Is decreased generalized immunity a cost of *Bt* resistance in cabbage loopers *Trichoplusia ni*?

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ARTICLE INFO

ABSTRACT

We studied the immune response to *Bacillus thuringiensis kurstaki* (*Btk*) in susceptible (*Bt*-RS) and resistant (*Bt*-R) *Trichoplusia ni* after exposure to low doses of *Btk* and injection with *Escherichia coli*. We measured the levels of resistance, the expression profiles of hemolymph proteins, the phenoloxidase (PO) activity, and the differential number of circulating hemocytes in resistant and susceptible individuals. Individuals from the *Bt*-RS line became more resistant following a previous exposure to sub lethal concentrations of *Btk*, but the resistance to *Btk* of the *Bt*-R line did not change significantly. Similarly the *Bt*-R strain showed no significant changes in any of the potential immune responses, hemolymph protein levels or PO activity. The number of circulating hemocytes was significantly lower in the *Bt*-R strain than in the *Bt*-RS strain. Exposure to *Btk* decreased the hemocyte counts and reduced PO activity of *Bt*-RS larvae. Hemolymph protein concentrations also declined significantly in the susceptible larvae continually exposed to *Btk*. Seven peptides with antibacterial activity were identified in the hemolymph of *Bt*-RS larvae after exposure to *Btk* and five were found in the *Bt*-R larvae. When exposed to a low level *Bt* challenge the susceptible strain increases in tolerance and there are concomitant reductions in hemolymph protein concentrations, PO activity and the number of circulating hemocytes.

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1. Introduction

Bacillus thuringiensis (*Bt*), is a microbial insecticide widely used to control lepidopteran pests of crops and forests. Resistance has developed in several Lepidopteran species after extensive use in post-harvest grain storage facilities (McGaughey, 1985), in the field (Kirsch and Schmutterer, 1988; Tabashnik et al., 1990), in the greenhouse (Janmaat and Myers, 2003) and following exposure in the laboratory (Ferré and Van Rie, 2002). Without continued exposure to *Bt*, resistance is lost in many of these species. In the cabbage looper, *Trichoplusia ni*, most populations revert from resistant to susceptible status within several generations when not exposed to *Bt*, and this suggests that maintaining *Bt* resistance is transient, and potentially costly (Janmaat and Myers, 2003).

Commercially, *Bt*-toxins are delivered in two ways; through transgenic crops (Vaeck et al., 1987), or through the spraying of Cry-toxin formulations that often contain viable *B. thuringiensis* spores. Toxicity occurs after *Bt*-endotoxins are ingested, solubilized in the alkaline midgut, and then proteolytically cleaved to release the active endotoxin (Haider et al., 1986; Jaquet et al., 1987; Brod-

erick et al., 2006). Once cleaved, the endotoxin binds to receptors on the midgut brush-border membrane vesicles (BBMV) (Hoffman et al., 1988; Zhang et al., 2005; Wang et al., 2007), which results in pore formation (Rausell et al., 2004), midgut paralysis (Gill et al., 1992; Pigott and Ellar, 2007), and cell death (Zhang et al., 2008). This midgut damage is thought to create a point of entry for enteric bacteria or *B. thuringiensis* to invade the hemocoel (Broderick et al., 2006). However, if the insect can interrupt or defer this complex mode of action at any step, either through behavioral (deterrence) or physiological mechanisms, *Bt* efficacy is likely to be reduced.

Resistance to *Bt*-endotoxins is due largely to mutations on midgut receptors that can be both unique to the insect, and specific to the endotoxins encountered (Van Rie et al., 1990; Pigott and Ellar, 2007). Because of this specificity, it is possible that the physiological costs of *Bt* resistance also will vary among host species. In greenhouse populations of *T. ni*, resistance appears to be due largely to a physiological incompatibility between the midgut BBMV receptors and the toxins they bind (Wang et al., 2007). These losses in binding are likely due to mutations in the toxin binding region of the receptors, but also may be due to changes in the abundance of receptors on the midgut epithelium. Other mechanisms, or combinations of mechanisms also may reduce the toxicity of *Bt* to resistant hosts. These alternate tolerance mechanisms include coagulation reactions that prevent solubilization of the *Bt*-toxins

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(Ma et al., 2005a), changes in the proteolytic processing of the toxins, and changes in pH of midgut lumen (Ma et al., 2005b). In addition, changes in immune system function have also been associated with *Btk* exposure (Tamez-Guerra et al., 2008; Rahman et al., 2004, 2007).

