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Jasmonic acid-induced plant defenses delay caterpillar developmental resistance to a baculovirus: Slow-growth, high-mortality hypothesis in plant–insect–pathogen interactions

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ABSTRACT

Plants damaged by herbivore feeding can induce defensive responses that reduce herbivore growth. The slow-growth, high-mortality hypothesis postulates that these non-lethal plant defenses prolong the herbivore's period of susceptibility to natural enemies, such as predators and parasitoids. While many juvenile animals increase their disease resistance as they grow, direct tests of the slow-growth, high-mortality hypothesis in the context of plant–herbivore–pathogen interactions are lacking. Caterpillars increase their resistance to lethal baculoviruses as they develop within and across instars, a phenomenon termed developmental resistance. Progression of developmental resistance can occur through age-related increases in systemic immune functioning and/or midgut-based resistance. Here, we examined the slow-growth, high-mortality hypothesis in the context of developmental resistance of caterpillars to baculoviruses. Intra-stadial (within-instar) developmental resistance of the fall armyworm, *Spodoptera frugiperda*, to an oral inoculum of the baculovirus SfMNPV increased more rapidly with age when larvae were fed on non-induced foliage than foliage that was induced by jasmonic acid (a phytohormone that up-regulates plant anti-herbivore defenses). The degree of developmental resistance observed was attributable to larval weight at the time of virus inoculation. Thus, slower growth on induced plants prolonged the window of larval susceptibility to the baculovirus. Developmental resistance on induced and non-induced plants was absent when budded virus was injected intrahemocoelically bypassing the midgut, suggesting that developmental resistance was gut-based. Addition of fluorescent brightener, which weakens midgut-based resistance mechanisms to oral virus challenge, abolished developmental resistance. These results highlight the impact of plant defenses on herbivore growth rate and consequences for disease risk.

1. Introduction

The ubiquity of pathogens can nearly guarantee that all organisms face risk of disease at some point in their life cycle. Susceptibility during an organism's life is variable, as investment in pathogen defense can depend on the resource needs of other life-history traits (Schmid-Hempel, 2011). One commonality among animal taxa is that juveniles are usually more susceptible to pathogens than adults, and juveniles usually become more resistant to pathogens as they age (Kloosterman et al., 1991; Ortega-Mora and Wright, 1994; Ryce et al., 2004). A prime example is the increasing resistance of the caterpillar stages of moths and butterflies to a group of viral pathogens, baculoviruses, both within and across larval instars, termed developmental resistance (Engelhard and Volkman, 1995; Grove and Hoover, 2007; Hoover et al., 2002; Teakle et al., 1986).

The mechanisms of caterpillar developmental resistance to baculoviruses can be system specific, depending on the effectiveness of host resistance mechanisms against a particular species of virus. The host's resistance mechanisms can interfere with virus pathogenesis at numerous points in the infection process. The infection process starts when viral occlusion bodies (OBs) are ingested on foliage and dissolve in the alkaline pH of the caterpillar's midgut, releasing infective virions (occlusion derived viruses) that bind to and infect midgut epithelial cells. Infected midgut cells then produce a second virus phenotype, budded virus (BV), which initially infects tracheolar cells and uses the tracheal system as a conduit to initiate a systemic infection (Harrison and Hoover, 2012). Most studies of caterpillar resistance mechanisms to baculoviruses have focused on noctuid caterpillars. In fully permissive

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(susceptible) noctuid–baculovirus systems, the host's anti-viral defenses are primarily restricted to blocking initial midgut infections, because most hosts do not show systemic resistance to viral infection (Engelhard and Volkman, 1995; Haas-Stapleton et al., 2003; Volkman et al., 2001). Midgut resistance mechanisms include the peritrophic matrix, which serves as a physical barrier (Wang and Granados, 2000, 1998, 1997), apoptosis of infected midgut cells (Dougherty et al., 2006) and the sloughing of infected midgut cells (Hoover et al., 2000; Washburn et al., 1998). In addition to midgut-based resistance, some lepidopterans that are non-permissive or semi-permissive to a certain baculovirus limit the systemic spread of virus infection by cellular encapsulation and melanization of infected host tissues (Trudeau et al., 2001; Washburn et al., 2000). Systemic resistance has also been shown in the permissive host, gypsy moth, *Lymantria dispar*, larvae, to its baculovirus, *L. dispar* nucleopolyhedrovirus (McNeil et al., 2010a,b).

The consensus mechanism of intra-stadial developmental resistance in permissive noctuid hosts studied to date appears to be the strengthening of the peritrophic matrix and apoptosis and subsequent sloughing of infected midgut cells for two main reasons: (1) larvae challenged with viral OBs later in the instar exhibited significantly fewer midgut infection foci than larvae challenged earlier in the same instar (Engelhard and Volkman, 1995; Volkman et al., 2001; Washburn et al., 1995) and (2) larvae did not exhibit developmental resistance if midgut resistance mechanisms were bypassed by injecting the BV viral phenotype directly into the hemocoel (Engelhard and Volkman, 1995; Washburn et al., 1995). Importantly, studies of developmental resistance have been conducted primarily on artificial diets, but caterpillars in the field feed on plants that vary widely in nutritional quality and allelochemical defenses. These qualitative differences among plants can influence larval growth rates and physical and immunological resistance mechanisms (Cory and Hoover, 2006; Lampert, 2012; Shikano, 2017). Noctuid caterpillars fed foliage from different plant species can differ markedly in resistance to oral inoculation of baculovirus OBs (Hoover et al., 2000; Plymale et al., 2008; Shikano et al., 2010). The mechanism for this is still unclear as plant-mediated variation in caterpillar physiological resistance to viral OBs has been linked to rates of midgut cell sloughing (Hoover et al., 2000), the thickness and structure of the peritrophic matrix (Plymale et al., 2008) and strength of systemic resistance (Shikano et al., 2010, 2015).

