

Article

Lack of Tolerance Development Following Oral Exposure To sublethal Cry1 and Vip3Aa Proteins in *Spodoptera exigua* (Hübner, 1808)

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Simple Summary

Farmers often rely on natural insecticidal proteins produced by the soil bacterium *Bacillus thuringiensis* (Bt) to protect crops from damaging caterpillars. These proteins have been incorporated into transgenic plants and have been highly effective. However, there are growing concerns because insect populations are developing tolerances and resistances to these toxins. Previous studies have suggested that exposure to low doses of Bt-derived products could give an advantage to insects, increasing their tolerance. In this study, we tested whether feeding neonate larvae of the beet armyworm (*Spodoptera exigua*) with Cry1 and Vip3Aa proteins would increase their tolerance and whether this effect would be transmitted to their offspring. Our results showed that feeding on neonates with these proteins did not lead to a biologically relevant increase in tolerance when larvae were later exposed to lethal doses. Moreover, the offspring of the exposed larvae did not show increased tolerance; in fact, they were in some cases slightly more susceptible. In conclusion, our findings indicate that it is highly unlikely that tolerance will develop in this species through this mechanism with the tested proteins. Therefore, sublethal exposure is not expected to compromise the long-term effectiveness of Bt-based pest management strategies, thus supporting the continued use of these proteins in agriculture.

Abstract

Insecticidal proteins derived from *Bacillus thuringiensis* (Bt) have been effectively employed in controlling lepidopteran pests, notably in transgenic crops targeting *Spodoptera* species. However, concerns have arisen regarding the long-term efficacy due to the emergence of tolerant and resistant insect populations. Prior research suggested that repeated exposures to Bt, which contains a mixture of spores and crystals, may contribute to the development of tolerance; however, the specific effects of sequential exposure to purified Cry1 and Vip3Aa proteins remain unclear. This study aimed to assess whether prior exposure of *Spodoptera exigua* neonate larvae to sublethal concentrations of Cry1Ab, Cry1Ca or Vip3Aa proteins would heighten their tolerance upon subsequent exposure, and whether such effects would extend to their offspring. Pre-exposure to Cry1Ab or Vip3Aa did not affect larval responses to the toxin. For Cry1Ca, a slight increase was observed under one treatment condition, but the effect was not considered biologically relevant. Transgenerational analysis revealed no enhancement of tolerance; rather, there was a negative impact on the offspring's response in some cases. These findings indicate that although previous studies have documented that sublethal contact with bacterial preparations may significantly affect insect tolerance, exposure to sublethal doses of purified Cry1 and Vip3Aa proteins is unlikely to lead to the development of tolerance in *S. exigua*.



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Keywords: *Bacillus thuringiensis*; Bt; insecticidal proteins; pesticidal resistance; transgenerational effects; resistance management

1. Introduction

Spodoptera exigua (Hübner, 1808), commonly known as the beet armyworm, is a polyphagous pest of global distribution that affects a wide variety of crops, including vegetables, fields, and flowers. The insect can feed on over 170 plant species, primarily consuming leaves and fruits, causing significant damage and substantial economic losses [1,2]. Among the strategies employed for its management, products derived from *Bacillus thuringiensis* (Bt) (Berliner, 1915) have proven to be effective. These include transgenic plants expressing Cry and Vip proteins, encoded by genes present in Bt, to control *Spodoptera* spp. [3–5]. By 2020, the global area of transgenic crops reached approximately 186 million hectares, mainly consisting of cotton, maize, soybeans, and canola [6]. Although transgenic crops have significantly benefited farmers by increasing production and profits, their extensive and, sometimes, indiscriminate use has provided selective pressure on pest populations, leading to the development of resistance. Until 2023, 26 cases of field-evolved practical resistance and 17 cases of early warning of resistance to Bt crops have been reported [7–28].

