virulent genotypes have the potential to rapidly spread in populations and in some cases have even been discovered before commercial release of resistant plant varieties, putting the long-term durability of host plant resistance management strategies in question (Michel et al. 2011). In response to threats posed by naturally occurring virulent aphid biotypes, it has been suggested that multiple *Rag* genes be combined or stacked in soybean varieties (Wiarda et al. 2012). If trade-offs exist involving fitness costs for virulent aphids exposed to multiple *Rag* genotypes, combining plant resistance traits could be an effective strategy to manage multiple virulent aphid biotypes. However, cross-virulence to multiple independent sources of host plant resistance could jeopardize the use of various resistant and stacked varieties. Mian et al. (2008a) found *Rag1* virulent aphids from Ohio (biotype 2) were unable to survive on *Rag2* soybean (PI243540), indicating biotype 2 aphids were not cross-virulent to *Rag2*. However, Hill et al. (2010) found mixed results, suggesting *Rag2*-virulent aphids (biotype 3) are also able to colonize different varieties containing the *Rag1* gene at levels similar to susceptible controls. As a result, it is unclear to what extent trade-offs involving fitness costs versus cross-virulence to host plant resistance exist across different virulent aphid biotypes. Furthermore, previous research identifying soybean aphid biotypes has primarily evaluated virulence at the population level, using groups of aphids that can be composed of multiple unique genotypes or has used only a single aphid clone (Kim et al. 2008, Hill et al. 2010). Therefore, it is unknown whether different unique aphid genotypes show quantitative variation in their response to plant defenses or if a dichotomous (virulent vs. avirulent) response is observed across different genotypes. Evidence from field collected aphids suggests response to *Rag1* may range from marginally to highly virulent (Michel et al. 2010), but the existence of a fitness gradient in response to additional sources of host plant resistance has not been investigated.

The sustainability and efficacy of plant resistance for soybean aphid management relies on a complete understanding of the relationship between plant defensive mechanisms and aphid response. Several key aspects of the soybean aphid’s response to plant resistance remain unexplored; therefore, this study aimed to better characterize aphid response to *Rag2* soybeans. In a series of three experiments we investigated: 1) the short- and long-term fitness consequences of exposure to *Rag2* plants, 2) clonal genetic variation in response to *Rag2* plant defenses, and 3) trade-offs and cross-virulence to additional antibiotic soybean varieties.

**Methods and Materials**

**Aphid Rearing and Maintenance.** Aphids collected in July 2011 from field locations in Concord, NE, and Madison, WI, were used to establish large mixed colonies and single aphid clonal lines. Large aphid colonies from each state were initially founded from 200 to 300 aphids and reared in separate growth chambers on 15-20 early vegetative stage plants (V3-V4) of the aphid tolerant soybean variety KS4202 (Pierson et al. 2010). Soybeans were rotated approximately once a week by adding 3-4 new V3-V4 vegetative stage plants to continuously maintain colonies of ~2,000-3,000 aphids at various life history stages. In addition, parthenogenetic aphid reproduction on soybean enabled the establishment of 16 clonal lines (8 per state), each founded from a single apterous female selected at random from the original field collection. It is presumed that each clonal line represents a unique genotype. Based on five microsatellite loci (Michel et al. 2009, Kim et al. 2010) and three SNP loci (Barker et al. 2011), we confirmed that each clonal line represented a unique genotype and not a mixture of genotypes (data not shown). Each clonal line was continuously maintained on a single KS4202 soybean plant grown in plastic Cone-tainers (Ray Leach Cone-tainer, Hummert International, Earth City, MO) and covered by a custom fitted cylindrical plastic cage (30.5 by 4.4 cm). Custom cages had two fine mesh
covered side panels and a mesh covered top that allowed air circulation and prevented aphid escape. Approximately every 2 wk 10–20 adults per clonal line were transferred to a new V1–V2 stage plant.

Soybean plants used for aphid maintenance and experiments were grown in a greenhouse using 15.2 cm diameter plastic pots and a potting medium comprised of peat moss, perlite, pine bark, and vermiculite (Fafard 3B Mix, Conrad Fafard Inc., Agawam, MA). All aphid maintenance and experiments were carried out in growth chambers at 24 ± 1°C and using a photoperiod of 16:8 (LD) h. Colony and clonal aphids were reared in growth chambers for 6–12 mo before the start of experiments.