Not only does plant quality vary between plants and spatially (e.g., foliage vs. reproductive parts) and temporally within a plant, damage to plant tissues during feeding can induce defensive responses in plants. Plants have a vast array of inducible anti-herbivore defenses, including alterations to primary and secondary chemical metabolites, physical resistance, gross morphology, life-history and physiology (Agrawal, 2011; Chen, 2008; Schaller, 2008; Zhu-Salzman et al., 2008). Many of these induced plant defenses are not immediately lethal to herbivores but rather reduce herbivore development by decreasing the nutritional quality, digestibility and palatability of the plant tissue. Ecologists have postulated that this sublethal form of plant defense indirectly increases herbivore mortality by increasing the herbivore's period of vulnerability to natural enemies, termed the slow-growth, high-mortality hypothesis (Feeny, 1976; Leather, 1985; Loader and Damman, 1991; Moran and Hamilton, 1980). Direct tests of this hypothesis have mainly focused on insect herbivores and their exposure to predators and parasitoids, with mixed support for the hypothesis (Benrey and Denno, 1997; Clancy and Price, 1987; Cornelissen and Stiling, 2006; Leather and Walsh, 1993; Williams, 1999). These varied findings stem from the fact that inter- and intraspecific variations in plant qualities influence a complex array of herbivore–predator/parasitoid interactions. Plants influence the herbivore's physical, immunological and chemical defenses, the nutritional quality of herbivores as a food source for the natural enemies, and the host searching and host handling abilities of the natural enemies (Hopkins et al., 2009; Lampert, 2012; Thaler, 1999, 2002).

Direct tests of the slow-growth, high-mortality hypothesis in the context of plant–insect–pathogen interactions are lacking. Since the susceptibility of caterpillars to baculoviruses is strongly dependent on developmental stage, it seems likely that plant-mediated retardation of caterpillar growth would keep the caterpillars at a more susceptible developmental stage for a longer period of time. Several studies have shown that caterpillar resistance to baculoviruses can vary after feeding on different food plant species (or artificial diet) for a set period of time within an instar (Hoover et al., 2000, 2002; Plymale et al., 2008). Whether this difference in intra-stadial developmental resistance associated with interspecific variation in plant quality was due to differential caterpillar growth is unknown, since the caterpillars were not weighed prior to virus inoculation. Similarly, no studies have examined whether the induced anti-herbivore defenses of plants serve to increase the period of susceptibility of insects to pathogens (i.e., delay developmental resistance).

Here, we investigated whether induced anti-herbivore defenses of soybean plants (*Glycine* max) inhibit the growth of fall armyworms (*Spodoptera frugiperda*) and increase their susceptibility to the baculovirus *S. frugiperda* multiple nucleopolyhedrovirus (SfMNPV). As potent natural mortality factors of lepidopteran larvae, infection rates by baculoviruses during epizootics can exceed 90% (Myers and Cory, 2016), and SfMNPV is known to cause epizootics in fall armyworm populations (Fuxa, 2004). We induced anti-herbivore defensive responses in soybean plants using jasmonic acid (JA), which is a phytohormone that upregulates plant defenses against chewing herbivores (Thaler et al., 1996). Importantly, we used a soybean genotype (Braxton) that, when induced, significantly prolongs fall armyworm development time, reduces feeding rate and the conversion of ingested foliage into body mass compared to non-induced foliage (Shikano et al., 2017a,b). By using this soybean genotype, we were able to untangle the effects of feeding time (i.e., time post molt), caterpillar weight and the quantity of ingested plant material on developmental resistance. This is important because previous studies of intra-stadial developmental resistance only used a single diet source, such that feeding time, larval weight and quantity of diet consumed were all dependent on one another. Thus, one of the key unanswered questions that we examined is whether the degree of intra-stadial developmental resistance is determined by the time since molting, amount of food consumed or the size (or growth) of the insect. Lastly, to determine whether the effects of induced plant defenses on developmental resistance was due to plant-mediated effects on midgut or systemic resistance mechanisms, we inoculated the caterpillars orally with virus OBs and intrahemocoelically with BVs.

2. Materials and methods

2.1. Insects and plants

Fall armyworm, *Spodoptera frugiperda*, eggs were purchased from Benzon Research (Carlisle, PA, USA) and larvae were reared individually on artificial diet (Southland Products Inc., Lake Village, AR, USA) at 29 °C and 16:8 h (L:D). Larvae were collected in the late third instar during head capsule slippage, and kept in individual wells of a 12-well cell culture plate to complete their molt without food. Newly molted fourth instar larvae were immediately presented with the food plant treatments described below.

Soybean, *Glycine max*, seeds (genotype: Braxton) were obtained from the National Plant Germplasm System (USDA-ARS). Seeds were sown individually in 10 cm plastic pots in professional growing mix (Sunshine Mix 4 Aggregate Plus). Plants were watered daily and maintained in a temperature-controlled greenhouse at 25 °C and 16:8 h (L:D) supplemented with high pressure sodium lights. Plants were fertilized with 3 g of Osmocote Plus (15–9–12; Scotts) when the first true leaves were fully expanded. For each trial, 60 plants (V5 stage) were divided equally into jasmonic acid (JA)-induced or non-induced treatments. Plants were sprayed with 2 mM JA until runoff (approximately

5 ml per plant) and used for experiments 48 h later (Shikano et al., 2017b). Non-induced control plants received the carrier solution. Previously, feeding by *S. frugiperda* on JA-induced foliage of the Braxton genotype was shown to significantly prolong development time, and reduce feeding rate, weight gain and the conversion of ingested leaf material into body mass, compared to feeding on non-induced foliage (Shikano et al., 2017a,b).