Immune responses by insects to microbial pathogens comprise humoral and cellular factors. The humeral component includes phenoloxidase mediated melanization, the production of proteases and bacteriolytic enzymes, as well as the synthesis of potent antimicrobial peptides (AMPs) (Boman, 1991; Brey et al., 1993; Gillespie and Kanost, 1997; Lowenberger, 2001). Phenoloxidase, an enzyme required for several aspects of insect development also catalyzes key steps in biochemical pathways that lead to the melanization of pathogens (Cotter and Wilson, 2002). Studies with the pyralid *Ephestia kueniella* have associated exposure to *Bt* with increases in phenoloxidase activity and induced tolerance to *Bt*-endotoxins (Rahman et al., 2004, 2007). Phenoloxidase, however, represents only one aspect of a potent and effective immune system (Soderhall and Cerenius, 1998; Gillespie and Kanost, 1997; Cerenius and Soderhall, 2004).

The cellular (hemocyte) response primarily involves the phagocytosis, nodulation or encapsulation of foreign microbes and debris (Lavine and Strand, 2002), but also may be involved in the rapid synthesis, expression and delivery of potent AMPs (Lavine et al., 2005). Hemocyte-mediated responses have been associated with significant changes in the number of circulating cells (Bidochka and Khachatourians, 1987; Nakahara et al., 2003), changes in abundance of particular hemocyte types, changes in phagocytic rates (Dubovskiy et al., 2008), and the differentiation of circulating cells (Strand, 2008). These different components of the insect's humoral and cellular immune system work in concert to eliminate microbial pathogens (da Silva et al., 2000).

Before a response can occur, however, the pathogen must be recognized by the insect immune system as non-self. In Gram-positive bacteria such as *B. thuringiensis*, peptidoglycan, a major constituent of the cell wall, is recognized by peptidoglycan receptors (PGRP) and this initiates a strong immune response (Leulier et al., 2003; Chang and Deisenhofer, 2007). These receptors are membrane bound or secreted into the hemolymph, such that if the midgut is breeched, or bacterial invasion of the hemocoel occurs, a rapid immune response takes place. What is unclear, however, is whether *B. thuringiensis* spores are recognized after ingestion, and if this can subsequently cause a systemic immune response that is detectable in the hemocoel.

In this study, we examined how *Bt*-resistant and -susceptible strains of *T. ni* differ in their immune response following exposure to low doses of a commercial formulation of *Btk* containing spores and five endotoxins (Cry1Aa, Cry1Ab, Cry1Ac, Cry2Aa, Cry2Ab). In addition to the susceptibility of pre-exposed hosts to *Btk*, we measured several parameters of the insect immune system including hemolymph phenoloxidase activity, the number of circulating hemocytes, and the presence of antimicrobial peptides in the hemolymph. Given the findings of Wang et al. (2007), that the loss of binding of *Bt*-endotoxins to the midgut receptors could only account for high-levels of tolerance to Cry1Ab and Cry 1Ac, we predicted that components of the innate immune response might contribute to the overall, total resistance to spore-crystal formulations.

2. Materials and methods

2.1. Rearing of Bt-resistant and Bt-susceptible T. ni line

A *Bt*-resistant (*Bt*-R) *T. ni* colony was initiated from 90 individuals collected from a commercial tomato greenhouse in British Columbia, Canada in 2001 (Janmaat and Myers, 2003). The *T. ni* population at collection was found to be 113-fold more resistant

in the first generation of laboratory culture than a reference susceptible laboratory colony. Two lines were established on a wheat-germ based diet (Ignoffo, 1963) and reared at 26 °C, a 16:8 (L:D) photoperiod. One line was reared without any *Btk* exposure and exhibited a significant decrease in resistance (change in LC_{50} from 256 to 2.7 kIU ml^{-1} diet where kIU is equal to 1000 International Units of *Bt* activity) (Janmaat and Myers, 2003). This is referred to as the reverted-susceptible line (*Bt*-RS). The resistant (*Bt*-R) line was exposed to *Btk* (DiPel WP, Valent Biosciences) periodically during laboratory culture to maintain resistance as described (Janmaat et al., 2004).