**Characterization of Short- and Long-Term Stress Induced by Rag2.** We measured the performance of adult aphids both during and after exposure to soybeans containing the Rag2 resistance gene (PI243540) and control soybeans (U06-607094), which lacked the Rag2 gene. Groups of 20 apterous adult aphids were confined to a single V1 trifoliate of either a Rag2 or control plant using a custom built plastic petri dish cage (8.9 by 2.5 cm). Each cage had two mesh panels (7 cm diameter) and was fastened to the trifoliate though a small hole in the side of the petri dish fitted to the stem using metal two-prong clips. Six replicate groups of 20 apterous adult aphids were taken from each large colony population (Wisconsin and Nebraska) and placed on Rag2 and control plants for 48 h. Adult survival and nymph production was measured at 48 h, after which 10 surviving adults were randomly selected from each treatment (Rag2 or control) and population (Nebraska and Wisconsin) for further analysis of long-term fitness. Selected adults were individually placed on a single leaf of a new V1 control plant (U06-607094) using small cages fastened to the leaf surface made from adhesive foam squares (2.5 by 2.5 cm square with a 1.3-cm-diameter mesh screen hole). This resulted in two treatment groups of aphids, those transferred from a control plant to a control plant and those transferred from a Rag2 plant to a control plant. Adult survival and nymph production were measured each day until all aphids from all treatments died. Nymphs were removed from cages each day to prevent overcrowding.

The performance of groups of aphids during exposure to Rag2 or control plants (48 h) and individual aphids post exposure to plant defenses (lifetime) were analyzed using analysis of variance (ANOVA) in the R statistical environment (R Development Core Team 2012, R Foundation for Statistical Computing, Vienna, Austria). Survival and nymph production were compared using the following fixed effects model: PLANT TYPE (Rag2 or Control), POP (Wisconsin or Nebraska), and POP × PLANT TYPE. We also calculated the level of stress imposed by plant defenses using the following equation adapted from Fox and Reed (2011):

\[
1 - \frac{\text{fitness}_{\text{Rag2}}}{\text{fitness}_{\text{CONavg}}} \]

where fitness was a composite measure (survival × nymph production) for aphids on Rag2 (fitness\textsubscript{Rag2}) or control (fitness\textsubscript{CONavg}) plants. This measure quantifies the relative reduction in fitness of aphids exposed to plant Rag2 defenses compared with those that were not. A stress level of zero indicates equivalent fitness on Rag2 and control plants. Stress level was calculated for each population separately (Wisconsin and Nebraska) at the two time points (during and post exposure) by dividing each Rag2 replicate (fitness\textsubscript{Rag2}) by the average control fitness (fitness\textsubscript{CONavg}). Finally, we used survival and nymph production to calculate the intrinsic rate of increase (Carey 1993, Walthal and Stark 1997):

\[
r_m = \frac{(\log R_0)}{T}
\]

where \( R_0 \) is the net reproductive rate and \( T \) is the mean generation time for aphids on Rag2 and control plants during the two time points (during and post exposure). Population growth was calculated for groups of aphids during exposure for 48 h, and then by combining data from individual aphids for each population (Wisconsin and Nebraska) post exposure.

**Aphid Clonal Variation in Response to Rag2.** From 16 aphid clonal lines, we randomly selected seven lines (NE2, NE3, NE5, WI2, WI3, WI4, and WI6) for further analysis of performance on Rag2 (PI243540) and control (KS4202) soybean varieties. KS4202 was selected as a control because it was shown to be susceptible to aphid feeding when infested in the early vegetative stages (VE, VC, and V1) based on damage ratings (da Silva Marchi 2012). We compared the survival and nymph production of groups of 20 age-synchronized apterous adult aphids (7–8 d old) from each of the seven clonal lines over 5 d. Replicate groups of aphids (two per clonal line) were placed on a single V1 trifoliate using plastic petri dish cages (described in previous section). Each day, surviving aphids and the total number of nymphs per group were counted and nymphs removed. Finally, we used survival and daily nymph production to calculate the growth rate \( r_m \) of each clonal line across the 5 d of the experiment (Carey 1993, Walthall and Stark 1997).