2.2. Baculovirus inoculations

Our original objective was to compare differences in the numbers of midgut infection foci and rates of disease progression in *S. frugiperda* larvae fed induced or non-induced soybean foliage. Therefore, for oral inoculation of virus, a droplet of OBs of recombinant SfMNPV expressing a LacZ reporter gene was injected directly into the insect's foregut rather than allowing the insect to consume OBs with food. We injected the OBs directly into the foregut to control timing of virus inoculation. However, we did not observe inhibition of virus-induced mortality when larvae had pre-fed on induced foliage (i.e., the gut contained induced foliage at the point of virus inoculation) compared to larvae pre-fed non-induced foliage. In our previous study, ingestion of OBs on induced leaf disks, compared to non-induced leaf disks, inhibited virus-induced mortality (Shikano et al., 2017b). The lack of inhibition in the present study might have occurred because injection of OBs directly into the foregut bypassed all potential interactions between phytochemicals and OBs anterior to the midgut, including on the leaf disk surface. Instead, what we observed was slower larval growth on induced than non-induced foliage, and this slower growth coincided with higher susceptibility of larvae to virus. Thus, we pursued the question of whether plant-mediated slower growth delays developmental resistance to baculovirus. Since the preliminary work had been conducted with the recombinant virus, we continued to use it for the entire experiment.

2.3. Construction of the recombinant SfMNPV

SfMNPV, isolate "Nicaragua" (SfMNPV-Nicaragua), DNA was purified from 2×10^8 polyhedral OBs using modifications of the method from O'Reilly et al. (1994). The *in vivo* OBs (Genbank Accession HM595733.1) were provided by Primitivo Caballero, Instituto de Agrobiotecnología, CSIC, Universidad Pública de Navarra, Spain. The purified DNA was then digested with HindIII (New England BioLabs [NEB], Ipswich, MA). The cloning vector, pBluescript II (SK+) was also digested with HindIII, followed by dephosphorylation using FastAP-thermosensitive alkaline phosphatase (Thermofisher Scientific, Waltham, MA). The digested DNAs were separated on a 1.2% Tris-borate-EDTA (TBE) agarose gel. Following electrophoresis, the bands were visualized using 0.001% nile blue as a post-stain. The SfMNPV 5.9 kilobase pair band (kb) and the linearized pBluescript II (SK+) were excised from the agarose, and were purified using GENECLEAN SPIN Kit (MP Biomedicals, Solon, OH). The 5.9 kb fragment contains SfMNPV sequences from nucleotides 78,927 to 84,812. The bands were then ligated at a 1:3 M ratio of vector: insert using T4 Ligase (NEB), following product protocol. Five µl of the ligation mix was used to transform competent DH5α cells using standard techniques. One hundred µl of the transformation was plated on LB agar plates containing 100 µg/ml of ampicillin. Correct colonies were 8.8 kb in total size, in which 5.9 kb was from SfMNPV. A clone containing a 5.9 kb insert was digested with HpaI (NEB), followed by dephosphorylation. HpaI linearizes the clone within the 5.9 kb insert at nucleotide position 81,245, an area that would not interrupt any SfMNPV gene, its promoter, or its termination sequence and polyadenylation signal. Plasmid p216.1 (from American Cyanamid) is derived from pAcDZ1, a plasmid that contains the *Escherichia coli* β-galactosidase gene (*lacZ*) that is transcribed from the *Drosophila melanogaster* heat-shock promoter (hsp70) and contains the simian virus 40 DNA fragment to act as terminator (Zuidema et al., 1990). Plasmid p216.1 was digested with XbaI and BamHI, end-filled using DNA polymerase I, large (Klenow) fragment (NEB). Both the SfMNPV clone digested with HpaI and the p216.1 clone digested with BamHI and XbaI were run on a 1.2% TBE agarose gel and post-stained with nile blue. The 8.8 kb SfMNPV band and the 3.7 kb band of p216.1 containing the *LacZ* cassette were excised and purified as previously described (McNeil et al., 2010a,b). The bands were ligated as described earlier; then 5 µl of the ligation mix was used to transform competent DH5-α cells using standard techniques. One hundred µl of the transformation mix was spread on LB-ampicillin plates containing 20 µg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) for visualization of colonies containing the *lacZ* cassette. Positive (blue) colonies were screened for correctness, as well as the orientation of the *lacZ* gene within the clone.

