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When herbivores come back: effects of repeated damage on induced resistance

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Summary

1. Plants are known to respond to damage with subsequent changes in resistance. The consequences of these changes for plant fitness and herbivore populations will depend on both the response of a plant to a particular attack at a given moment and on how plants respond through time to varying levels of damage and varying numbers of attacks. While a small number of studies document how induced resistance changes with time after attack (time course of induction) and different levels of damage, few studies have examined the cumulative level of resistance after multiple attacks, and little is known about how the plant's response to damage changes with subsequent attacks.

2. Two experiments were conducted to address the consequences of repeated damage for resistance of tomato (*Solanum lycopersicum,* var Castlemart) to a common pest, the beet armyworm (*Spodoptera exigua)*. The first experiment documented the time course of resistance following a single damage event using both growth and choice bioassays. The second experiment examined whether the plant responded differently to one versus two damage events.

3. Results show that plants were significantly induced by day 1 and remained induced until 15 or 20 days later, suggesting that repeated damage during the response to initial damage is possible. Plants receiving a second bout of damage were able to further increase their resistance level over the level reached in response to a first bout of damage, but the magnitude of response to the second damage event was initially smaller and slower than the response to a single damage event.

4. There was no evidence in this study for immune-like memory in induced resistance. Results of this study suggest that plants can respond to repeated damage, but that there is some limit on responses to repeated damage. Such limits on total plant resistance will affect the influence of induced resistance on herbivore populations and are consistent with assumptions of existing models of induced resistance and herbivore population dynamics, although models have not yet considered the consequences of slower rather than smaller responses to repeated damage.

Key-words: induced defence, *Lycopersicon esculentum*, memory, performance, preference, *Solanum lycopersicum*, *Spodoptera exigua*, timing, tomato

Introduction

Most plants respond to damage by herbivores with some degree of induced resistance or susceptibility, which is a positive or negative change in chemical or physical resistance traits after damage. Plants in nature can be subject to different levels of damage at any one time and to repeated damage by herbivores during their lifetimes. The level of resistance maintained by a plant through time will thus be affected by the speed and duration of the plant's response, the relationship between the amount of damage and response, and whether the response varies with initial and repeated damage. The mean and variance of resistance over time will, in turn, be critical for determining how induced resistance interacts with population-level processes such as the spatial distributions of herbivores, herbivore population dynamics and natural selection on plant defence. How induced resistance changes over time and as a function of the amount and frequency of attack are key assumptions in models that link induced resistance to population-level effects (Edelstein-Keshet & Rausher 1989; Underwood 1999; Underwood, Anderson & Inouye 2005), but our empirical knowledge of these

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relationships, especially the consequences of repeated attacks, is still limited.

For herbaceous plants, studies in a variety of systems find that induced resistance is relatively rapid (occurs in one or a few days) and decays in the absence of further damage. Although few studies have characterized the full relaxation time of induced resistance (Karban 2011), in herbs, responses can last at least 28 days (Gomez, Dijk & Steufer 2010). The fact that resistance can last for a substantial period of time suggests that plants will often be damaged by the same type of herbivore again before the response to previous damage has fully decayed. For a few systems, it is also known that the level of induced response to a single damage event can vary with the amount of damage (e.g. Baldwin & Schmelz 1994; Underwood 2000, 2010), suggesting that responses to subsequent damage events might depend on how damaged plants were to begin with. However, we know little about whether new damage occurring during responses to previous damage provokes different plant responses than initial damage events, or how the amount of initial damage influences subsequent responses.

When plants are attacked by herbivores multiple times, there are several possible outcomes. If plants possess some type of 'memory' similar to animal immune systems, responses to subsequent damage might be faster, larger, or longer lasting (Baldwin & Schmelz 1996). On the other hand, there are at least two reasons why subsequent responses could be smaller than initial responses, especially if attacks are close together in time. First, the resources necessary for induction (either for production of resistance traits or for components of signalling pathways) might be depleted by earlier responses. Second, if subsequent damage occurs before previous induced resistance has fully decayed, and if there is some maximum resistance state possible as has been assumed in some models of induced resistance (Edelstein-Keshet & Rausher 1989; Morris & Dwyer 1997; Underwood 1999), then the response of a plant to subsequent attack might be a decreasing function of the plant's resistance state at the time of attack.

The idea that induced resistance might become faster or stronger with subsequent attacks on the same individual plant was raised in the mid-1990s (Karban & Niiho 1995; Baldwin & Schmelz 1996), but there have still been few studies on how plants respond to repeated damage by the same herbivore. Some studies have shown that levels of resistance after repeated attacks can be higher (Agrawal 1998; Poelman *et al.* 2008) than resistance after a single attack, that resistance can be produced more quickly after repeated attacks (Baldwin & Schmelz 1996) and that transcriptomes can differ after multiple versus single attacks (Poelman *et al.* 2008). Two studies have focused on jasmonic acid (JA) accumulation (a step in the production of resistance) after repeated attacks on a very short timescale (between 1 and 4 h after damage); one found that accumulation was the same as JA accumulation in response to the initial damage event (Ziegler, Keinanen & Baldwin 2001) and another found that accumulation was less than additive (Stork *et al.* 2009). Recent studies have also found that attacks on one part of a shrub can prime defences in other parts of the same plant which are not connected through plant vasculature (Frost *et al.* 2008; Rodriguez-Saona, Rodriguez-Saona & Frost 2009) resulting in faster or stronger responses to damage in those parts. Such priming is clearly a kind of change in response with multiple attacks to an individual plant, but will likely be distinct in its dynamics from responses to multiple attacks within a single completely physiologically integrated plant unit. An additional type of study relevant to responses to repeated attack is studies of how attack by one herbivore influences responses to other herbivore species. These studies also suggest that one damage event can influence plant resistance level after later damage by a different herbivore species (Voelckel & Baldwin 2004; Viswanathan, Lifchits & Thaler 2007).