Aphid survival and nymph production on Rag2 and control plants were analyzed by fitting linear mixed-effects models, where the dependent variable was either the ln(x) transformed number of aphids or number of nymphs and fixed factors included PLANT TYPE (Rag2 or Control), clonal line (LINE), DAY (days 1–5), and all interactions. Repeated aphid counts on the same plant violated the assumption of independent observations. Therefore, we treated unique plant identity as a random effect and selected the error correlation structure that provided the best model fit using the Akaike information criteria corrected for sample size (AICC; Pinheiro and Bates 2000). Inclusion of population did not improve model fit for either measure, and therefore was not included in the final models. Post hoc multiple comparisons across clonal lines were performed using Tukey honestly significant difference (HSD) tests on least squared means, where \( P \) values were adjusted for multiple testing. We compared \( r_m \) across clonal lines and plant types using the following ANOVA model: PLANT TYPE (Rag2 or Control), POP (Wisconsin or
Nebraska), clonal line nested within populations (LINE(POP)) and interactions. All analyses were implemented in R using the nlme package (R Development Core Team 2012).

**Aphid Performance on Multiple Antibiotic Soybean Varieties.** Results from the above experiment examining clonal variation in response to Rag2 indicated WI6 aphids were Rag2 virulent. We identified several clones with significantly lower survival on Rag2 relative to controls (i.e., Rag2 avirulent) and selected avirulent clone WI3 for further analysis. First, we confirmed the virulence status of clones WI3 and WI6 by measuring performance on Rag2 and control KS4202 plants over 4 d (two to four replicates per clone and plant type; methods described above). These two clones were subsequently exposed to the following soybean varieties: Jackson (PI548657), Dowling (PI548663), K1621, and KS1639. Jackson and Dowling both contain the Rag1 resistance gene (Hill et al. 2006a,b), whereas K1621 and KS1639 have reported antibiotic effects of unknown genetic origin (Diaz-Montano et al. 2006, Pierson et al. 2010). KS4202 was used as a control with no known antibiotic effects. Survival and nymph production were measured for groups of 20 apterous age-synchronized adults placed on antibiotic soybean varieties and control plants over 4 d (four replicates per clone and plant type). Each day, surviving aphids were counted, and the total number of nymphs per group were counted and removed. We used survival and daily nymph production to calculate the growth rate ($r_m$) of both clonal lines across the 4 d of the experiment on each soybean variety (Carey 1993, Walthall and Stark 1997).

We compared survival and reproduction on Rag2 and control plants by fitting linear mixed-effect models with the following factors: PLANT_TYPE (Rag2 or Control), CLONE (WI6 or WI3), DAY (days 1–4), and all interactions. The dependent variable was ln($x$) transformed number of aphids or number of nymphs. We treated unique plant identity as a random effect, and all interactions. Post hoc multiple comparisons across soybean plant types were performed using Tukey HSD tests on least squared means and P values were adjusted for multiple testing. All analyses were implemented using the R nlme package (R Development Core Team 2012).

**Results**

**Characterization of Short- and Long-Term Stress Induced by Rag2.** Rag2 soybeans significantly reduced aphid survival and reproduction relative to control plants, both during direct exposure and after transfer from resistant to susceptible plants (Table 1). Short-term exposure to Rag2 plants for 48 h reduced aphid survival by 33% relative to control KS4202 plants ($F = 80.00; df = 1.20; P < 0.001$) and adults produced on average 2.5 times fewer offspring on Rag2 soybeans ($F = 12.09; df = 1.20; P < 0.01$). A significant interaction between population and plant type ($F = 7.81; df = 1.20; P < 0.05$) for survival indicated there were population level differences in the effects of Rag2, with Wisconsin colony aphids suffering greater reductions in survival on Rag2 plants than Nebraska colony aphids (Table 1). In the long-term, aphids that were exposed to Rag2 plants for the first 48 h of their adult lives and then moved to susceptible control plants also had significantly reduced lifetime survival and nymph production (Table 1). Aphids exposed to Rag2 plants lived on average 3 d less ($F = 5.12; df = 1.35; P < 0.05$) and produced approximately half as many offspring compared with aphids that were on control plants their entire lifetime ($F = 6.35; df = 1.35; P < 0.05$). In addition, Wisconsin aphids overall had significantly reduced lifetime survival ($F = 5.04; df = 1.35; P < 0.05$) and reproduction ($F = 5.57; df = 1.35; P < 0.05$) relative to Nebraska aphids, living 3 d less and producing half as many offspring on average across both plant types (Table 1). We used aphid performance measures during and post exposure to Rag2 plants to quantify the levels of stress induced by plant defenses. We used the relative fitness of aphids exposed to Rag2 for 48 h versus unexposed aphids as a proxy for stress level (see methods). In general, exposure to resistant plants was highly stressful, reducing cumulative fitness (survival and reproduction) on average across populations by 74% (stress level = 0.74) during the 48 h aphids were in direct contact with Rag2 plants (Table 1). Even after aphids were transferred from resistant to susceptible plants, the brief prior exposure to Rag2 proved to be stressful by reducing the lifetime cumulative fitness by 61% (stress level = 0.61; Table 1). The effect of stress imposed by plant defenses was also evident when comparing population growth rates for Rag2 and control populations with lower $r_m$ for populations on Rag2 plants during and after exposure (Table 1). In particular, the negative value for $r_m$...
during exposure to Rag2 indicates aphid populations decline, whereas aphids on control plants show positive population growth.