DNA from the SfMNPV-*LacZ* clone was ultra-purified using GeneJET Plasmid Midiprep Kit (ThermoFisher). This DNA was then co-transfected with genomic SfMNPV-Nicaragua DNA into the *Spodoptera frugiperda* cell line IPLB-Sf-21 (Vaughn et al., 1977) (provided by Robert Harrison, ARS, Beltsville, MD), using Cellfectin-II® (ThermoFisher) as the transfection reagent. When the transfection showed the completion of the viral infection, the media containing budded virus (BV), was diluted (10^{-4}, 10^{-5}, and 10^{-6}) with ExCell 420 media (Sigma-Aldrich, St. Louis, MO) that contained 5% Serum Plus Media Supplement (Sigma-Aldrich) and was plaque-purified by standard plaque assay techniques. The agarose overlay contained ExCell 420, 5% Serum Plus, sterile BD BaculoGold Plaque Assay Agarose (BD Biosciences, Franklin Lakes, NJ), final concentration 0.8%, and 20 µg/ml X-gal dissolved in dimethyl sulfoxide (Sigma-Aldrich). The blue plaques that were the most separated from other plaques in the highest dilution were transferred to a p96 well culture plate (Corning Life Sciences Inc., Acton, MA) seeded with 10^4 cells/well and 200 µl of media (ExCell 420, 5% Serum Plus) per well. Infections in the p96 wells were allowed to develop for seven days; at which time, 100 µl of medium was removed from each well and was transferred to a new p96 plate containing 100 µl sterile, deionized water and 20 µg/ml of X-gal solution. The wells that displayed the darkest level of blue color in the shortest time were identified and the BV from their original wells were once again diluted (10^{-4}, 10^{-5}, 10^{-6}) and plaqued-out for a second time. The same protocol was followed in the selection of the second round of blue plaques. The BV from five of the blue plaques, which were the darkest blue in the fastest time, were transferred to T25 flasks seeded at 10^5 Sf-21 cells/ml. The infections continued for one week, at which time, 1 ml of medium from each flask was used to infect T150 flasks (two T150s for each blue plaque) seeded at 2×10^5 cells/ml. Following one week of infection, the entire contents of the flasks (BV and the cell debris containing OB) were transferred to sterile 50 ml centrifuge tubes (Corning Life Sciences) and spun on a clinical centrifuge at 5125*g*, room temperature for five minutes. The clear supernatant containing BV was transferred to a sterile 50 ml tube and stored at 4 °C. This BV suspension was diluted to produce the BV dose used in the intrahemocoelic inoculation experiment described below. The concentrations of BVs in the stock solution and subsequent dilutions were determined by virus plaque assays. Ten ml of sterile deionized water was added to the pellets remaining in the 50 ml tube, which contained the OBs. Each OB pellet was sonicated (20 W for 30 s) and stored at 4 °C. The resulting OB suspension, which also contained cell debris, was diluted to produce the OB doses for the experiments described below (i.e., OBs were not re-amplified in larvae). The concentrations of OBs in the stock suspension and subsequent dilutions were counted under a phase contrast microscope using an improved Neubauer brightline haemocytometer.

BV DNA was prepared from 25 ml of clear supernatant of each of the five samples. DNA was isolated by the method from O'Reilly et al. (1994). The final purified DNA pellet was re-suspended in 200 µl TE and digested along with SfMNPV DNA with various restriction endonucleases to determine the purity of the recombinants by observing shifts in predicted band sizes, as well as for the presence of sub-molar bands.

2.4. Experiment 1: Larval feeding rate, weight and susceptibility to oral virus challenge

The experiments described below (*experiments 1, 2* and *3*) were conducted independently from one another. Leaf disks were excised from the youngest fully opened trifoliate leaf on each plant and placed individually in 30 ml containers lined with moistened paper towel. A single, newly molted fourth instar larva was placed in each cup and allowed to feed for 2, 4, 6, 8 or 12 h (designated 4^2, 4^4, 4^6, 4^8, 4^{12}, respectively) at 29 °C. The feeding time also corresponds to the time post molt. The remaining leaf disk area was measured using ImageJ 1.48 (NIH, http://imagej.nih.gov/ij/) to determine the amount of foliage consumed. After feeding, larvae were weighed to the nearest 0.1 mg and orally challenged with SfMNPV OBs. Two trials were conducted; each trial consisted of 30 virus-challenged and 10–12 mock-challenged larvae at each time point per plant treatment (JA-induced or non-induced). A micro-applicator (ISCO) fitted with a blunt 32-gauge needle was used to control the timing and amount of oral virus-challenge. A 1 µl suspension of virus OBs was administered through the mouth and deposited into the anterior midgut. The OB suspension contained 440 OBs μl^{-1} of the recombinant SfMNPV in 60% glycerol. Control larvae received 1 µl of 60% glycerol without virus. Larvae were then maintained individually with *ad libitum* artificial diet and mortality was recorded every 8 h. Since our objective was to assess the effects of plant induced defenses on intra-stadial developmental resistance, larvae were only fed their respective plant treatments from the start of the fourth instar until immediately prior to virus inoculation.

2.5. Experiment 2: Intrahemocoelic virus inoculation

To determine if plant-mediated within-instar resistance was due to midgut-based or systemic resistance, we intrahemocoelically injected larvae with the non-occluded phenotype (budded virus; BV) of the recombinant virus (3160 $TCID_{50}$ units μl^{-1} of Excell 420 + 5% serum medium). Larvae were fed leaf material from the plant treatment groups as described above for 12 h. Larvae were then weighed and injected with BV using a sharp-tip 33-gauge needle. The needle was inserted through the base of one of the prolegs and a 1 µl virus suspension was delivered directly into the hemocoel. The larvae were then maintained on artificial diet as described above. Two trials were conducted; each trial consisted of 30 virus-challenged and 15 mock-challenged larvae (i.e., injected with media only) per plant treatment. Thirty newly molted larvae (4^0) were inoculated with budded virus (and 15 mock-challenged) to provide a baseline susceptibility level to assess the degree of within-instar developmental resistance (i.e., positive control).