Such cases have been primarily attributed to modifications in target receptors [29], although it has also been less frequently linked to other mechanisms such as toxin sequestration, alterations in proteolytic processing, and more efficient repair of damaged midgut epithelial cells [30,31]. In addition, the potential involvement of the immune system in the development of resistance to Bt has been examined. While the evidence remains inconclusive, elevated immune activity appears to play an important role in enhancing survival rates against Bt proteins [32]. It has been suggested that when an insect is exposed to sublethal doses of Bt vegetative cells, spores and endotoxins or heat-killed bacterial cells it can mount a stronger immune response upon subsequent exposure. This enhanced response, known as immune priming, can lead to increased survival or tolerance to subsequent lethal treatments. Evidence of immune priming has been observed in species such as *Bombyx mori* [33], *Ephesia kuehniella* [34], *Galleria mellonella* [35], *Rhynchophorus ferrugineus* [36], *Tribolium castaneum* [37,38], and *Tenebrio molitor* [39]. This increased tolerance has been associated with changes in the regulation of specific immune components, such as the Toll and IMD pathways, hemocyte proliferation, enhanced phagocytic activity, and higher expression of antimicrobial peptides [34–36,38,40]. Furthermore, this decrease in susceptibility to Bt can be inherited by offspring, a phenomenon known as transgenerational immune priming (TgIP). TgIP has been reported in *T. castaneum* [41,42], *T. molitor* [43–45] and *Trichoplusia ni* [46].

In the case of *S. exigua*, knowledge remains limited. Recent studies have stated that priming with heat-killed *Escherichia coli* via hemocoelic injection enhances survival upon subsequent challenges with lethal doses of *Acidovorax citrulli*, *Beauveria bassiana*, and *Xenorhabdus hominickii*. These findings suggest that prior exposure to non-pathogenic bacteria can indeed elicit an immune-priming effect in *S. exigua* [47]. While immune priming in *S. exigua* has been documented, its relevance to *B. thuringiensis* and its insecticidal proteins remains unclear. Moreover, most existing studies involve hemocoelic inoculation, leaving the effects of oral exposure largely unexplored in this context. Previous research has demonstrated that sublethal exposure to isolated Bt proteins, such as Cry and Vip, in *S. exigua* larvae induces changes in the expression of immune-related genes [48–50]. However, whether these responses can trigger similar priming effects, altering the tolerance

to Bt proteins and potentially undermining the effectiveness of Bt-based pest control strategies, needs further clarification. Therefore, this study aimed to assess whether prior exposure of neonate *S. exigua* larvae to purified Cry1Ab, Cry1Ca and Vip3Aa toxins would heighten their toxicological response upon subsequent exposure and whether such effects would extend to their offspring.

2. Materials and Methods

2.1. Insects

All the experiments were conducted using a laboratory strain of *S. exigua* reared on an artificial diet [51] under controlled conditions of 16/8 h light/dark at 25 ± 3 °C and $70 \pm 5\%$ of relative humidity. This strain has been maintained for a minimum of 10 years without exposure to *B. thuringiensis* insecticidal proteins.

2.2. Expression and Purification of Cry1 and Vip3Aa Proteins

Cry1Ab and Cry1Ca proteins were obtained from recombinant *Escherichia coli* strains carrying plasmids pBD140 (Cry1Ab) and pBD150 (Cry1Ca). Protein production, inclusion bodies purification, solubilization, and protoxin activation by trypsin were performed according to the protocol described by Sayyed et al. [52]. For the Cry1Ca protein, an additional purification step was necessary. The protein was purified by anion-exchange chromatography utilizing the ÄKTA Explorer 100 System (GE Healthcare Life Sciences, Uppsala, Sweden), as described previously by Crava et al. [48]. Fractions were collected and screened for the presence of Cry1Ca using 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The activated proteins were analyzed by 12% SDS-PAGE and were subsequently stored at -20 °C until further use.