Although the existing studies thus suggest that induced resistance can vary between initial and subsequent attacks (but see Karban & Niiho 1995), most of these studies measured the level of resistance or state of the plant reached with repeated attack rather than how initial damage might alter the plant's *ability to respond* to subsequent damage. Because the level of resistance after multiple attacks is a function of both the magnitude of response to new damage and any resistance remaining from the initial attack, the ability to respond to subsequent damage can only be measured with reference to the resistance level of plants that received initial damage but no subsequent damage (the baseline level of resistance at the time of subsequent damage). Such a baseline would also account for ontogenetic changes in resistance (Barton & Koricheva 2010; Quintero & Bowers 2011) occurring between initial and subsequent damage. Most previous studies lack such a baseline treatment, although in some cases (e.g. Baldwin & Schmelz 1996) the time course of induced resistance was known, and the second damage event was inflicted after previous induction should have decayed.

In this study, I address induced responses to subsequent damage events in a system where induced resistance is well studied: tomato (*Solanum lycopersicum*) and the generalist herbivore *Spodoptera exigua*. I characterized the time course of induced resistance and then examined induced responses to a second damage event occurring after different levels of previous damage. In particular, I asked:

1. What is the time course of induced resistance?
2. Does the strength of resistance over time vary between high and low initial damage?
3. Do plants increase their level of resistance in response to a second bout of damage and does this increase depend on previous damage?
4. Is the magnitude of response to subsequent damage (difference in resistance between damaged and undamaged plants) different from the magnitude of response to initial damage?

Materials and methods

SYSTEM

Induced responses to damage in tomato are well studied (Howe & Ryan 1999; Orians, Pomerleau & Ricco 2000) and known to affect insect herbivores (e.g. Thaler *et al*. 2001). *Spodoptera exigua* is a broad generalist and can be an economic pest on tomato (Lange & Bronson 1981). Tomato variety Castlemart (used in this study) is known to have resistance induced by *S. exigua* feeding (Broadway *et al*. 1986; Stout *et al*. 1996; Thaler *et al*. 1996), with significant increases in resistance within 24 h after the onset of damage and peak resistance at 3 days after damage (Edwards *et al*. 1985). Plants for the experiments described here were grown in the Florida State University greenhouses in 5-inch (1·68 L) pots with water and fertilizer provided as needed. *Spodoptera* were reared in Percival growth chambers (Percival Scientific Inc., Perry, IA, USA) with 12 : 12 day length at 28 °C and fed artificial diet (Southland Products Inc., Little Rock, AR, USA).

EXPERIMENT 1: TIME COURSE OF INDUCED RESISTANCE

Two treatments were applied to plants at the beginning of the experiment: no damage (controls) and damage (approximately 20–25% of leaf area consumed). Resistance was measured by growth bioassay (based on larval relative growth rate, RGR) and choice bioassay (based on relative damage to leaves from different plants) at 1, 3, 5, 10, 15 and 20 days after damage was complete (see Bioassays section for details). This experiment was carried out in two temporal blocks (March and May of 2009) with roughly equal replicates of all treatments in each block. The total replication for each treatment at each assay was 13–15. Plants had 4–5 fully expanded leaves at the beginning of the experiment. Leaf area (sum of all leaflet lengths) was measured, and a mesh bag was placed on their second youngest fully expanded leaf. Third- and fourth-instar *Spodoptera* larvae were added to the bags on plants in the damage treatment. Enough larvae were used to completely consume the bagged leaf area within 24 h (larvae were added or removed as needed during the day). When damage was complete, the bags were removed, and plants were held in the greenhouse until leaf samples were taken for bioassay. Separate plants were used for each treatment and bioassay, and plants were discarded after being sampled, so no plant was sampled more than once.

Analysis

All analyses for both experiments were conducted in SAS (SAS Institute 2003). To examine the pattern of induced resistance over time as measured by growth bioassay, I used ANOVA (Proc GLM), with RGR modelled as a function of temporal block, time since damage, treatment and all possible interactions. All factors were treated as fixed, and residuals were approximately normally distributed. Planned contrasts between least squared means were used to assess whether there was significant induced resistance (difference between damaged and control treatments) at each day since damage. To determine whether the baseline quality of undamaged plants varied over the experiment, I also examined a model including block, time since damage and their interaction for only undamaged plants. In this case, planned contrasts were used to look for differences in RGR between different days since damage. To examine induced resistance as measured by choice bioassay, I used *t*-tests to determine whether the preference of larvae differed from 0·5 (equal preference for discs from damaged and control plants) at each sample date (data appeared to fit assumptions for a *t*-test, and a signed-rank test produced equivalent results).