2.2. Bt-induction bioassay: changes in susceptibility

The induction bioassay was repeated three times within the period of a year. In the first and third replicates, a response to pre-exposure was evaluated in both *Bt*-R and *Bt*-RS strains. In the second replicate, a pre-exposure response was evaluated only in the *Bt*-RS line. In all experiments, groups of 25 *T. ni* larvae were reared in 175 ml Styrofoam cups containing 15–20 ml of artificial diet. After 4 days of growth, larvae in the treatment group were transferred to cups containing 10 ml of a *Btk*-diet mixture with a sublethal *Btk* dose (0.6 kIU ml^{-1}) diet as defined by preliminary experiments. Larvae in the control group were transferred to cups containing 10 ml of artificial diet without *Btk*, in the first two replicates of the induction assay. In the third replicate, larvae in the control group were not transferred but were maintained on *Bt*-free diet. Following 18–24 h on the pre-treatment dose of *Btk*, larvae were transferred in groups of five to 59.2 ml plastic soufflé cups (Solo Cup Company, Highland Park, IL, USA) containing 3 ml of fresh diet with a range of *Btk* concentrations (0.625–200 kIU ml^{-1}) or a control containing no *Btk*. Larval mortality was observed 3 days following exposure to treatments and susceptibility to *Btk* was determined by probit analysis of a concentration-mortality assay as described previously (Janmaat and Myers, 2003). Unique dose ranges were required for each line due to their inherent differences in susceptibility to *Btk*.

2.3. Phenoloxidase assay and hemolymph protein concentration

Additional *T. ni* larvae were raised as described above, and after 4 days of growth were transferred in groups of 20 larvae to 175 ml Styrofoam cups with the appropriate diet treatment. Control larvae were transferred to freshly prepared artificial diet without *Btk*, and larvae in the *Bt*-exposure group were transferred to fresh diet containing *Btk*. We collected hemolymph from these insects in the fifth larval instar. Because the treatment groups developed at different rates, hemolymph samples were collected at different times. Hemolymph samples were collected by first excising a proleg with fine scissors and allowing the exuding hemolymph to pool onto parafilm. Ten-μl of this hemolymph were diluted in 240 μl of Dulbecco's phosphate buffer saline (DPBS) and samples were frozen at −20 °C for 24–48 h to disrupt hemocyte membranes (Wilson et al., 2001). Triplicate 50 μl hemolymph/buffer mixtures were transferred into 96-well microtitre plates and 150 μl of 15 mM dopamine HCl (Sigma-Aldrich, St. Louis, MO, USA) were added to each well. Absorbance was measured at 492 nm on a Spectramax 190 microplate reader (Molecular Devices Corporation, Sunnyvale, CA, USA). Preliminary experiments indicated that the linear phase of the reaction began shortly after the addition of dopamine and continued for 40 min. The kinetic activity of phenoloxidase per μl hemolymph sample was expressed as change in optical density units per minute (δOD min^{-1}), at VMAX.

Hemolymph protein concentrations were measured using the Bradford protein assay (Bio-Rad Laboratories, Hercules, CA, USA) with bovine serum albumin (BSA) used as a protein standard. Trip-

licate 5 μl samples of each hemolymph/buffer mixture was transferred into a 96-well microtitre plate and 200 μl of Bradford reagent were added to each well. Absorption was measured at 595 nm on a Spectramax 190 microplate reader (Molecular Devices Corporation, Sunnyvale, CA). The amount of protein in each sample was then calculated by extrapolation to a standard curve of BSA that ranged from 0 to 2 mg BSA/ml sample.

2.4. Changes in immune parameters

To test the role of the immune response in *Bt*-tolerance, we measured the number of circulating hemocytes in these insects and the presence of induced hemolymph proteins with antimicrobial activity. To simulate the enteric sepsis known to be associated with *Btk* degradation of midgut tissues, *Escherichia coli* was injected into the hemocoel. This injection also would act as a standardized immune insult, and a positive control for immune stimulation.