**Aphid Clonal Variation in Response to Rag2.** Aphid survival rate averaged across all seven aphid clonal lines was significantly lower on Rag2 (PI243540) compared with control soybeans (KS4202; plant type \( \times \) day: \( F = 5.50; \ df = 4.16; P < 0.01; \) Fig. 1a and b). This translated to 70.7 ± 0.06% of aphids surviving to day 5 on control plants compared with only 31.4 ± 0.07% surviving on Rag2 plants. A significant effect of plant type indicated that daily nymph production was significantly reduced on Rag2 plants across all seven clonal lines (\( F = 61.26; \ df = 1.27; P < 0.001 \)), and this difference was equivalent across each day (plant type \( \times \) day: \( F = 1.90; \ df = 4.36; P > 0.05; \) Fig. 1c and d). Groups of aphids produced an average of 30 ± 7.8 nymphs per day on control plants, but only 14.5 ± 4.9 on Rag2 plants. Significant clonal variation was found for both survival (\( F = 4.08; \ df = 6.14; P < 0.05 \)) and reproduction (\( F = 6.60; \ df = 6.27; P < 0.001 \)). Post hoc paired comparisons between all combinations of the seven clonal lines indicated that on control plants all clones had equivalent survival (Tukey HSD, \( P > 0.05 \)). In contrast, clonal variation in response to Rag2 plants was driven by one clone (WI6), which had significantly higher survival (Tukey HSD, \( P < 0.05 \); Fig. 1b) and daily nymph production (Tukey HSD, \( P < 0.05 \); Fig. 1d) compared with all other clones. The remaining six clones had equivalent survival and nymph production on Rag2. After being exposed to Rag2 plants for 5 d, clone WI6 had 62.5% survival versus an average of 23.4% for all other clones. WI6 aphids also produced on average 31.4 nymphs per day on Rag2 compared with an average of 11.7 for the other clones.

We used survival and nymph production to calculate the intrinsic rate of increase for populations of each clonal line on control and Rag2 plants (Table 2). ANOVA revealed significant variation in population growth rates across clonal lines (\( F = 5.78; \ df = 5, 14; P < 0.01 \)) and plant type (\( F = 50.82; \ df = 1, 14; P < 0.001 \)). This equated to an average 0.08 ± 0.02 reduction in population growth rate across all clonal lines on Rag2 plants compared with control susceptible plants. Clonal line WI6 showed only a 0.01 difference in population growth on Rag2 and control plants (Table 2).

**Aphid Performance on Multiple Antibiotic Soybean Varieties.** Based on results from experiments to measure clonal variation in response to Rag2, we identified one Rag2 virulent clone (WI6) and one avirulent clone (WI3). We first verified these results by performing an additional experiment with greater replication that compared performance of these two clones on Rag2 and control soybeans. We found that the relative performance of Rag2 virulent (WI6) and avirulent (WI3) clones varied depending on whether they were on Rag2 (PI243540) or control (KS4202) plants, as indicated by a significant three-way interaction between clone, plant type, and day for survival (\( F = 28.97; \ df = 1, 8; P < 0.001 \)) and a significant clone by plant type interaction for daily nymph production (\( F = 21.14; \ df = 1, 8; P < 0.01 \)). Post hoc paired comparisons revealed that on control plants both clones had equivalent survival rates (\( t = 0.63; \ df = 10; P > 0.05 \)) and daily nymph production (\( F = 0.69; \ df = 8; P > 0.05 \)). In contrast, on Rag2 plants WI6 aphids had significantly greater survival (\( t = 16.93; \ df = 10; P < 0.0001 \)) and daily nymph production (\( t = 5.67; \ df = 8; P < 0.0001 \)) than WI3 aphids (Fig. 2). After 4 d of exposure to Rag2 plants, WI6 aphids showed only a slight reduction in survival compared with control plants (86.3% vs. 97.5%), and produced equivalent numbers of offspring on both plant types (37.5 ± 9.3