Vip3Aa was expressed and purified from a recombinant *E. coli* BL21 strain using the procedure described by Chakroun [53], with minor modifications. Protein expression was induced with 1 mM Isopropyl- β -D-thiogalactopyranoside (IPTG; Fisher bioreagents, Geel, Belgium) when the culture reached an OD600 of 0.9, followed by overnight incubation at 29 °C with agitation at 180 rpm. Cells were harvested by centrifugation at $8800 \times g$ for 30 min at 4 °C. The cell pellet was resuspended in phosphate-buffered saline (PBS; Fisher bioreagents, Geel, Belgium) and lysed according to the referenced protocol. The soluble fraction containing the target protein Vip3Aa was purified using a HisTrapTM FF crude 1 mL column (Cytiva, Marlborough, CA, USA) and subsequently dialyzed overnight against 20 mM Tris, 150 mM NaCl, pH 8.6. The quantification of Cry1Ab, Cry1Ca and Vip3Aa used in bioassays was performed via densitometry following SDS-PAGE electrophoresis, using bovine serum albumin (BSA) as a standard (Figure S1) and TotalLab 1D (v13.01) software.

2.3. Intragenerational Bioassays

The impact of prior exposures to sublethal concentrations of Cry1 and Vip3Aa proteins on subsequent challenges was analyzed by determining the percentage of growth inhibition. First-instar (L1) larvae were initially exposed to several sublethal concentrations of the proteins using the surface contamination method [54]. Larvae were maintained on this treated diet for five days. Mortality and weight of surviving larvae were recorded before being transferred to a non-treated diet until the fourth instar, at which point they were exposed to the protein again. For the challenge, larvae were exposed to different protein concentrations for 24 h using the same surface contamination methodology. Subsequently, their weights were recorded to calculate the percentage of growth inhibition (%GI) following the methodology described by Herrero et al. [55]. Additionally, a control group was included, wherein larvae were exposed only to the solubilization buffer. Three independent biological replicates were performed for each protein.

For the Cry1Ab protein, two different concentrations, 7.5 ng/cm² and 75 ng/cm², were used for the first exposure, and three different concentrations, 7.5 ng/cm², 75 ng/cm² and 750 ng/cm², for second exposures, based on the IC₅₀ values (concentration that produces 50% growth inhibition relative to untreated controls) reported by Hernández-Martínez et al. [56]. For the Cry1Ca protein, neonates were initially exposed to 1 ng/cm² and 10 ng/cm², considering the IC₅₀ values. Subsequently, the second exposure was performed using three different concentrations, 10 ng/cm², 100 ng/cm², and 1000 ng/cm², to evaluate the effect on growth inhibition. In the case of the Vip3Aa protein, a single concentration of 0.5 ng/cm² was used for the first exposure, as higher doses resulted in more than 50% of mortality. The second exposure was conducted using concentrations of 0.1 ng/cm², 1 ng/cm², 10 ng/cm² and 100 ng/cm².

2.4. Transgenerational Bioassays

To evaluate whether there was an enhancement in toxicological response among larvae originating from a pre-exposure generation, the weight in neonates (L1) offspring and percentage of growth inhibition in L4 offspring were measured. Following the methodology as previously described, neonate larvae were exposed to sublethal concentrations of the protein for five days. Then, larvae were transferred to a non-treated diet and allowed to produce the F1 generation. The offspring were then divided into two groups: neonate larvae designated for the challenge and the larvae to be maintained until reaching the L4 instar, where another challenge would be performed. The neonate larvae in the first group were exposed to different concentrations of the proteins for 5 days. After exposure, they were weighed and compared with untreated larvae. The second group of larvae, upon reaching the L4, was treated with different concentrations of the protein for 24 h, and growth inhibition was calculated.

Three independent biological replicates were performed for each Cry1 protein, whereas two replicates were carried out for Vip3Aa. A schematic overview of the methodology described above is presented in Figure 1.