2.6. Experiment 3: Oral virus inoculation with fluorescent brightener (M2R)

The fluorescent brightener, Calcofluor white M2R, was added to the virus OB suspension. Previous studies showed that co-inoculation of baculovirus OBs and M2R produce greater numbers of virus infection foci in the midgut, compared to inoculation of virus alone (Hoover et al., 2000; Washburn et al., 1998). M2R increases midgut infection foci in numerous ways, including the disruption of the integrity of the peritrophic matrix (Bartnicki-Garcia et al., 1994; Wang and Granados, 2000), inhibition of apoptosis of infected midgut cells (Dougherty et al., 2006) and inhibition of sloughing of infected midgut cells (Hoover et al., 2000; Washburn et al., 1998).

Larvae were weighed and consumption measured after feeding for 6 or 12 h. Thirty larvae, per plant treatment by time combination, were orally inoculated with 316 OBs suspended in 1 µl of 60% glycerol, with or without M2R (1% w/v), using a blunt needle as described above. Thirty newly molted larvae (4^0) were virus-challenged without M2R to provide a baseline mortality level to compare the effects of M2R on within-instar resistance (i.e., positive control). Fifteen larvae per treatment group were mock-challenged with each carrier solution. Oral inoculation with just 1% M2R in glycerol solution (mock-challenge) is not known to cause mortality (Hoover et al., 2000). After challenge, larvae were fed artificial diet. In all experiments (*experiments 1, 2* and *3*), virus-infection was confirmed in all cadavers and no control larvae showed signs of virus infection.

2.7. Statistical analyses

Experiment 1. Larval weight and the area of leaf disk consumed over time, and the relationship between larval weight and consumption were analysed by multiple linear regression (MLR). Only data from larvae that were subsequently challenged with virus were used for MLR analyses. Virus-induced mortality in relation to feeding duration, leaf area consumption and larval weight was analysed using a generalized linear model (GLM; binomial distribution, logit link). Trial and plant treatment and interactions were included as variables in each analysis.

Experiment 2. Mortality induced by BV injection was analysed by GLM as described above, with treatment (positive control and JA-induced and non-induced plant treatments) and trial included as variables. Differences in BV-induced mortality between larvae pre-fed on JA-induced and non-induced foliage and differences in mortality between the positive control and larvae fed the plant treatments were assessed by Chi square contrasts.

Experiment 3. Mortality induced by oral inoculation with virus OBs alone and OBs plus 1% M2R were analysed separately using GLM. Differences in mortality between each treatment (6 h on JA-induced plant, 12 h on JA-induced plant, 6 h on non-induced plant and 12 h on non-induced plant) and the positive control were assessed by Chi square contrasts. All analyses were performed on JMP 12.1 (SAS Institute, Cary, NC, USA).

3. Results

3.1. Experiment 1: Larval feeding rate, weight and susceptibility to oral virus challenge

JA-induced soybean defense was evident as larvae ate less and grew slower over time on JA-induced than non-induced foliage (Table 1; Fig. 1a, b). Larvae in the first trial ate faster and were larger at the time of virus-challenge (trial by time by plant treatment interaction). Moreover, at a given amount of ingested foliage, larvae feeding on JA-induced foliage weighed less than those feeding on non-induced foliage (Table 2; Fig. 1c). This effect increased as more foliage was consumed.

Larval susceptibility to virus decreased with the time spent feeding prior to oral virus inoculation. Susceptibility to virus decreased more over time in larvae that consumed non-induced compared to JA-induced foliage (Fig. 2a; time × plant treatment: $X_1^2 = 5.39$, $p = 0.02$; trial: $X_1^2 = 0.43$, $p = 0.51$). The probability of virus-induced death

Table 1
Results from multiple linear regression analyses for the area of leaf disk consumed and larval weight at the time of virus-challenge. Trial, time and plant treatment (JA-induced vs. non-induced) and their interactions were included as predictor variables. Significant results are in bold font.

Source	Sqrt leaf area consumed (mm^2)			Sqrt larval weight (mg)		
	DF	*F*	*p*	*DF*	*F*	*p*
Trial	1, 579	**74.31**	**< 0.0001**	1, 579	**11.17**	**0.0009**
Time	1, 579	**3631**	**< 0.0001**	1, 579	**1319**	**< 0.0001**
Plant	1, 579	**447.6**	**< 0.0001**	1, 579	**337.9**	**< 0.0001**
Trial by time	1, 579	**15.73**	**< 0.0001**	1, 579	0.56	0.45
Trial by plant	1, 579	**13.87**	**0.0002**	1, 579	**4.25**	**0.040**
Time by plant	1, 579	**67.67**	**< 0.0001**	1, 579	**99.61**	**< 0.0001**
Trial by time by plant	1, 579	**11.50**	**0.0007**	1, 579	**9.79**	**0.002**

Plant = JA-induced vs. non-induced.