EXPERIMENT 2

Plants in the second experiment received one of the five damage treatments (Table 1). Two levels of initial damage (low and high) were crossed with the presence or the absence of a second low-damage event to produce four damage treatments (L, LL, H and HL), and a fifth undamaged group served as a control (C). Systemic induced resistance was measured by bioassay at three times during the experiment (see Bioassay section for details); different individual plants were used for each measurement of resistance, so plants were measured only once during the experiment. The first bioassay was at 3 days after initial damage and measured the response to initial high versus low damage. The second damage event began 3 days after initial damage and ended the next day (4 days after initial damage). The second bioassay occurred 7 days after initial damage (3 days after the end of the second damage event) and measured the response to the second damage event. The third bioassay occurred 10 days after initial damage (6 days after the end of the second damage event). The experiment was conducted in four temporal blocks differing only in that the fourth block had higher replication ($N = 4$ per treatment X assay combination in blocks 1–3, $N = 6$ for block 4) and different levels of imposed damage. Low damage was 20% of leaf area for blocks 1–3 and 10% for block 4; high damage was 40% for blocks 1–3 and 50% for block 4. At the second damage event, all damaged plants received the initial low level of damage for their block (20% for blocks 1–3 and 10% for block 4). Damage levels were chosen based on data showing that these high and low damage levels provoke significantly different induced responses in tomato var Castlemart (Underwood 2010).

At the beginning of the experiment, each plant had approximately four true leaves. At each damage event, each plant's relative leaf area was determined by measuring the lengths of all undamaged leaflets (initially the entire plant, and the uneaten part at the second bioassay). Damage was then inflicted by placing third- and fourth-instar *Spodoptera* larvae on appropriate numbers of leaflets to achieve high versus low damage and confining them with mesh bags. Enough larvae were used to complete damage within approximately 24 h, with larvae added or subtracted as needed. Control plants had the same number of leaves bagged without larvae. After damage was complete, bags were removed. Damage was made to lower leaves, leaving the most recently expanding leaves undamaged for use in bioassays, so that bioassay larvae were fed leaves of similar age and degree of expansion.

Analysis

All questions were addressed with ANOVA (proc GLM). Initial models included all interactions; three-way interactions were dropped as they were never significant. All factors in models were treated as fixed, and residuals were always approximately

Table 1. Treatments in experiment 2. Low damage was 20% of leaf area in blocks 1, 2 and 3 and 10% of leaf area in block 4. High damage was 40% of leaf area in blocks 1–3 and 50% in block 4. Subscripts on treatments indicate the time point of the bioassay (first, second or third)

Treatment	Damage at first event	Damage at second event
C_1, C_2, C_3	None	None
L_1, L_2, L_3	Low	None
LL_2, LL_3	Low	Low
H_1, H_2, H_3	High	None
HL_2, HL_3	High	Low

normally distributed. Planned contrasts of least-squares means were used to examine the differences between particular treatments at particular times. To address whether high and low initial damage caused different levels of resistance over time (question 2), I modelled relative larval growth rate as a function of initial damage level, block and assay (days since damage), using only data for the controls (C) and once-damaged (L and H) plants. To address whether plants were able to reach a higher level of resistance with two damage events than with one, and whether this level depended on initial damage (question 3), I focused only on the damaged treatments and coded them based on their initial level of damage (high (H) or low (L)) and whether they received two (HL and LL) or one (H and L) damage events. I then modelled RGR as a function of block, assay, initial damage level and number of damage events. An effect of damage events would indicate different resistance levels between one and two damage events, and an interaction of damage events and initial damage would indicate that this level of resistance depended on initial damage.

Finally, to address whether the *change* in resistance in response to a second damage event differs from the response to a first damage event (question 4), I created models allowing comparison of resistance in particular treatments with appropriate baselines (Table 2). The change in resistance in response to the first damage treatment is the difference in resistance between the controls and damaged plants at the first assay (3 days after damage). The change in resistance after the second event is the difference between the once-damaged and twice-damaged treatments at the second or third assay (7 or 10 days after initial damage). Because there was no significant difference in the resistance induced by low and high initial damage, I pooled the two initial damage treatments for this analysis. To ask whether the response after 3 days to a second damage event differs from the response after 3 days to the first damage event, I thus used data for controls and damaged plants from the first assay, and once- and twice-damaged plants from the second assay. I coded C_1, L_2 and H_2 as baseline (level of resistance before damage) and L_1, LL_2 and HL_2 as response (level of resistance after damage); C_1 and L_1 were coded as the first damage event, and the rest as the second damage event (Table 2). An interaction of baseline/response and damage events would indicate that the magnitude of response after 3 days depended on whether the plant received one or two rounds of damage. A similar model was used to examine the response to the second damage event after 6 days; here, the closest appropriate baseline for comparison was the difference between controls and once-damaged plants from the second assay (the response to initial damage after 7 days, Table 2).

Although initial damage treatments did not differ significantly, because high initial damage plants consistently tended to be more induced than initial low-damage plants, I conducted an exploratory analysis of whether the magnitude of response to repeated damage might have varied with initial damage. These tests used models similar to the model for both damage treatments combined (Table 2). For all these models, I used only the first two assays and included block and all two-way interactions (three-way interactions were never significant and thus dropped).

BIOASSAYS

In both experiments, bioassays based on the growth rate of *Spodoptera* larvae were used to measure systemic induced resistance. For these assays, two or more leaflets from undamaged, recently fully expanded leaves on each plant were excised by cutting the petiole with a razor. In tomato, leaves at different distances from a damaged leaf achieve different levels of resistance, creating within-plant heterogeneity in resistance (Orians, Pomerleau & Ricco 2000). Because it was not possible to keep the distance between damaged and sampled leaves constant, multiple leaflets and when possible multiple leaves were sampled, generating a sample of plant quality averaging over at least some of the likely variation in resistance among plant parts. Leaflets from each plant were placed into two 2-oz plastic cups (Solo Cup Company, Highland Park, IL, USA) with a piece of moist filter paper. When there was only one undamaged fully expanded leaf above any treated leaves on a plant, leaflets in both cups came from that leaf. When there was more than one expanded leaf newer than any treated leaves, leaflets in the cups came from the newest and second newest leaves.