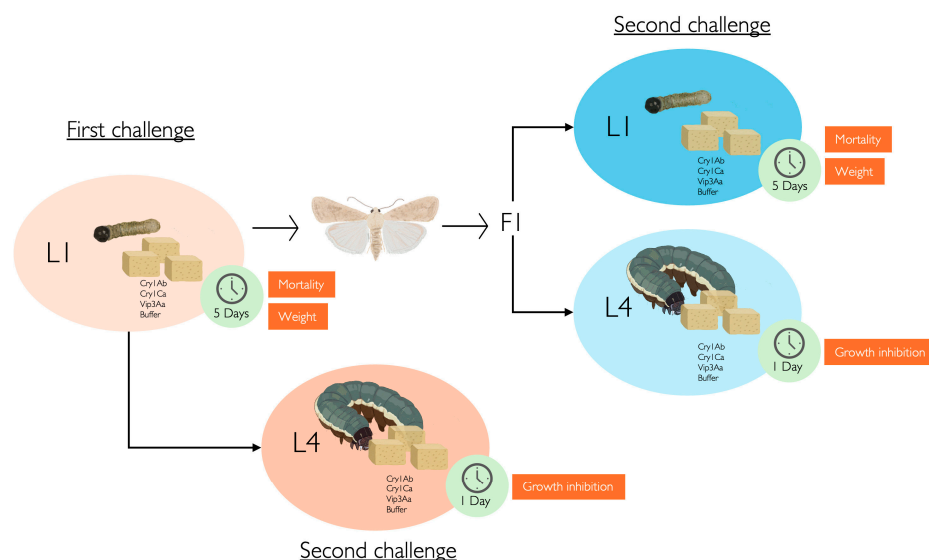


Figure 1. Experimental design used in this study. L1 larvae were subjected to a sublethal exposure using Bt insecticidal proteins. After the 5-day exposure period, larvae were transferred to untreated diet and divided into two groups. In the first group, a second exposure was applied once larvae reached the fourth instar (intragenerational assays). Larvae in the second group were allowed to develop to adulthood, and the resulting F1 generation was exposed to Bt proteins during either the first or fourth larval instar (transgenerational assays). Growth inhibition and mortality values were recorded for those larvae that underwent the second exposure.

2.5. Statistical Analyses

Normality was tested using the Shapiro–Wilk test, and homogeneity of variances was evaluated using Brown–Forsythe test. Differences in developmental time were assessed using one-way ANOVA with Dunnett’s multiple comparisons correction and emergence rates were analyzed using t-tests. Mortality rates were adjusted using Abbott’s correction formula. Statistical analyses of mortality and growth inhibition were performed employing a nonparametric method: the Mann–Whitney U test for two-group comparisons and the Kruskal–Wallis test followed by Dunn’s multiple comparisons tests for analyses involving more than two groups. All analyses were carried out using GraphPad Prism version 9.4.1 (GraphPad Inc., La Jolla, CA, USA).

3. Results

3.1. Sublethal Effects on *Spodoptera exigua* L1 Instar Larvae Exposed to Cry1 and Vip3Aa Proteins During First Exposure

To observe the sublethal effects on L1 larvae caused by toxin exposure, larvae were exposed for 5 days to different concentrations below the expected LC₅₀ (concentration causing mortality of 50% of the exposed insects) for each protein (Table S1 [56,57]). As anticipated, the mean Abbot-corrected mortality levels observed for the L1 larvae exposed to the different concentrations of Cry1Ab or Cry1Ca did not exceed 15% (Figure 2A,C), whereas the average mortality for Vip3Aa reached 18% (Figure 2E). Mortality in the control groups is represented in Figure S2.

After 5 days of exposure, relative larval weight was used as an indicator of sublethal intoxication. Exposure to Cry1Ab resulted in a weight reduction greater than 50%, with a progressive decline in mass as the protein concentration increased (Figure 2B). On the other hand, larvae exposed to Cry1Ca exhibited a moderate weight reduction with approximately a 50% decrease only at the highest concentration used (Figure 2D). In the case of Vip3Aa, the single concentration evaluated caused the largest decrease in relative weight, resulting in more than an 80% reduction (Figure 2F).