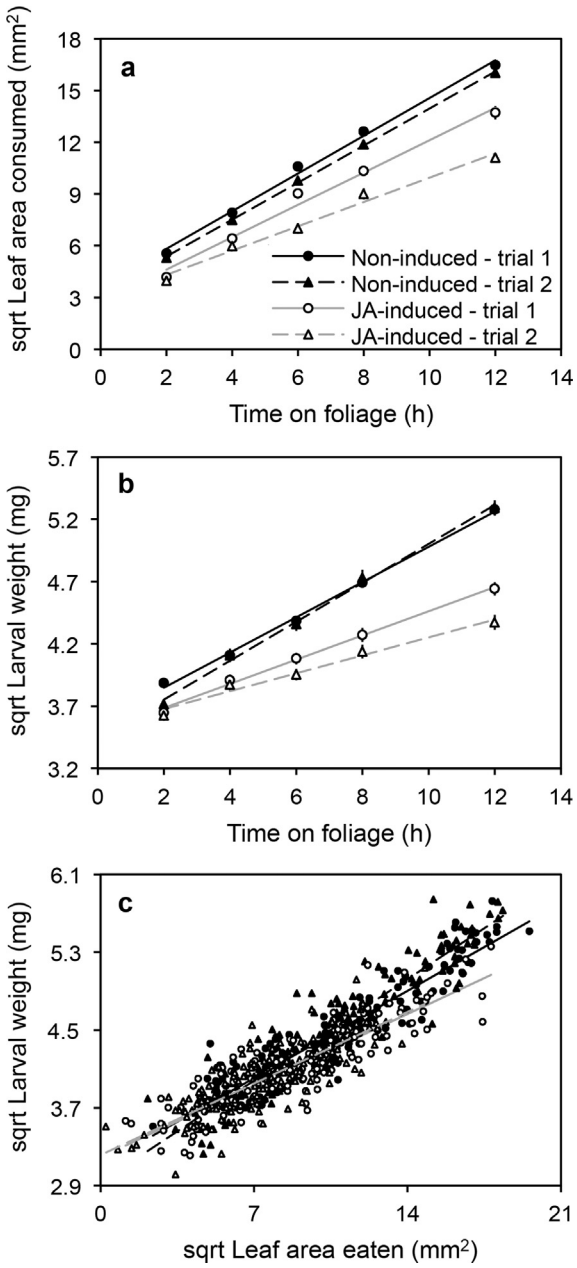

Fig. 1. (a) Area of leaf disk (mm^2) consumed by *S. frugiperda* larvae and (b) weight of larvae (mg) after 2, 4, 6, 8 and 12 h of feeding. (c) The relationship between the area of leaf disk consumed and larval weight. Symbols indicate mean values (± SE) and lines represent fitted lines from the statistical models.

decreased with increasing amount of foliage larvae had consumed before virus-challenge (Fig. 2b; consumption: $X_1^2 = 40.73$, $p < 0.0001$). The extent of this effect depended on the plant treatment ingested before virus challenge. At an equivalent consumed amount of JA-induced or non-induced foliage, the probability of death was significantly higher for larvae that had consumed JA-induced foliage (plant treatment: $X_1^2 = 4.04$, $p = 0.04$; trial: $X_1^2 = 1.25$, $p = 0.26$; no interactions). The probability of virus-induced death also decreased with increasing larval weight at the time of oral virus inoculation (Fig. 2c; weight: $X_1^2 = 52.08$, $P < 0.0001$). However, weight influenced larval susceptibility to virus irrespective of the plant treatment consumed before virus challenge (plant treatment: $X_1^2 = 0.97$, $p = 0.32$; trial: $X_1^2 = 2.10$, $p = 0.15$; no interactions). No mock-challenged larvae died in the two plant treatments at any time point.

Table 2
Results from multiple linear regression analyses for the effect of leaf area consumed on larval weight. Trial, leaf area consumed and plant treatment (JA-induced vs. non-induced) and their interactions were included as predictor variables. Significant results are in bold font.

Source	Sqrt larval weight (mg)		
	DF	*F*	*p*
Trial	1, 579	1.40	0.24
Sqrt leaf area	1, 579	**2420**	**< 0.0001**
Plant	1, 579	**49.77**	**< 0.0001**
Trial by sqrt leaf area	1, 579	**4.49**	**0.035**
Trial by plant	1, 579	0	0.98
Sqrt leaf area by plant	1, 579	**45.31**	**< 0.0001**
Trial by sqrt leaf area by plant	1, 579	**4.42**	**0.036**

Plant = JA-induced vs. non-induced.

3.2. Experiment 2: Intrahemocoelic virus inoculation

The proportion of larvae killed by intrahemocoelic injection of BV did not differ significantly between larvae that were fed JA-induced or non-induced foliage for 12 h prior to injection ($X_1^2 = 0.59$, $p = 0.44$; Fig. 3). There was also no evidence of developmental resistance to BV when proportional mortalities of foliage-fed larvae were compared to 4^0 larvae (positive control) (positive control vs. JA-induced plants: $X_1^2 = 0.03$, $p = 0.85$; positive control vs. non-induced plants: $X_1^2 = 0.35$, $p = 0.55$). More larvae died from BV-challenge in Trial 1 than in Trial 2 ($X_1^2 = 7.85$, $p = 0.005$). No mock-challenged larvae died.

3.3. Experiment 3: Oral virus inoculation with fluorescent brightener

As expected from experiment 1, the proportion of larvae killed by oral inoculation of virus OBs decreased significantly with increasing duration of feeding on the plant treatments prior to inoculation ($X_1^2 = 39.81$, $p < 0.0001$; Fig. 4); more so on non-induced than JA-induced foliage (see supplementary material Appendix 1 for detailed pairwise contrasts). When fluorescent brightener (1% Calcofluor white M2R) was added to the virus OB suspension, levels of mortality across treatments (feeding duration and plant treatment) were not significantly different ($X_1^2 = 1.52$, $p = 0.82$; Fig. 4), indicating that the addition of M2R abolished intra-stadial developmental resistance to oral virus inoculation. No larvae that were mock-challenged, with the carrier solutions in the two plant treatments, died.

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.jip.2018.09.001.