### Table 1. Aphid performance (± SEM) during and post exposure to Rag2 (PI243540) and control (KS4202) soybeans

<table>
<thead>
<tr>
<th>Performance measure</th>
<th>During exposure</th>
<th>Post exposure</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aphid death (d)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nebraska</td>
<td>81.7 ± 0.03</td>
<td>15.8 ± 0.30</td>
</tr>
<tr>
<td>Wisconsin</td>
<td>80.0 ± 0.03</td>
<td>10.9 ± 1.28</td>
</tr>
<tr>
<td>Overall avg</td>
<td>80.8 ± 0.02</td>
<td>13.4 ± 0.85</td>
</tr>
<tr>
<td>Nymph production (nymphs per day)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nebraska</td>
<td>32.5 ± 5.74</td>
<td>18.0 ± 0.71</td>
</tr>
<tr>
<td>Wisconsin</td>
<td>35.5 ± 5.29</td>
<td>13.3 ± 1.49</td>
</tr>
<tr>
<td>Overall avg</td>
<td>35.5 ± 5.29</td>
<td>13.3 ± 1.49</td>
</tr>
<tr>
<td>Cumulative stress level</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nebraska</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Wisconsin</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Overall avg</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Population growth (rm)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nebraska</td>
<td>0.08 ± 0.04</td>
<td>0.07 ± 0.03</td>
</tr>
<tr>
<td>Wisconsin</td>
<td>0.74 ± 0.03</td>
<td>0.00 ± 0.03</td>
</tr>
<tr>
<td>Overall avg</td>
<td>0.08 ± 0.04</td>
<td>0.07 ± 0.03</td>
</tr>
</tbody>
</table>

*Survival and nymph production of groups of 20 aphids taken from large colony populations was measured at 48 h (During Exposure). Individual aphids were removed from Rag2 and control plants after 48 h and placed on new control plants, where lifetime survival and reproduction was measured (Post Exposure). Stress level was calculated for two populations (Wisconsin and Nebraska) using a multiplicative measure of fitness combining survival and nymph production (see methods).*

*Adult survival was measured as either % alive at 48 h (during exposure) or total no. of days an individual aphid lived (post exposure).*

*Intrinsic rate of growth averaged across WI and NE populations during the time period of exposure (2 d) or post exposure until the last aphid died (14 d).*
and 40.5 ± 9.2, respectively), indicating WI6 clone was resistant to the Rag2 gene. Exposure to Rag2 caused a 62.5% reduction in survival (32.5% vs. 95% survival on day 4) and a 63.5% reduction in daily nymph production (16.5 ± 4.3 and 45.3 ± 9.5, respectively) for susceptible WI3 aphids.

After confirming that clone WI6 was virulent to Rag2 and clone WI3 was not, we compared performance of these two clones across four soybean varieties previously identified as having antibiotic type resistance and a susceptible control (KS4202). Table 2. Population growth $r_{m}$ (average ± SEM) of seven aphid clonal lines from Wisconsin and Nebraska calculated over 5 d on control susceptible (KS4202) and Rag2 (PI243540) plants

<table>
<thead>
<tr>
<th>Aphid clonal line</th>
<th>Control ($r_{m}$)</th>
<th>Rag2 ($r_{m}$)</th>
<th>Difference (control–Rag2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NE2</td>
<td>0.19 ± 0.003</td>
<td>0.14 ± 0.06</td>
<td>0.05</td>
</tr>
<tr>
<td>NE3</td>
<td>0.26 ± 0.004</td>
<td>0.15 ± 0.001</td>
<td>0.10</td>
</tr>
<tr>
<td>NE5</td>
<td>0.25 ± 0.03</td>
<td>0.13 ± 0.04</td>
<td>0.12</td>
</tr>
<tr>
<td>WI2</td>
<td>0.20 ± 0.03</td>
<td>0.11 ± 0.02</td>
<td>0.09</td>
</tr>
<tr>
<td>WI3</td>
<td>0.24 ± 0.01</td>
<td>0.13 ± 0.01</td>
<td>0.11</td>
</tr>
<tr>
<td>WI4</td>
<td>0.16 ± 0.02</td>
<td>0.10 ± 0.02</td>
<td>0.06</td>
</tr>
<tr>
<td>WI6</td>
<td>0.24 ± 0.01</td>
<td>0.23 ± 0.02</td>
<td>0.01</td>
</tr>
<tr>
<td>Overall avg</td>
<td>0.22 ± 0.01</td>
<td>0.14 ± 0.02</td>
<td>0.08 ± 0.02</td>
</tr>
</tbody>
</table>