Seventy-five 3rd instar larvae from each colony were challenged with an injection of fluorescein isothiocyanate (FITC)-labeled *E. coli* (Sigma-Aldrich, St. Louis, MO, USA), and 75 additional larvae were set aside as controls and received no immunological challenge. After 24-h, mortality was recorded and survivors were transferred in groups of five to the *Btk*-diet regimes. These consisted of 2 ml of synthetic diet at *Btk* concentrations previously determined to be sublethal for each insect line (*Bt*-RS: 0.6 kIU ml^{-1} diet; *Bt*-R: 5.0 kIU ml^{-1} diet) in a 59.2 ml plastic soufflé cup (Solo Cup Company, Highland Park, IL, USA). Higher concentrations were used with the *Bt*-R line in these experiments to provide a challenge of similar magnitude (LC15-20) as that given to *Bt*-RS, and to ensure that survivors selected for the hemolymph analysis were indeed tolerant to the specified concentrations. Thirty replicate cups were prepared for each *Btk* treatment, and 30 cups containing diet with no *Btk* were included as a control for each line. After 48 h, cabbage loopers from each group (control, injected, *Btk*, *Btk* + injection), were observed for mortality and hemolymph from survivors was collected for hemocyte and antimicrobial activity analyses.

2.5. Differential hemocyte counts

Hemolymph samples were collected by removing a proleg from a cold-anesthetized larva, and collecting hemolymph with a pipette as it pearled from the wound. A 3 μl aliquot of hemolymph was added to 10 μl of Schneider's insect medium supplemented with sodium bicarbonate and calcium carbonate (pH: 6.8; Sigma-Aldrich, St. Louis, MO, USA). Samples were mixed and immediately mounted on an Improved Neubauer hemocytometer. The number of hemocytes per microliter of hemolymph was determined for each larva.

2.6. Hemolymph protein analysis

For each treatment group approximately 100 μl of hemolymph from 10 loopers were pooled in 1 mL ice-cold acidified HPLC grade water (0.1% trifluoroacetic acid-TFA, Sigma-Aldrich), containing 100 μl of protease inhibitor cocktail (Roche, 10× dilution, Basel, Switzerland), and stored at −80 °C. Subsequently samples were thawed on ice, vortexed, and centrifuged at 10,000 rpm at 4 °C for 30 min. The supernatant was collected and concentrated using solid-state extraction via Sep-Pak C-18 cartridges (Waters Corporation, Milford, MA, USA). One ml of each sample was loaded onto a C-18 cartridge, and was subsequently eluted with an 80% acetonitrile (ACN; 0.05% TFA) solution. The eluates were concentrated using an automatic Environmental SpeedVac 1000, (Savant Instruments, Hicksville, NY, USA) at 4 °C until the final volume was approximately 15 μl. Samples were then re-suspended in 100 μl

of 0.05% TFA, vortexed, and centrifuged briefly to ensure all liquid was collected at the bottom of the tube.

A 100 μl sample was loaded into a C18 analytical column (Xbridge 4.6× 250 mm; 300 Å pore size, 5 μm particle size; Waters Corporation, Milford, MA, USA) for reverse-phase high performance liquid chromatography (RP-HPLC) via a manual injector (Beckman Coulter Gold, Fullerton, CA, USA). The detector wavelength was set at 225 nm. The mobile phase solvent gradient ranged from 0% to 80% ACN over 85 min at a flow rate of 1 ml/min and fractions were collected every minute.

2.7. Antimicrobial activity assay

From each treatment group RP-HPLC fractions with an absorbance greater than 0.05 AU (at 225 nm) were concentrated in a vacuum centrifuge as described above to an approximate volume of 1–5 μL at 4 °C to remove most of the mobile phase solvents. HPLC fractions were then re-suspended in 25 μL ddH$_2$O, vortexed for 1 min, and immediately used in an antimicrobial disc-diffusion assay. In these assays lawns of the Gram-positive bacterium *Staphylococcus epidermidus* and the Gram-negative bacterium *E. coli* were plated on Luria–Bertani (LB) agar plates. Sterile paper discs (6 mm diameter), on which 10 μL of the HPLC fractions, water, or the control antibiotic ampicillin (100 mg/ml) had been aliquoted, were placed on the plates. HPLC mobile phase solvents (sol A; sol B) were also included as solvent controls. The plates were placed at 37 °C and zones of inhibition (clear halos around the disks) were examined 18–24 h later.