4. Discussion

Our findings demonstrate that induced plant defenses can increase the susceptibility of an herbivorous insect to a key natural enemy, baculovirus, by slowing down the insect's growth. The use of a soybean genotype that when induced, reduces the insect's ability to convert ingested foliage into body mass, revealed that the level of developmental resistance depended on the weight of the insects at the time of oral virus inoculation. Duration and amount of feeding were also associated with the level of developmental resistance, but these factors were significantly influenced by the defensive status of the plants (i.e., JA-induced or non-induced). Fall armyworms do not respond to induced defenses of this soybean genotype by initiating an earlier molt, and instead prolong the duration of the instar (Shikano et al., 2017a). Thus our findings indicate that the induced plant defenses kept the insects in a state that was more susceptible to baculovirus infection (i.e., smaller) for a longer period of time, consistent with the slow-growth, high-mortality hypothesis. In nature and even in monoculture agro-ecosystems, there is great spatial variability in plant quality, both nutritionally and in levels of defenses, which will create heterogeneity

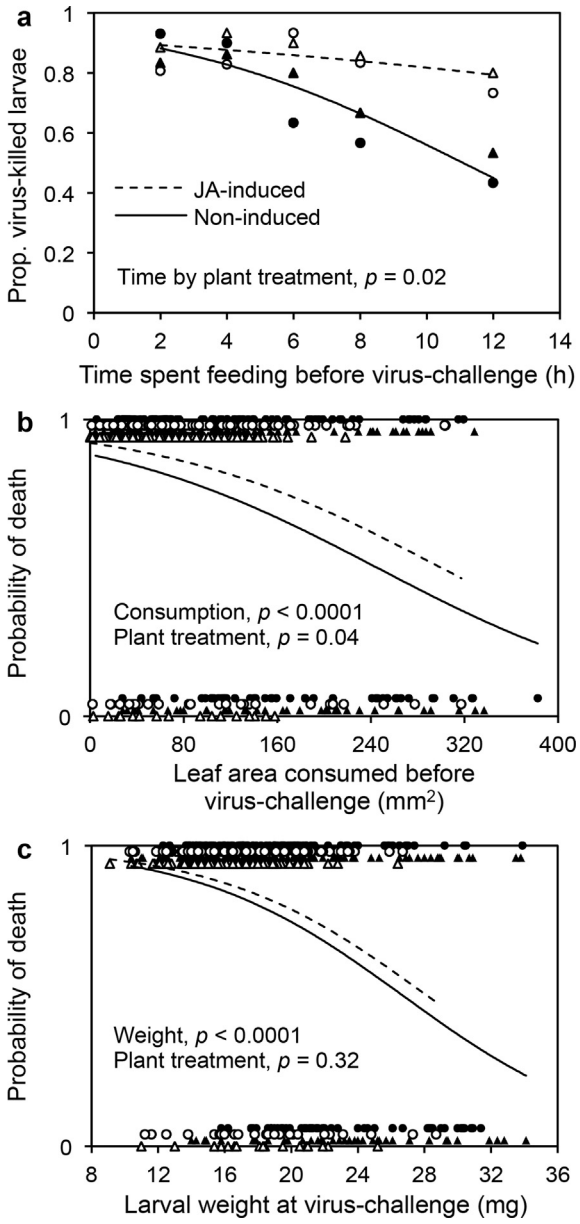

Fig. 2. (a) The relationship between the proportion of virus-challenged larvae that died and the amount of time these larvae spent feeding on JA-induced and non-induced foliage before virus-challenge. The probability of virus-induced death in relation to (b) area of JA-induced and non-induced foliage consumed before virus-challenge and (c) larval weight at virus-challenge (1 = died; 0 = survived). Different symbols represent trial 1 (circle) and trial 2 (triangle) and different colours represent JA-induced (white) and non-induced (black) plant treatments. Lines represent fitted models.

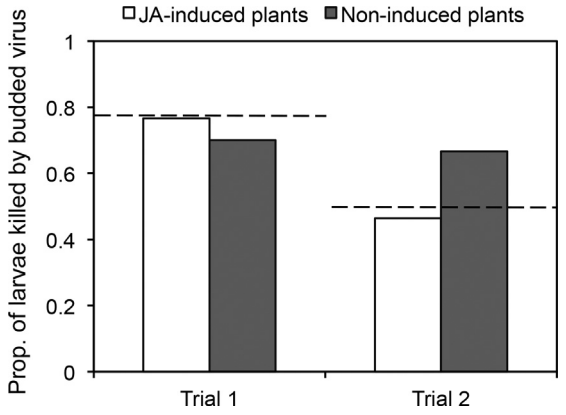

Fig. 3. The proportion of larvae killed by intrahemocoelic injection of budded virus when challenged after 12 h of feeding on JA-induced and non-induced foliage. Proportional mortality of newly moulted 4th instar larvae (4^0; positive control) are represented by a dashed line for each trial. Levels of mortality did not differ significantly between plant treatments or between plant treatments and the positive control.

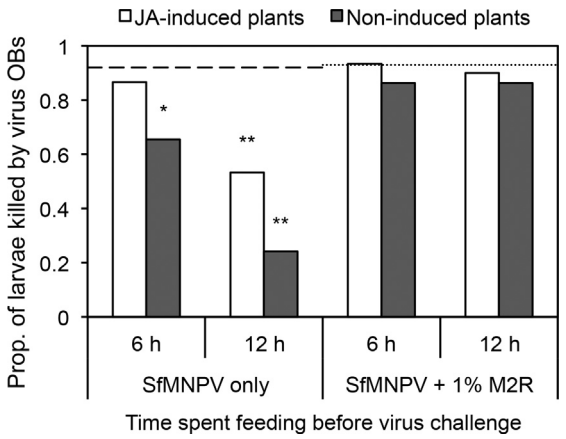

Fig. 4. The proportion of larvae killed by oral virus inoculation (with or without 1% M2R) when challenged after 6 or 12 h of feeding on JA-induced or non-induced foliage. Proportional mortalities of newly moulted 4th instar larvae (4^0; positive control) are represented by dashed (virus only) and dotted (virus with 1% M2R) lines. Asterisks above bars indicate a significant difference in mortality relative to the positive control (*$p < 0.05$, **$p < 0.005$).

in developmental resistance within insect herbivore populations. Since the occurrence and intensity of epizootics are influenced by the degree of heterogeneity in infection risk (Elderd et al., 2013; Hall et al., 2009), plant-mediated changes in developmental resistance are likely to play an important role in disease outbreaks.