Sublethal exposures to the toxins significantly impacted larval size and weight, resulting in developmental delay. After exposure, treated larvae remained at earlier instars (L1–L2), whereas control larvae had reached the third-instar larvae (L3), except in the treatment with 1 ng/cm² of Cry1Ca, where the larval development was comparable to the control. After transferring the exposed larvae to a non-treated diet, the exposed larvae resumed normal behavior (Table 1), indicating that the negative effect caused by toxin exposure was not permanent. The mean duration of each life stage, as well as the proportion of individuals that successfully reached the pupal and adult stages, did not differ among treatments, indicating no associated fitness costs. However, some exceptions were observed, such as larvae treated with 75 ng/cm² of Cry1Ab and 0.5 ng/cm² of Vip3Aa, where development to the pupal stage was delayed.

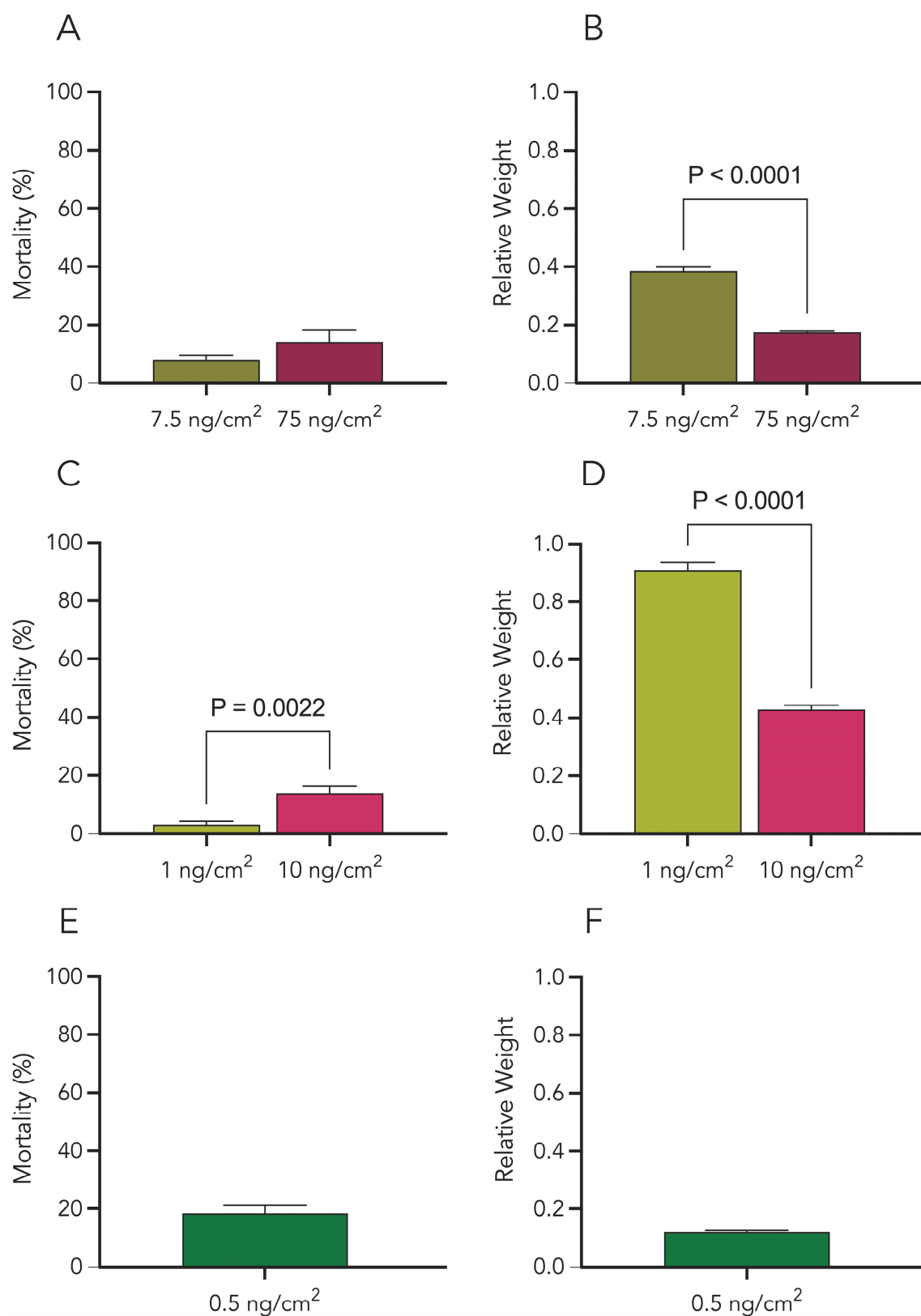


Figure 2. Effect of initial Cry1Ab, Cry1Ca, or Vip3Aa exposure on larval weight and mortality in *S. exigua*. Larval mortality (A,C,E) and relative weight (B,D,F) of *S. exigua* after five days of exposure to different protein concentrations of Cry1Ab (A,B), Cry1Ca (C,D) or Vip3Aa (E,F). Data are expressed as the mean $\pm$ SE. Values were compared by Mann–Whitney U test.