2.8. Statistical analyses

Larval mortality in the induction bioassay was analyzed using the probit procedure in SAS 9.1 ((c) 2002–2003 by SAS Institute Inc., Cary, NC, USA) with the *Bt* concentration (natural logarithm transformed), line (*Bt*-RS; *Bt*-R), induction treatment, and assay date as main effects. LC$_{50}$ values and slopes of concentration-mortality lines for each Line-Induction Treatment combination were estimated separately using the probit procedure. All LC$_{50}$ values in the text and tables are represented as kIU ml^{-1} diet and are rounded to the nearest hundredth. Phenoloxidase activity and hemolymph protein concentrations were analyzed using the proc mixed procedure in SAS 9.1 with the *T. ni* line, *Bt*-exposure and their interaction as main effects. Assay plate was included as a random factor. Differences between assay plates were due to variation in temperature during absorption measurements, and variation in experimental procedures during preparation of the plate. Plates were numbered sequentially across the different sampling dates and assay times in order to include the variation due to date and time of phenoloxidase assay in the assay plate factor. Phenoloxidase activity raw data were natural logarithm transformed to normalize the data (Zar, 1996). The numbers of circulating hemocytes were analyzed using the proc mixed procedure in SAS 9.1 with *Bt*-treatment, *E. coli* injection, and their interaction as main effects. Six contrasts were performed, and significant differences were determined using a Bonferroni adjusted $p = 0.008$ ($\alpha = 0.05/6$). The percentage mortalities from treatments in the immune parameter assay were compared using a *Chi*-squared analysis.

3. Results

3.1. Bt-induction bioassay: changes in susceptibility

The effects of exposure to sublethal *Btk* concentrations varied between the *T. ni* lines. For the *Bt*-RS line, all three replicates indicated that the LC$_{50}$ of the induced treatment group was significantly higher than the LC$_{50}$ of the *Bt*-RS control group (Table 1 and Fig. 1).

Table 1
A comparison of LC_{50}'s from resistant and susceptible lines following induction with sublethal levels of *Btk*. The variation in control mortality and the slope are represented by the standard error of the mean.

Line	Treatment	n	Control mortality	Slope	LC_{50} (95% CI)	Induction-ratio[a]
Bt-R	Control	600	3.00 ± 3.00	0.75 ± 0.08	39.4 (28.3–53.7)	
	Induced	600	2.02 ± 2.02	1.08 ± 0.13	54.2 (40.3–70.1)	1.4
Bt-RS	Control	900	2.30 ± 2.29	0.84 ± 0.07	2.1 (1.3–3.2)	
	Induced	900	6.44 ± 4.87	0.77 ± 0.06	18.4 (12.9–25.8)	8.7

[a] The induction-ratio is defined as the ratio between the LC_{50} of the induced treatment group relative to the control treatment group of each *T. ni* line.

There was a mean 9-fold increase in the LC_{50} between the induced and control groups. In contrast, there was no significant difference between the LC_{50} of the induced group relative to the control group for the *Bt*-R line although the LC_{50} of the induced treatment did tend to be higher (1.4×) than that of the control group.

3.2. Phenoloxidase assay and hemolymph protein concentration

In the overall analysis, no significant differences in hemolymph phenoloxidase activity were detected between the susceptible and resistant lines (Table 2). Differences between lines were detected in a post-hoc analysis on only the control larvae of both lines, such that the *Bt*-R control larvae exhibited lower hemolymph phenoloxidase activity than the *Bt*-RS control larvae ($F = 5.51$, $df = 63$, $p = 0.006$). Significant differences were detected between control and exposed larvae and we identified a significant interaction between the *T. ni* line and induction treatment. For the *Bt*-RS line, the larvae continuously exposed to *Btk* exhibited a significant reduction in hemolymph PO activity when compared to the control larvae ($F = 16.6$; $df = 63$; $p < 0.0001$). For the *Bt*-R line, no significant differences in PO activity were detected when compared to control larvae ($F = 1.05$; $df = 63$; $p = 0.35$) (Fig. 2).
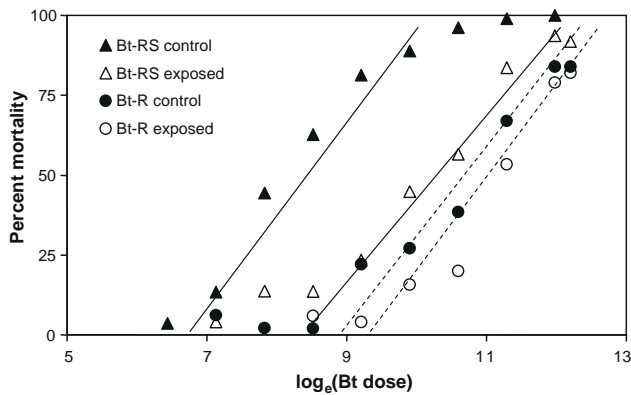

Fig. 1. The concentration-mortality curves of *Bt*-resistant (*Bt*-R) and *Bt*-susceptible (*Bt*-RS) *T. ni* lines previously exposed or unexposed to a sublethal dose of *Bt* for 24 h. Solid lines represent *Bt*-RS colony, and dashed lines represent the *Bt*-R colony.