The delay in developmental resistance inflicted by induced plant defenses is also likely to act in concert with other plant-mediated effects on caterpillar–baculovirus interactions (Shikano, 2017). For example, herbivore-induced plant defenses can prompt herbivore movement away from feeding sites to different plants or plant parts (Roslin et al., 2008), which might increase the probability that caterpillars will contact baculovirus-contaminated foliage. Moreover, feeding by virus-infected fall armyworms on induced foliage significantly reduced the numbers of virus OBs produced compared to infected fall armyworms feeding on non-induced foliage (Shikano et al., 2017a), which is likely to influence levels of secondary cycling of virus. Lastly, mixing of virus OBs with induced phytochemicals in the midgut can significantly inhibit virus-induced mortality (Hoover et al., 1998a, 1998b, 1998c; Shikano et al., 2017b). Though not observed in the present study, this inhibitory effect of induced foliage on virus efficacy was evident when we previously allowed fall armyworms to ingest SfMNPV OBs on induced soybean leaf disks (Shikano et al., 2017b). The lack of inhibition of virus in our present study may have resulted from the injection of virus OBs into the midgut, thereby bypassing any plant–baculovirus interactions that could have occurred anterior to the midgut. Altogether, the occurrence and intensity of baculovirus outbreaks will be a product of complex interactions between multiple plant-mediated effects that work in concert and antagonistically with one another.

Similar complex plant-mediated effects have been shown to influence the developmental resistance of caterpillars to another food-borne pathogen, *Bacillus thuringiensis*. For example, resistance of the spruce budworm, *Choristoneura fumiferana*, to *B. thuringiensis* increased with larval age when the ingested dose of *B. thuringiensis* was controlled (Frankenhuyzen et al., 1997). However, developmental resistance to *B.*

thuringiensis was not clearly evident in other lepidopteran species when *B. thuringiensis* was homogeneously incorporated into artificial diet or applied to foliage, because variation in feeding rates and behaviors among larval instars strongly influenced the ingested dose, and consequently mortality (Ali and Young, 1996; Li et al., 1995; Liu et al., 1995).

An important finding in our present study was that, regardless of the quality of the food plant, no developmental resistance was detected when budded virus was injected directly into the hemocoel to bypass midgut resistance mechanisms. Thus, developmental resistance of fall armyworms to SfMNPV appears to depend on gut-based resistance mechanisms, such as apoptosis and sloughing of infected midgut cells (Dougherty et al., 2006; Hoover et al., 2000; Washburn et al., 1998), rather than immunological resistance mechanisms in the hemolymph. This conclusion was supported when developmental resistance was abolished in both food plant treatments with the addition of a fluorescent brightener (1% Calcofluor white M2R) to the virus suspension. M2R has been studied intensively as a UV protectant and enhancer of virus pathogenicity, and has numerous activities in the midgut that could have influenced the establishment of infection. It can prevent the apoptosis and sloughing of infected midgut cells (Dougherty et al., 2006; Hoover et al., 2000; Washburn et al., 1998) and prevent the formation of the peritrophic matrix by inhibiting chitin synthetase *in vitro* (Bartnicki-Garcia et al., 1994; Wang and Granados, 2000). M2R is also suggested to inhibit plant oxidative enzymes assayed *in vitro* (Hoover, unpublished data), such as peroxidases and polyphenol oxidases which can influence baculovirus efficacy (Felton and Duffey, 1990; Hoover et al., 1998a, 1998b, 1998c; Shikano et al., 2017b). In insect–baculovirus systems where the host has effective systemic resistance mechanisms, plant-mediated variation in the strength of insect cellular and humoral immune responses may have some influence on developmental resistance, especially since immunocompetence tends to increase with insect body weight (Vogelweith et al., 2013). Weight-dependent immunocompetence might also play an important role in developmental resistance against other pathogens.

To conclude, plants rely heavily on indirect defenses from the natural enemies of herbivores for protection. The slow-growth, high-mortality hypothesis postulates that the direct defenses of plants, which slow the development of herbivores, enhance these indirect defenses. We recently demonstrated that caterpillar feeding damage induces higher activity levels of defensive enzymes in tomato plants (i.e., induced direct defenses). These induced plant enzymes, particularly polyphenol oxidase, enhanced the plants' indirect defenses by increasing the lethality of *Bt* when the *Bt* was ingested with the induced plant tissues (Shikano et al., 2018). Another recent study has shown that induced direct plant defenses can even indirectly enhance plant protection by encouraging caterpillars to eat conspecific caterpillars on the same plant (Orrock et al., 2017). Here, we demonstrated that the direct induced defenses of plants alter caterpillar disease risk by prolonging their susceptibility to a lethal pathogen through reduced weight gain. Plant-mediated delay in developmental resistance of caterpillars against baculoviruses, which are key natural mortality factors and biological control agents, could be mediating the effectiveness of pest management efforts and the frequency and intensity of baculovirus disease outbreaks in nature.

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