Post hoc testing revealed that survival and nymph production did not differ between the susceptible control (KS4202) and K1621 (Tukey HSD, $P > 0.05$). All the remaining three antibiotic varieties (Jackson, Dowling, and KS1639) significantly reduced survival and nymph production compared with controls (KS4202) and K1621 (Fig. 3; Tukey HSD for all, $P < 0.001$). However, there was no significant interaction between clone and plant type (Table 3 (A)), indicating the virulent and avirulent aphid clones showed equivalent patterns of survival and reproduction across the five types of soybean (antibiotic and susceptible control combined). Differences in survival and reproduction translated to variation in growth rates across soybean varieties, with controls (KS4202) and K1621 showing higher aphid population...
growth compared with the remaining antibiotic varieties (Table 4).

Equivalent performance on control plants (KS4202) across the abovementioned two experiments (see methods) allowed for a combined analysis of response to soybean varieties containing the \textit{Rag1} (Jackson and Dowling) and \textit{Rag2} (PI243540) genes. Performance of WI6 and WI3 clones was found to vary significantly across \textit{Rag} types (Table 3 (B); Figs. 2 and 3). A significant three-way interaction between \textit{Rag} type, clone, and day was found for survival, and a significant \textit{Rag} type by clone interaction for reproduction, indicating reduced performance caused by \textit{Rag} soybean varieties was different between the two aphid clones (Table 3). Post hoc paired comparisons of aphid performance on the \textit{Rag1} soybean varieties showed that the \textit{Rag2} avirulent clone (WI3) preformed better than the \textit{Rag2} virulent clone (WI6; Fig. 3; Tukey HSD, $P < 0.05$). WI3 aphids had a greater overall survival on \textit{Rag1} plants (51% vs. 40%; $t = 3.29; df = 20; P < 0.05$) and produced more average nymphs per day (11.7 vs. 7.3; $t = 3.99; df = 20; P < 0.01$). These results suggest increased susceptibility for \textit{Rag2} virulent WI6 relative to avirulent WI3 aphids when exposed to \textit{Rag1} plants.

**Discussion**

In this study, we aimed to better understand several aspects of plant–insect interactions: 1) levels of stress induced by plant defenses over the lifetime of an aphid, 2) plasticity in aphid virulence to plant defenses, and 3) the existence of fitness trade-offs versus cross-virulence using two major aphid resistance genes (\textit{Rag1} and \textit{Rag2}) in soybean. First, our results indicate that soybean plant defenses inict substantial levels of stress with persistent effects on aphid survival and reproduction, resulting in reduced population growth both during and after exposure. Second, we found an overall dichotomous response to the \textit{Rag2} gene in soybean, with one virulent and six avirulent aphid clones. Finally, reduced performance of \textit{Rag2} virulent aphids relative to \textit{Rag2} avirulent aphids on \textit{Rag1} soybeans suggests independent virulence mechanisms and/or a fitness cost to \textit{Rag2} virulence. Our results also indicate that the mechanisms of resistance differ between the two \textit{Rag} soybean varieties.

We measured levels of stress induced by soybean plant defenses by evaluating the short- and long-term consequences of brief exposure to a major effect aphid resistance gene (\textit{Rag2}) in two important components of aphid fitness, survival, and reproduction. Previous work has shown \textit{Rag2} reduces aphid colonization and adversely affects survival, fecundity, and nymph development (Hill et al. 2004a,b; Li et al. 2004). The current study furthers the characterization of soybean
aphid response to soybean resistance by considering how limited exposure to plant defenses affects aphid performance over its lifetime. It was previously unknown whether soybean aphids could recover from brief periods of exposure to plant defenses or incurred long-term damage that affected performance even in the absence of plant induced stress. The current study did not include tolerant soybean varieties because tolerance has not been shown to impose significant levels of stress on the aphid via reduced fitness (Pier-son et al. 2010, da Silva Marchi 2012). Overall, Rag2 plant defenses imposed high levels of stress that reduced fitness on average across populations during (74%) and after (61%) exposure (Table 1). We found that aphids were not only highly susceptible to Rag2 defenses when directly exposed, but their performance was also severely affected after removal from Rag2 plants. Negative effects on fitness translated to populations that were not growing during exposure to Rag2 and reduced population growth rates after exposure compared with nonstressed aphids (Table 1). Population growth rates measured in this study are comparable to those reported by Wiarda et al. (2012) for susceptible and resistant varieties with Rag1 and Rag2 under field conditions. Overall, the high stress levels observed in this study both during and after exposure suggest aphids are unable to counteract the negative effects of plant-induced stress associated with the Rag2 gene. Once aphids were removed from Rag2 plants, stress levels decreased slightly (74–61%).