Table 2. Models comparing the magnitude of response to one versus two bouts of herbivore damage. Baselines estimate the level of resistance in plants when damage (initial or second bout) occurred

Model	Baseline, first damage	Baseline, second damage	Response to first damage	Response to second damage
Effect of number of damage events on response, regardless of initial damage; 3 days after damage	C_1	$\overline{(L_2 + H_2)}$	$\overline{(L_1 + H_1)} - C_1$	$\overline{(LL_2 + HL_2)} - \overline{(L_2 + H_2)}$
Effect of number of damage events on response, regardless of initial damage; 7 (for one event) or 6 (for two events) days after damage	C_2	$\overline{(L_3 + H_3)}$	$\overline{(L_2 + H_2)} - C_2$	$\overline{(LL_3 + HL_3)} - \overline{(L_3 + H_3)}$
Effect of number of damage events on response, high initial damage plants only	C_1	H_2	L_1–C_1	HL_2–H_2
Effect of number of damage events on response, low initial damage plants only	C_1	L_2	L_1–C_1	LL_2–L_2

C, control (no damage); L, low damage; and H, high (40–50%) damage. Subscripts indicate the time point of each bioassay; 1 = 3 days after end of first bout of damage, 2 = 3 days after end of second bout of damage (7 days after initial damage), 3 = 6 days after end of second bout of damage (10 days after initial damage). Bars over expressions in the tables indicate averages. Models separating initial low and initial high damage should be treated as exploratory because low and high initial damage did not produce significantly different responses.

Individual second- or third-instar *Spodoptera* larvae were starved for 2 h to clear their guts and weighed before being placed in the cups. After 48 h, the larvae were removed, starved and reweighed. Cups all contained sufficient leaf material so that larvae did not run out of food. The relative growth rate of larvae was calculated as RGR = ln(final size/initial size). RGR was used as a measure of plant resistance, where higher RGRs indicate less-defended plants. The two cups for each plant were averaged to form one observation per plant for analysis.

In experiment 1, a choice bioassay was also used to assess induced resistance. For this assay, four 9-mm-diameter leaf discs were cut from leaflets from the same leaves used in the growth bioassays using a cork borer. Control and damage treatment plants were paired by size at each sample date, and each pair of plants was used in choice tests with two separate *Spodoptera* larvae. Two leaf discs from each plant (control and damaged) were placed in a petri dish lined with moist filter paper; discs from the two treatments alternated around the outside of the dish. One early third-instar larva was placed in the centre of each dish, dishes were placed in a growth chamber at 28 °C and the larvae were allowed to consume approximately one-fourth of the total leaf area in the dish. The area of each disk missing was quantified from digital images using Image J (National Institutes of Health, Bethesda, MD, USA). The preference of each larva was calculated as the amount of damage to the control discs divided by damage to all the discs combined. The two dishes for each plant pair were averaged to form a single observation. A preference value of 0·5 indicates no induced response perceived by the larvae (no preference for leaf discs from control versus damaged plants), and values >0·5 indicate induced resistance (preference for discs from control plants).

Results

EXPERIMENT 1

When plant resistance was measured in terms of larval growth, undamaged plants increased in resistance (RGR declined) over the course of the experiment, except for a temporary decrease in resistance at day 15 (significant main effect of days since the beginning of the experiment $F_{5,86} = 17{\cdot}53$, $P < 0{\cdot}0001$, Fig. 1). Damage caused induced resistance (main effect of damage treatment $F_{1,171} = 37{\cdot}69$, $P < 0{\cdot}0001$), and average plant resistance changed over time (main effect of days since damage $F_{5,171} = 17{\cdot}68$, $P < 0{\cdot}0001$, Fig. 1). A marginally significant three-way interaction between treatment, days since damage and block ($F_{5,171} = 2{\cdot}0$, $P = 0{\cdot}08$) suggests that the degree of induced resistance varied over time, more in block two than block one. Planned contrasts between damaged and control treatments at each number of days since damage indicate significant induction at 1, 3, 5 and 15 days after damage ($P < 0{\cdot}02$ for these days, Fig. 1). When induced resistance was measured as larval preference for damaged versus undamaged plants, there was significant induced resistance (preference for eating undamaged discs) on days 1, 5, 15 and 20 (Fig. 2).

EXPERIMENT 2

Main effects of temporal block were consistently significant across all models; relative growth rates of larvae were higher in blocks 1 and 2 than in blocks 3 and 4. Considering only plants that were damaged once, both low and high initial damage caused significant increases in plant resistance as measured by declines in *Spodoptera* larval growth rates (main effect of treatment $F_{2,160} = 43{\cdot}46$, $P < 0{\cdot}0001$, Fig. 3, the three points on day 3). Induced resistance was maintained through 10 days after high initial damage (contrasts between control and damaged treatments at each assay, all $P < 0{\cdot}01$, Fig. 3 top line versus next two lines down). Although the high- and low-damage treatments used in this study should have caused significant differences in induced responses (Underwood 2010), there was no significant difference in resistance between once-damaged plants with high and low damage at any of