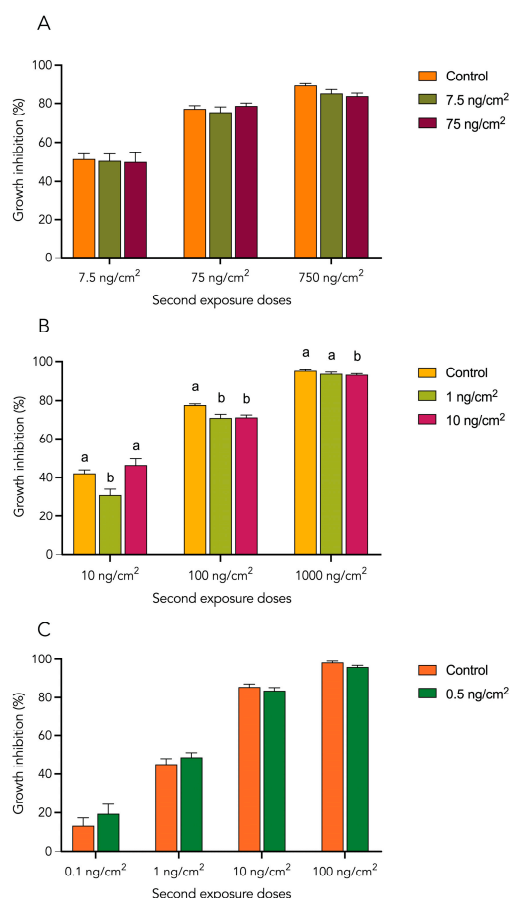
Table 1. Developmental parameters and survival of *Spodoptera exigua* after first exposure to Cry1Ab, Cry1Ca or Vip3Aa.

Parameter	Control	Exposure to Cry1Ab ¹		Exposure to Cry1Ca ¹		Exposure to Vip3Aa ¹
		7.5	75	1	10	0.5
Development time						
Days to pupation from neonate	13.7 ± 0.4	13.7 ± 0.3	16.0 ± 0.6 *	15.0 ± 0.6	15.7 ± 0.9	17.0 ± 0.0 **
Days to adulthood from pupa	8.4 ± 0.4	9.0 ± 1.0	7.7 ± 1.2	8.7 ± 0.7	8.7 ± 0.7	7.5 ± 0.5
Days to egg-laying after adulthood	3.0 ± 0.3	2.0 ± 0.0	2.3 ± 0.8	3.0 ± 1.0	3.3 ± 0.9	3.5 ± 0.5
Days to hatching from egg	2.6 ± 0.2	2.7 ± 0.3	2.7 ± 0.7	2.3 ± 0.3	2.7 ± 0.7	2.5 ± 0.5
Pupal and adult emergence rates						
Percentage of pupae from larvae	87 ± 4	85 ± 9	85 ± 5	86 ± 2	72 ± 4	74 ± 6
Percentage of adults from pupae	90 ± 2	91 ± 5	90 ± 7	92 ± 3	92 ± 4	84 ± 10

* Indicates significant differences ($p < 0.05$) and ** ($p < 0.01$) between the control and treatment. ¹ Values are presented as mean ± SE., and protein concentration are in ng/cm².