Table 2
ANOVA results outlining the effects of *Trichoplusia ni* line, and sublethal *Bt*-exposure on hemolymph phenoloxidase activity and protein concentration.

Factor	df	F ratio	p
Phenoloxidase			
T. ni line	1, 117	0.02	0.88
Exposure	1, 117	7.85	0.0005
Line * exposure	2, 117	9.67	0.0007
Protein concentration			
T. ni line	1, 117	0.28	0.59
Exposure	1, 117	28.19	<0.0001
Line * exposure	2, 117	5.83	0.02

Hemolymph protein content varied between the susceptible and resistant lines, and between the control and exposed larvae in the overall analysis (Fig. 3). Significant differences between insect lines were identified in a post-hoc analysis of the control larvae of both lines. *Bt*-R larvae had lower hemolymph protein concentrations than *Bt*-RS larvae ($F = 3.82$, $df = 63$, $p = 0.05$). The *Bt*-exposure significantly reduced the total hemolymph protein concentration (Table 2), resulting in a 37% decrease in protein concentration in the *Bt*-RS line and a 15% decrease in the -R line.
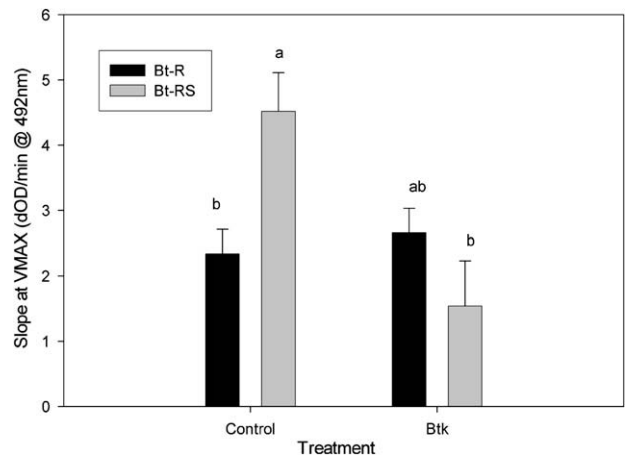

Fig. 2. The hemolymph phenoloxidase activity (slope at VMAX) in *Bt*-susceptible (*Bt*-RS; grey) and *Bt*-resistant (*Bt*-R; black) lines after continuous exposure to sublethal concentrations of *Btk*. Results represent mean values, and error bars represent the standard error of the mean (SEM). Means denoted by the same letter are not significantly different (Tukeys HSD; $\alpha = 0.05$).
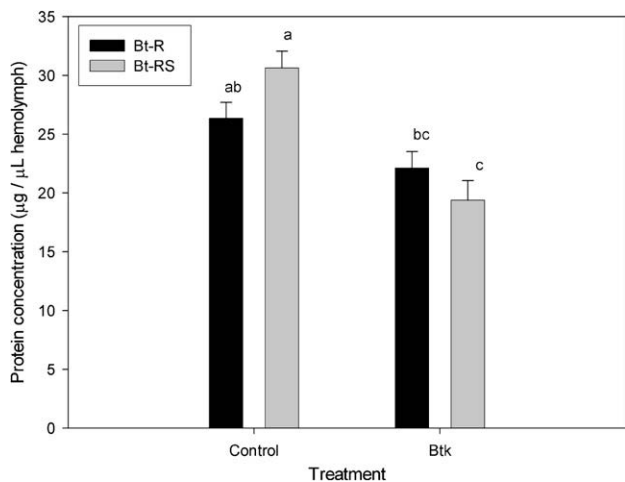