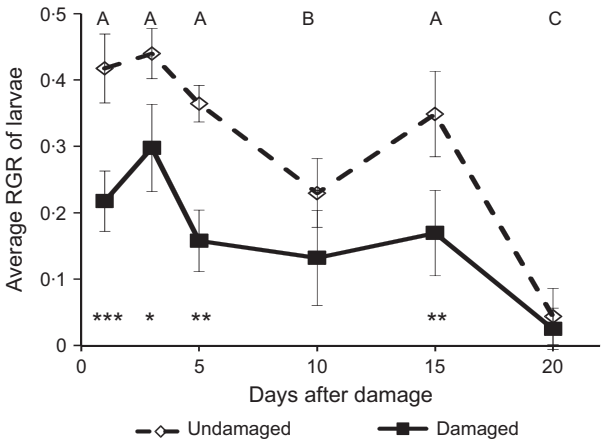

Fig. 1. Time course of plant quality as measured by the relative growth rate (RGR) of larvae on undamaged leaves from undamaged (dashed line) and damaged (solid line) plants in experiment 1. Different letters indicate significantly different relative growth rates among sample days for undamaged plants at $P < 0{\cdot}01$. Asterisks indicate *P*-values for contrasts between undamaged and damaged plants at each sample day (*$P < 0{\cdot}05$, **$P < 0.01$, ***$P < 0{\cdot}002$). *N* for each point = 13–15 and error bars indicate 1 SE.

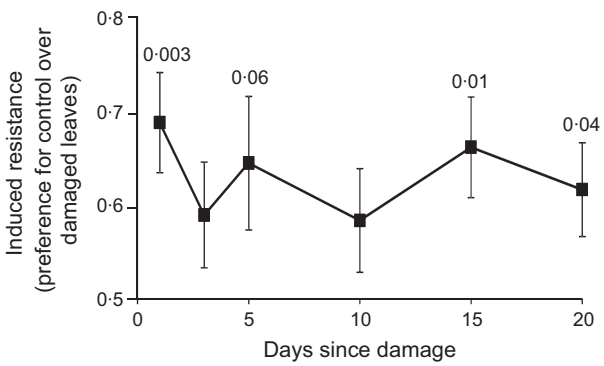

Fig. 2. Time course of induced resistance as measured by *Spodoptera* larva preference for undamaged over damaged leaves. Induced resistance calculated as % control leaves eaten/(% control +% damaged leaves eaten). A value of 0·5 indicates no preference between damaged and undamaged leaves, and values over 0·5 indicate preference for undamaged leaves (induced resistance). *N* for each point = 14 or 15, error bars indicate 1 SE.

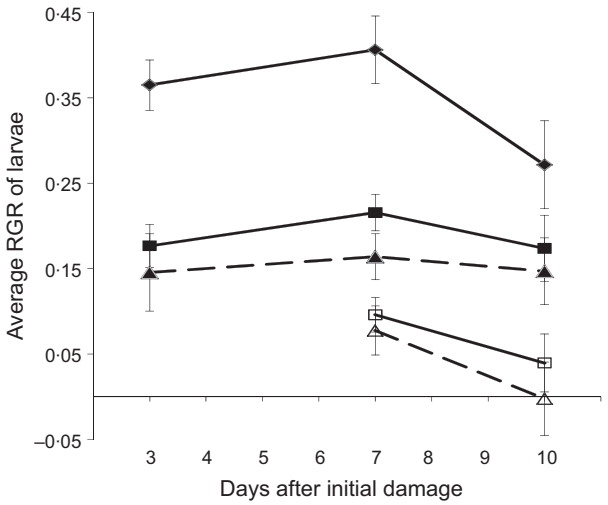

Fig. 3. Time course of plant quality as measured by larval relative growth rate (RGR) in response to five different damage treatments: no damage (♦), a single low-damage event (■), a single high damage event (▲), low damage after low damage (□) and low damage after high damage (△). All damage treatments were completed in 24 h. Larval relative growth rate on all plant treatments (recently damaged, control or damaged at some previous time) was measured by bioassay 3 days after each damage event ended. The second damage event began on day 3 and ended on day 4. Error bars indicate 1 SE. *N* for each point = 18.

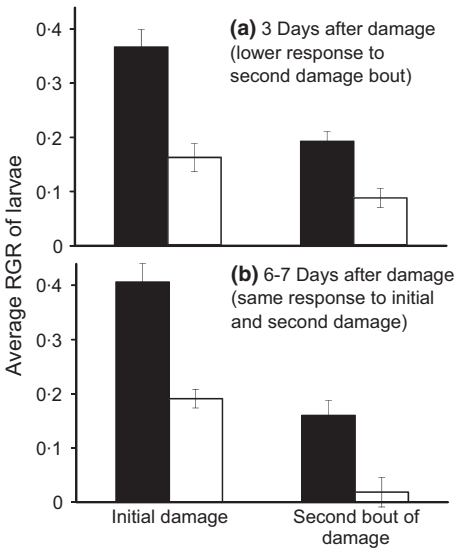

Fig. 4. The induced response to a second damage event was smaller than the response to a single-damage event 3 days after damage (panel a); there was no difference in the response to two versus one damage events by 6–7 days after damage (panel b). Baseline (filled bars) indicates estimated resistance of plants before the associated damage treatment and response (open bars) indicates the resistance level after damage. The baseline for the initial damage treatment is undamaged plants, while the baseline resistance for plants receiving a second round of damage is plants that had received one previous damage treatment (Table 2). *N* for the baselines for initial damage = 18; *N* for remaining bars = 33–36. Error bars indicate 1 SE.

the three bioassays (P's for least squared means contrasts between the two damage treatments, all >0·2). There was however a consistent pattern of plants with low damage being less resistant than plants with high damage (Fig. 3, lines with solid squares and solid triangles).

Considering only damaged plants after the second damage event (Fig. 3 bottom four points on days 7 and 10), plants did respond to a second round of damage with increased resistance (main effect of whether plants were damaged once or twice, $F_{1,175} = 44{\cdot}98$, $P < 0{\cdot}0001$). The level of resistance reached was the same whether the plants initially received high or low damage (no interaction between number of damage events and initial damage level).