![Fig. 3. Performance of Rag2 virulent (WI6) and avirulent (WI3) clones on soybean varieties with reported antibiosis (Jackson, Dowling, KS1639, and K1621) and control (KS4202). Survival (A, B) and daily nymph production (C, D) were measured over 4 d.](image-url)

Table 4. Population growth $r_m$ (average ± SEM) of Rag2 virulent (WI6$_{Rag2-V}$) and avirulent (WI3$_{Rag2-AV}$) clones calculated over 4 d on four antibiotic varieties (Dowling, Jackson, K1639, and K1621) and one susceptible control (KS4202)

<table>
<thead>
<tr>
<th>Soybean variety</th>
<th>WI6$_{Rag2-V}$ ($r_m$)</th>
<th>WI6$_{Rag2-AV}$ ($r_m$)</th>
<th>Difference (WI3–WI6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dowling (Rag1)</td>
<td>0.13 ± 0.01</td>
<td>0.06 ± 0.02</td>
<td>0.07</td>
</tr>
<tr>
<td>Jackson (Rag1)</td>
<td>0.08 ± 0.03</td>
<td>0.06 ± 0.02</td>
<td>0.02</td>
</tr>
<tr>
<td>K1639</td>
<td>0.13 ± 0.06</td>
<td>0.08 ± 0.03</td>
<td>0.05</td>
</tr>
<tr>
<td>K1621</td>
<td>0.23 ± 0.00</td>
<td>0.21 ± 0.02</td>
<td>0.02</td>
</tr>
<tr>
<td>KS4202 (control)</td>
<td>0.25 ± 0.01</td>
<td>0.24 ± 0.02</td>
<td>0.01</td>
</tr>
</tbody>
</table>
and populations began to grow (see Table 1); however, in general, results indicate plant defenses inflicted irreversible damage that aphids were unable to recover from completely. By transferring aphids from \textit{Rag2} to susceptible plants, we simulated local dispersal of aphids between plants, which reduces exposure to plant defenses. Our results indicate that the insect management benefits of \textit{Rag2} can extend to nearby plants with susceptible genotypes, given the observed long-term stress effects of even brief exposure to \textit{Rag2}. However, the current study evaluated plant-induced stress for components of adult aphid fitness, but did not include effects on the next generation of offspring. Establishing whether additional lingering effects of antibiosis affect future parthenogenetic generations is essential to understanding the overall significance of antibiosis on aphid evolution and adaptation to plant defenses.

The existence of virulent aphid biotypes in natural populations is a serious concern for the sustainability and efficacy of incorporating host plant resistance into management practice. Little is known about the mechanisms that lead to biotype evolution in the soybean aphid or how prevalent virulent genotypes are in field populations (Michel et al. 2011). This study evaluated aphid clonal variation in response to \textit{Rag2} resistant soybeans to determine the extent to which quantitative variation in virulence exists across unique genotypes. Previous work has shown significant clonal variation in response to host plant resistance for various life history and behavioral traits in \textit{Aphis gossypii} Glover (Lombaert et al. 2009) and \textit{Myzus persicae} Sulzer (Cardoza et al. 2006). We found a dichotomous response to \textit{Rag2} across seven clonal populations collected from two states (Nebraska and Wisconsin), with six avirulent clones showing equivalent reduction in survival and nymph production, while the seventh virulent clone was unaffected by exposure to \textit{Rag2} (Figs. 1 and 2). Virulence to \textit{Rag2} indicates clone WI6 would be classified as biotype 3 (Hill et al. 2010). Our results suggest that the frequency of virulent biotypes may be quite high, given that a virulent genotype was identified among just seven clones. However, it should be noted that while the Wisconsin isolate appeared to have a high frequency of \textit{Rag2} virulent genotypes, the sample of clones from the Nebraska isolate was too small to conclude that the isolate was homogeneous for \textit{Rag2} avirulence. In general, further work is needed to identify genetic markers specific to virulent genotypes for use in measuring and monitoring frequencies in field populations over time.