Fig. 3. The hemolymph protein concentration ($\mu g\ \mu l^{-1}$) in *Bt*-susceptible (*Bt*-RS; grey) and *Bt*-resistant (*Bt*-R; black) lines after continuous exposure to sublethal concentrations. Results represent mean values, and error bars represent the standard error of the mean (SEM). Means denoted by the same letter are not significantly different (Tukeys HSD; $\alpha = 0.05$).
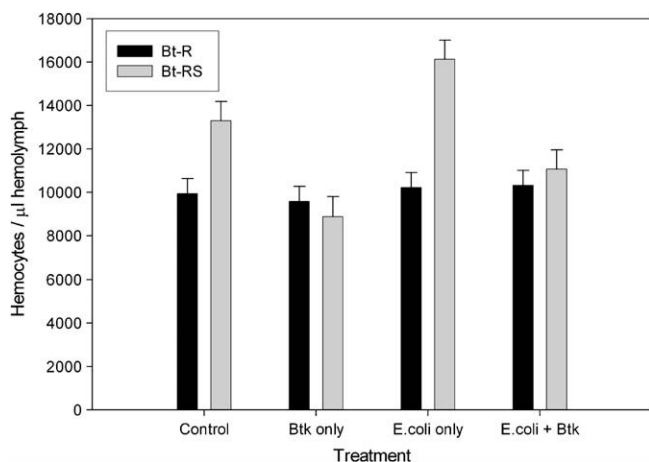
Fig. 4. The total number of circulating hemocytes (cells μl^{-1} hemolymph) in *Bt*-resistant (*Bt*-R; black) and *Bt*-susceptible (*Bt*-RS; grey) *T. ni* after exposure to *Btk*, after injections of *E. coli*, and after combined treatments. Results represent mean values, and error bars represent the standard error of the mean (SEM).

3.3. Changes in immune parameters: treatment mortality

After 24 h, the injection of *E. coli* caused 6% mortality in larvae from *Bt*-RS lines and 10% mortality in *Bt*-R larvae. There was only 1% mortality observed in the control groups. After 48 h exposure to the *Btk* treatment (0.6 kIU ml^{-1} diet), mortality in *Bt*-RS groups previously injected with *E. coli* was 14%, and that of uninjected groups on *Btk* was 21%. After 48 h exposure to the *Btk* treatment (5.0 kIU ml^{-1} diet), mortality in *Bt*-R groups previously injected with *E. coli* averaged 41.5%, and was 45.0% in uninjected groups.

3.4. Differential hemocyte counts

The mean number of circulating hemocytes in *Bt*-R individuals was significantly lower than that of *Bt*-RS individuals ($F = 8.52$, $df = 1, 76$; $p = 0.0046$) (Fig. 4). In the *Bt*-RS line the number of circulating hemocytes were similar between controls and larvae injected with *E. coli*, whereas hemocyte counts were significantly lower in those exposed to *Btk* ($F = 11.36$, $df = 1, 154$; $p = 0.001$) treatments. In the *Bt*-R lines there were no significant differences in the number of circulating hemocytes in any of the treatment groups ($F = 0.16$, $df = 3, 154$, $p = 0.923$).

3.5. Soluble protein analysis and antimicrobial activity assays

HPLC chromatograms of collected hemolymph proteins were generated for each treatment and differentially occurring peaks were compared by their solid phase retention times. Several peaks were common to all groups, but there were also distinct peaks present after each treatment.

Each HPLC fraction was dried, re-suspended in ddH$_2$O, and tested for antimicrobial activity against Gram-positive and Gram-negative bacteria by their ability to generate a zone of inhibition in plate assays. In larvae exposed to *Btk* we identified 7 fractions with antimicrobial activity in *Bt*-RS loopers compared with 5 fractions in *Bt*-R loopers. In *Bt*-RS loopers, 3 fractions had activity only against the Gram-positive *S. epidermidis*, and 4 had activity against both Gram-negative *E. coli* and *S. epidermidis*. Of the 5 fractions obtained from the *Bt*-R loopers, 2 fractions were active against *S. epidermidis*, 1 fraction was active against *E. coli*, and 2 were active against both bacteria. Several fractions had the same retention times suggesting that their presence was not related to the treatments. Unique antimicrobial fractions also were detected in both

lines after exposure to *Btk* treatments. When the effects of the *Btk* exposure were compared to the *E. coli* injection treatment, antimicrobial fractions that differed in their retention times were found in the hemolymph in both *Bt*-R and *Bt*-RS lines. Although the injection of bacteria caused an increase in the number of antimicrobial fractions in the hemolymph in both lines, the retention times of these fractions varied, suggesting that the expression of different molecules or a differential cleavage of precursor molecules may have occurred.