The magnitude of change in resistance 3 days after the second damage event was different from the change induced 3 days after the first damage event (Fig. 4a). Three days after damage, there was a significantly smaller overall response to a second low-damage event (both initial damage treatments combined) than to the initial damage event (both initial damage treatments combined) (interaction of baseline/response and damage event, $F_{1,122} = 6{\cdot}14$, $P = 0{\cdot}01$). This interaction is also significant using only low initial damage at the first damage event. However, the change in resistance 6–7 days after the second damage event did not differ from the change induced 7 days after the first damage event (interaction of baseline/response and damage event, $F_{1,124} = 2{\cdot}75$, $P = 0{\cdot}1$, Fig. 4b). In an exploratory analysis, plants with initially low damage had the same response 3 days after damage to the second and initial bouts of low damage (no significant interaction ($P = 0{\cdot}1$) between baseline versus damaged and one versus two damage events), while plants with high initial damage had a smaller response 3 days after damage to low damage than plants that had only one bout of low damage (significant interaction between baseline versus damage and one versus two damage events; $F_{1,69} = 4{\cdot}09$, $P = 0{\cdot}048$).

Discussion

Relatively few studies have followed non-volatile components of induced resistance in herbaceous plants long enough to document full decay of resistance (Karban 2011), but effects of damage on resistance lasting up to 3 weeks have been found (Underwood 1998 in soybean; Gomez, Dijk & Steufer 2010 in clover). In this study, I found that induced resistance in tomato var Castlemart lasted at least 2 weeks and perhaps longer as measured by the behaviour of *Spodoptera* larvae (Fig. 2). Although both growth and choice bioassays indicate long-lasting induced resistance in this study, these two measures were not always in agreement (Pearson correlation $r = 0{\cdot}86$, $P = 0{\cdot}06$); it appears that while the larvae do perceive changes in plant quality which influence their performance, they may also sometimes perceive differences that do not influence their performance (cf. no significant difference in larval growth with damage treatment (Fig. 1) but a clear larval preference for undamaged plants (Fig. 2) at day 20).

Studies in other plant–insect systems that have measured larval preference and performance on damaged and undamaged plants have found that while larval behaviour can match performance (e.g. van Dam, Hadwich & Baldwin 2000; caterpillars leave and have low growth on damaged plants), the two can also be decoupled (e.g. Wise & Weinberg 2002; beetle larvae grow slower on damaged plants but have no preference for damaged versus undamaged plants). The fact that resistance can last a fairly long time suggests that plants may be damaged again before induced responses to initial damage have fully decayed (although the signalling pathways for producing resistance traits may turn off sooner). *Spodoptera* larvae do leave damaged plants at a higher rate than undamaged plants, but when placed between damaged and undamaged plants in an experimental arena, they arrive at both plant types with equal frequency (N. Underwood, unpublished data), suggesting that larvae may well return to previously damaged plants, although later bouts of damage may be smaller than initial bouts.

Only one previous experiment (Stout & Duffey 1996) has followed induced resistance in tomato longer than this study; that study found, also in Castlemart, that induced resistance lasted as long as 23 days in the damaged leaf, but had decayed between 13 and 23 days for undamaged leaves. Results of this study thus suggest similar or longer systemic induction. It is possible that longer systemic induction might arise from a higher level of initial damage (approximately 20%) in this experiment than in Stout and Duffey (which used 10% damage). The level of induced resistance in tomato is known to differ with the amount of initial damage (Underwood 2010), but little is known about whether higher initial levels of damage lead to longer lasting induced responses. Both this study and Stout & Duffey (1996) found a substantial decline in undamaged plant quality with plant age. In this study, this decline was interrupted by a temporary increase in plant quality at day 15. It is unclear what might have caused this; an external influence is unlikely because the experiment was carried out across multiple temporal blocks, and no obvious phenological event occurred at that time. Regardless of their origin, these changes in undamaged plant quality suggest that it is critical in any study of repeated damage to have controls that account for such background changes in resistance.

When induced resistance lasts for more than a few hours or days, repeated damage during responses to initial damage is possible. In this study, tomato plants were able to increase their resistance in response to a second bout of damage, building on induced resistance to previous damage; in experiment 2, *Spodoptera* larvae grew more slowly on plants that were damaged a second time than on plants that had only been damaged a single time, although the once-damaged plants were still significantly more resistant than control plants at the time of second damage (Fig. 3). Put another way, one bout of damage reduced larval growth approximately 50% relative to control plants at the same time, and a second bout of damage reduced growth a further 50% relative to plants damaged only once. This result is similar to responses to second rounds of damage in other plants, where resistance was higher after two rounds of damage than after one round (Agrawal 1998; Poelman *et al.* 2008).

To understand how total plant resistance might change through time in response to multiple attacks, we also need to know whether subsequent attacks provoke the same *response* as initial attacks, or whether, as has been suggested, later attacks might provoke either larger (in the case of immune-like memory) or smaller (in the case of physiological limits) responses. Measuring the response to attack, as opposed to the level of resistance reached, requires having a baseline for comparison. In this study, those controls were provided by following damaged and undamaged treatments through multiple sample dates (Table 2). I found that 3 days after damage, the change in resistance provoked by a second bout of damage was significantly smaller than the change in resistance provoked by the initial bout of damage (Fig. 4a). By six (in the case of two damage events) to seven (in the case of one damage event) days after damage, however, there was no difference in the magnitude of induced response between plants that received one bout of damage and plants that received two bouts of damage (Fig. 4b). These results suggest two things: first, there may be limits on the plant's ability to produce induced resistance with repeated damage, and second, the response to a second damage event can be slower than the response to the first event, rather than ultimately smaller in magnitude.