Identification of unique aphid clonal lines that were distinctly virulent or avirulent to \textit{Rag2} enabled the evaluation of genotypic interactions with multiple soybean varieties previously reported to exhibit antibiotic-type resistance. It should be noted that plant defense to insects can express varying levels of both antibiotic- and antixenotic-type resistance. The current study used soybean varieties that exhibit primarily antibiotic effects (see Table 1; in review by Hill et al. 2012), although in several cases antixenotic effects have also been observed (Li et al. 2004, Hesler et al. 2007, Zhu et al. 2011). Experiments conducted in this study focused on antibiotic effects using no-choice tests and did not measure antixenotic effects on aphid behavior separately. Overall, our results agree with previous reports of antibiotic resistance in Dowling, Jackson, and KS1639 (Hill et al. 2004a,b; Diaz-Montano et al. 2006). However, in this study K1621 was not resistant to aphids when infested in the early vegetative stage (Fig. 3), suggesting antibiotic effects could be age-dependent, as has been reported in other soybean varieties (Pierson et al. 2010). For example, sources of the \textit{Rag1} gene, Jackson and Dowling, were shown to be moderately susceptible to aphid damage in the reproductive stages (Pierson et al. 2010), but highly resistant when infested in the seedling stages (Hill et al. 2004b, Diaz-Montano et al. 2006). We did not find evidence for cross-virulence, as indicated by the overall low survival and reproduction of the \textit{Rag2} virulent clone (WI6) on three additional antibiotic varieties (Fig. 3b and d). When the performance of WI3 and WI6 clones was compared specifically on \textit{Rag1} and \textit{Rag2} soybean varieties, we found: 1) WI6 was clearly \textit{Rag2} virulent whereas WI3 was avirulent (Fig. 2) and 2) \textit{Rag2} virulent clone (WI6) performed significantly worse on \textit{Rag1} soybeans than the \textit{Rag2} avirulent clone (WI3; Fig. 3; Table 4). Recent work by Hill et al. (2010) indicates similar patterns exist in the opposite direction as well, with biotype 2 aphids virulent to \textit{Rag1} showing reduced colonization in no-choice tests on \textit{Rag2} soybeans relative to biotype 3 but not biotype 1 aphids. Two nonmutually exclusive mechanisms could explain these observed patterns: 1) fitness trade-offs for \textit{Rag} virulence involving greater fitness costs for virulent genotypes relative to avirulent genotypes when exposed to additional \textit{Rag} varieties or 2) independent virulence mechanisms for \textit{Rag1} and \textit{Rag2}. In this study, the observed differences in performance of WI3 and WI6 clones on \textit{Rag1} suggest either a fitness cost to \textit{Rag2} virulence or could result from inherent genetic differences between the clones related to \textit{Rag1} virulence. Recent population genetic analysis of virulent and avirulent aphids exposed to \textit{Rag1} soybeans found biotypes were genetically indistinguishable, suggesting either a complex polygenic genetic basis to aphid virulence or that nongenetic sources could contribute to biotype evolution (Wenger and Michel 2013). Either of these mechanisms could contribute to variation in the response of the two clones used in this study on \textit{Rag1} plants. To gain a more complete understanding of the role of fitness costs versus independent mechanisms of virulence, further research is needed comparing the performance of various aphid biotypes exposed to a suite of existing \textit{Rag} genes.

Wiarda et al. (2012) showed that \textit{Rag} genes alone and in combination were equally effective in preventing significant yield loss, but only a modest reduction in aphid growth rate was observed on \textit{Rag1}/\textit{Rag2} soybeans relative to genotypes with a single resistant gene. Our results suggest this stacked variety could be effective against biotype 3 aphids, given the generally poor performance of the biotype 3 clone (WI6) on
Genetic mapping revealed concentration of amino acids (Chiozza et al. 2010). This may involve a nutritive effect via reduction in plant from changes in molecular processes associated with Rag1 genes in general (Hill et al. 2012). Recent work in- anisms contributing to resistance in soybean with is known about the genetic and physiological mech- Engineering of Plant Defenses. Funct. Ecology. 25: 420 – 432. 

Plant defenses may inflict irreversible damage on aphids through the ingestion of toxic compounds, dis- Plophorinae to pyrethroid, organophosphate, and neonicotinoid insecticides. J. Econ. Entomol. 104: 1357–1363. 


Hesler, L. S., and K. E. Dashiel. 2007. Character- 

Hill, C., A. Chirumamilla, and G. Hartman. 2012. Resis- 
tance and virulence in the soybean-Aphis glycines interaction. Euphytica 186: 635–646. 