4. Discussion

In the reverted-susceptible strain of *T. ni*, exposure to a low dose of *Btk* increased the tolerance to *Btk* to a level similar to that observed in a strain selected for *Bt* resistance. Although the origin of this inducible trait is unknown, it is possible that the original *T. ni* colony, that had evolved resistance to *Btk* in the greenhouse, had both an inducible and fixed mechanism of resistance to *Btk*. If this were the case, then low levels of tolerance conferred by inducible mechanisms would be expected to have a proportionally smaller influence on the induction-ratio in the *Bt*-R. However, given the absence of an inducible trait in the *Bt*-R line in response to the sublethal *Btk* concentration, it is possible that a certain amount of binding of the *Btk* toxins is required, and hence a certain amount of gut damage is experienced before an inducible response can occur.

After exposure to *Btk*, the total hemolymph protein concentration decreased for both resistant and susceptible loopers. The reductions were less in resistant lines, however, which may be associated with reduced damage to the gut occurring from *Btk* exposure in that strain. *Bt*-R loopers have lower hemolymph phenoloxidase activity, lower hemolymph protein content, lower hemocyte counts, and fewer induced antimicrobial hemolymph fractions than do *Bt*-RS loopers. We may speculate that a reduced PO activity and hemolymph protein concentration is directly associated with the increased resistance of the induced *Bt*-RS. Freitak et al., 2007 found that PO activity was reduced in *T. ni* larvae after the consumption of nonpathogenic bacteria. Thus rather than an increase in PO activity being an indication of increased tolerance through immunity, it appears that PO activity is reduced or that PO is being used resulting in a reduction in the levels of available PO circulating in the hemolymph. Although the extent of the immune response in *Bt*-R insects is reduced compared with *Bt*-RS, *Bt*-R insects remain able to clear microbial infections. Alternatively the presence of *Bt* in the *Bt*-R insects may not cause a breech in the midgut and a sepsis of the hemolymph, and therefore may not activate hemolymph immune responses.

The hemocyte data indicate that the *Bt*-R and *Bt*-RS lines maintain significantly different numbers of circulating hemocytes per μl hemolymph, and that the two lines respond differently to both *E. coli* injections and *Btk* treatments. Studies with *Galleria mellonella* have shown that significant increases in phagocytic and encapsulation rates occur in response to sublethal *Bt*-exposure (Dubovskiy et al., 2008), and this enhanced cellular immunity is likely associated with changes in total hemocyte numbers. Although other studies have reported changes in the number of circulating hemocytes after challenge with a pathogen (Bidochka and Khachatourians, 1987; Christensen et al., 1989; Beetz et al., 2008), it is not clear what these reductions in cell number after *Btk* treatment in *Bt*-RS larvae indicate. Thus, identifying the changes in hemocyte populations and characterizing the functional role in the *Bt* response is likely to be a fruitful area of research, but more work is needed to characterize further the changes in hemocyte number found here.

Although the *E. coli* injections and *Btk* treatments caused the number of antimicrobial HPLC fractions to increase in the hemo-

lymph in both resistant and susceptible lines, the retention times of various fractions varied between treatments. These changes may indicate that novel peptides are being expressed, that inactive precursors are being cleaved for activation, or that some activated molecules contain post-translational modifications that alter their retention times. This will be clarified in future work as we purify, identify and characterize these differentially expressed proteins.

We have observed differences in the immune responses of our selected lines to immune stimuli (the parameters measured here indicate differences in cellular and humoral immune factors), however, we cannot determine differences in the competency of these different levels of response. A 'reduced' immune response may be sufficient for the normal functioning of Bt-R loopers. If, however, Bt resistance causes an overall reduction in immunological competence, then their susceptibility to other biological controls might be increased. This concept of increased susceptibility has been supported by studies demonstrating that Bt-R loopers are more susceptible to nucleopolyhedrovirus (Cervantes, 2006). These studies highlight the potential of using multiple pathogen treatments to manage Bt resistance, and of exploiting the changes in immune-competency that may be generated in Bt-R insects by extensive use of the insecticide. Significantly more work must be done to investigate changes in immune responses in resistant insects to confirm this postulate, to identify the differentially expressed molecules, and to determine how we might use this knowledge to reduce pest populations.

Acknowledgments

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