The fact that the response to a second damage event is initially smaller and slower to develop than the response to a single-damage event suggests that plants that are damaged multiple times may lack the resources to immediately produce larger responses. There is also a suggestion in the data from this study that the degree of constraint on the plant's response may depend on the level of previous damage received (exploratory analysis suggests that the response to two versus one low-damage events 3 days after damage is lower for plants with initially high damage but not for plants with initially low damage). Constraints on responses to cumulative damage have been assumed in models of induced resistance (Edelstein-Keshet & Rausher 1989; Underwood 1999; Underwood, Anderson & Inouye 2005); this study supports those assumptions. While studies have shown a limit to plant responses to increasing damage at one time (e.g. Underwood 2010) and constraints on responses to different types of damage arising from crosstalk between jasmonate and salicylate pathways (e.g. Thaler, Humphrey & Whiteman 2012), this study is the first to provide evidence of a constraint in response to repeated damage by the same herbivore. It might also be possible that smaller or slower responses are adaptive because of diminishing benefits from higher and higher levels of resistance; this would be an interesting direction for future research. If the pattern observed in this study turns

out to be present in other systems, future models should consider the consequences of slower responses to repeated damage. Slower plant responses to repeated damage will require simulation rather than analytical models because number and timing of damage events will have to be taken into account.

There is no evidence from this study that tomato plants exhibit immunological memory in the magnitude or speed of their response to damage. The level of induced resistance reached with repeated damage was higher than with one bout of damage (Fig. 3), but this was because the response to repeated damage built on previously existing resistance rather than because of an increased magnitude of response. The clearest previous demonstration of 'memory' in induced resistance to a single herbivore involved a faster rather than a larger response to damage (Baldwin & Schmelz 1996). There is speculation about what mechanisms might allow memory (Gális *et al.* 2009), but still little known about how common effects of previous damage on the speed or magnitude of response in the damaged plant might be. In this study, more sample dates would have been required to determine whether repeated damage might provoke a larger early response (before 3 days after damage), or whether plants with repeated damage might eventually produce a larger response (after 6 days after damage). Given that in this study the response increased between 3 and 6 days after damage, a transient early response is unlikely, but a continued increase in resistance after 6 days seems possible, because the response to a single damage event can last at least 15 days (Fig. 1). It is also important to note that herbivore traits (growth rate, survival, etc.) are in general an imperfect measure of memory in the plant's response to damage. Depending on the relationship between the herbivore trait and a particular plant trait, it is possible that there might be memory operating in the production of the plant trait that is not reflected in the herbivore, for example, if the herbivore trait reaches an asymptote as the plant trait changes. In this study, herbivore growth rate did not reach any natural lower limit (i.e. growth rates can be negative during a short bioassay), so it is likely that larger responses resulting from memory could have been detected if they occurred. However, there can be an asymptote in the relationship between damage to tomato and *S. exigua* growth rate (Underwood 2010), so some caution is needed regarding interpreting memory in the magnitude of production of resistance traits in this study.

Summary

Studies from many systems indicate that induced resistance can last long enough that plants are likely attacked again before previous induced responses have decayed. However, few studies have measured how plants respond to sequential attacks, especially by the same herbivore. In this study, I found that induced resistance was fairly long-lasting and that plants were able to increase their level of resistance in response to the second bout of damage, consistent with the results of previous studies. However, there also appears to be some limit to the plant's ability to respond to repeated damage as the response to a second damage event was initially smaller and slower than the response to the first damage event. Understanding how plants respond to repeated attacks will be important for determining how induced responses influence herbivore movement, performance and damage to plants through time. Although initial damage level was not a significant factor in this experiment, evidence from other studies and a suggestive pattern from this study suggest that the amount of damage may interact with the response to repeated damage. Fully characterizing how plants respond to damage through time should include measuring the magnitude of response (with appropriate controls) over time and over different levels of damage.

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References

Agrawal, A.A. (1998) Leaf damage and associated cues induce aggressive ant recruitment in a neotropical ant-plant. *Ecology*, **79**, 2100–2112.

Baldwin, I.T. & Schmelz, E.A. (1994) Constraints on an induced defense: the role of leaf area. *Oecologia*, **97**, 424–430.

Baldwin, I.T. & Schmelz, E.A. (1996) Immunological 'memory' in the induced accumulation of nicotine in wild tobacco. *Ecology*, **77**, 236–246.

Barton, K.E. & Koricheva, J. (2010) The ontogeny of plant defense and herbivory: characterizing general patterns using meta-analysis. *American Naturalist*, **175**, 481–493.

Broadway, R.M., Duffey, S.S., Pearce, G. & Ryan, C.A. (1986) Plant proteinase inhibitors: a defense against herbivorous insects? *Entomologia Experimentalis et Applicata*, **41**, 33–38.

Edelstein-Keshet, L. & Rausher, M.D. (1989) The effects of inducible plant defenses on herbivore populations. 1. Mobile herbivores in continuous time. *American Naturalist*, **133**, 787–810.

Edwards, P. J., Wratten, S. D. *et al.* (1985) Wound-induced changes in the acceptability of tomato to larvae of *Spodoptera littoralis*: a laboratory bioassay. *Ecological Entomology*, **10**, 155–158.

Frost, C.J., Mescher, M.C., Carlson, J.E. & De Moraes, C.M. (2008) Plant defense priming against herbivores: getting ready for a different battle. *Plant Physiology*, **146**, 818–824.

Gális, I., Gaquerel, E., Pandey, S.P. & Baldwin, I.T. (2009) Molecular mechanisms underlying plant memory in JA-mediated defence responses. *Plant, Cell & Environment*, **32**, 617–627.

Gomez, S., van Dijk, W. & Steufer, J.F. (2010) Timing of induced resistance in a clonal plant network. *Plant Biology*, **12**, 512–517.

Howe, G.A. & Ryan, C.A. (1999) Suppressors of systemin signaling identify genes in the tomato wound response pathway. *Genetics*, **153**, 1411–1421.

Karban, R. (2011) The ecology and evolution of induced resistance against herbivores. *Functional Ecology*, **25**, 339–347.

Karban, R. & Niiho, C. (1995) Induced resistance and susceptibility to herbivory: plant memory and altered plant development. *Ecology*, **76**, 1220–1225.

Lange, W.H. & Bronson, L. (1981) Insect pests of tomatoes. *Annual Review of Entomology*, **26**, 345–371.

Morris, W.F. & Dwyer, G. (1997) Population consequences of constitutive and inducible plant resistance: herbivore spatial spread. *American Naturalist*, **149**, 1071–1090.

Orians, C.M., Pomerleau, J. & Ricco, R. (2000) Vascular architecture generates fine scale variation in systemic induction of proteinase inhibitors in tomato. *Journal of Chemical Ecology*, **26**, 471–485.

Poelman, E.H., Broekgaarden, C., van Loon, J.J.A. & Dicke, M. (2008) Early season herbivore differentially affects plant defence responses to subsequently colonizing herbivores and their abundance in the field. *Molecular Ecology*, **17**, 3352–3365.

Quintero, C. & Bowers, M.D. (2011) Plant induced defenses depend more on plant age than previous history of damage: implications for plant-herbivore interactions. *Journal of Chemical Ecology*, **37**, 992–1001.

Rodriguez-Saona, C.R., Rodriguez-Saona, E.R. & Frost, C.J. (2009) Herbivore induced volatiles in the perennial shrub, *Vaccinium corymbosum*, and their role in inter-branch signaling. *Journal of Chemical Ecology*, **35**, 163–175.

SAS Institute (2003) *The SAS System for Windows 8.02*. SAS Institute, Cary, North Carolina, USA.

Stork, W., Diezel, C., Halitschke, R., Gális, I. & Baldwin, I.T. (2009) An ecological analysis of the herbivory-elicited JA burst and its metabolism: plant memory processes and predictions of the moving target model. *PLoS ONE*, **4**, 1–15.

Stout, M.J. & Duffey, S.S. (1996) Characterization of induced resistance in tomato plants. *Entomologia Experimentalis et Applicata*, **79**, 273–283.

Stout, M.J., Workman, K.V., Workman, J.S. & Duffey, S.S. (1996) Temporal and ontogenetic aspects of protein induction in foliage of the tomato, *Lycopersicon esculentum*. *Biochemical Systematics and Ecology*, **24**, 611–625.

Thaler, J.S., Humphrey, P.T. & Whiteman, N.K. (2012) Evolution of jasmonate and salicylate signal crosstalk. *Trends in Plant Science*, **17**, 260–270.

Thaler, J.S., Stout, M.J., Karban, R. & Duffey, S.S. (1996) Exogenous jasmonates simulate insect wounding in tomato plants (*Lycopersicon esculentum*) in the laboratory and field. *Journal of Chemical Ecology*, **22**, 1767–1781.

Thaler, J.S., Stout, M.J., Karban, R. & Duffey, S.S. (2001) Jasmonate-mediated induced plant resistance affects a community of herbivores. *Ecological Entomology*, **26**, 312–324.

Underwood, N.C. (1998) The timing of induced resistance and induced susceptibility in the soybean Mexican bean beetle system. *Oecologia*, **114**, 376–381.

Underwood, N. (1999) The influence of plant and herbivore characteristics on the interactions between induced resistance and herbivore population dynamics. *American Naturalist*, **153**, 282–294.

Underwood, N. (2000) Density dependence in induced plant resistance to herbivore damage: threshold, strength and genetic variation. *Oikos*, **89**, 295–300.

Underwood, N. (2010) Density dependence in insect performance within individual plants: induced resistance to *Spodoptera exigua* in tomato. *Oikos*, **119**, 1993–1999.

Underwood, N., Anderson, K. & Inouye, B.D. (2005) Induced versus constitutive resistance and the spatial distribution of insect herbivores among plants. *Ecology*, **86**, 594–602.

van Dam, N.M., Hadwich, K. & Baldwin, I.T. (2000) Induced responses in *Nicotiana attenuata* affect behavior and growth of the specialist herbivore *Manduca sexta*. *Oecologia*, **122**, 371–379.

Viswanathan, D.V., Lifchits, O.A. & Thaler, J.S. (2007) Consequences of sequential attack for resistance to herbivores when plants have specific induced responses. *Oikos*, **116**, 1389–1399.

Voelckel, C. & Baldwin, I.T. (2004) Herbivore-induced plant vaccination: part II. Array-studies reveal the transience of herbivore-specific transcriptional imprints and a distinct imprint from stress combinations. *Plant Journal*, **38**, 650–663.

Wise, M.J. & Weinberg, A.M. (2002) Prior flea beetle herbivory affects oviposition preference and larval performance of a potato beetle on their shared host plant. *Ecological Entomology*, **27**, 115–122.

Ziegler, J., Keinanen, M. & Baldwin, I.T. (2001) Herbivore-induced allene oxide synthase transcripts and jasmonic acid in *Nicotiana attenuata*. *Phytochemistry*, **58**, 729–738.

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