3.2. Effects of Prior Exposure to Sublethal Concentrations of Cry1 and Vip3Aa Proteins on Growth Inhibition in L4 Instar *S. exigua* Larvae After a Second Exposure

As expected, Cry1 and Vip3Aa proteins inhibited *S. exigua* larval growth in fourth-instar larvae (L4), in a dose-dependent manner, although the magnitude of the inhibition varied among proteins, with Vip3Aa exhibiting a greater inhibitory effect at comparable doses (Figure 3). Specifically, exposure to 7.5 ng/cm² of Cry1Ab resulted in a 52% inhibition, reaching 89% at 750 ng/cm². For Cry1Ca, growth inhibition was 42% at 10 ng/cm² and increased to 95% at 1000 ng/cm². In contrast, Vip3Aa caused 13% inhibition at 0.1 ng/cm², rising to 98% at 100 ng/cm².

**Figure 3.** Growth inhibition (mean ± SE) of the 4th instar *S. exigua* larvae following second exposure to different concentrations of Cry1Ab (A), Cry1Ca (B), and Vip3Aa (C). Each bar represents the first

treatment administered to L1 larvae, while each group corresponds to the concentrations used to expose L4 larvae for 24 h within the same generation. Control: L1 larvae exposed to the buffer used for protein solubilization. Lowercase letters indicate significant differences ($p < 0.05$) between the control and different concentrations of the protein, according to Kruskal–Wallis and Dunn’s tests for (panels A,B), and the Mann–Whitney U test for (panel C).

In the case of Cry1Ab and Vip3Aa (Figure 3A,C), a comparative analysis between the control group (larvae without prior exposure) and larvae previously exposed as neonates revealed no statistically significant differences, indicating that early-stage exposure did not alter tolerance levels in the same generation. By contrast, Cry1Ca exhibited a different pattern (Figure 3B). As a general trend, larvae treated as neonates showed a significant reduction in growth inhibition compared to the control group. Neonates initially exposed to 1 ng/cm² and then to 10 ng/cm² exhibited a decrease in growth inhibition from 42% to 31% relative to the control and from 77% to 71% when exposed to 100 ng/cm². Conversely, neonates initially exposed to 10 ng/cm², showed a slight change in terms of growth inhibition from 77% to 71% at the 100 ng/cm² treatment, and from 95% to 93% at the 1000 ng/cm² treatment.

3.3. Changes in the Toxicological Response of *S. exigua* Larvae Over Time After Sublethal Exposure to Cry1 and Vip3Aa Proteins of Parental Larvae

To evaluate potential changes in the toxicological response following exposure to the proteins, descendant larvae were exposed to the same sublethal concentrations used for the parentals, as well as to an additional, higher concentration.

The average weight and mortality in L1 instar larvae were measured after five days of exposure (Figure 4). For Cry1Ab and Vip3Aa exposure (Figure 4A,B,E,F), the results revealed no significant differences between control groups and descendants of treated parents across all tested concentrations, with both weight and mortality rates remaining consistent. Similar results were observed after exposure of *S. exigua* descendant L1 larvae to Cry1Ca (Figure 4C,D). Interestingly, a reduction in weight, approximately 30% compared to the control group, in larvae descended from parents exposed to 10 ng/cm² was observed. This weight reduction occurred only when larvae were exposed to 10 ng/cm² and not to the other two concentrations tested. No significant differences in terms of mortality were observed between treatments and the control group.

For the assays carried out using descendant larvae at L4 instar, larvae derived from parents exposed to higher concentrations of Cry1Ab exhibited different responses (Figure 5A). When the offspring were exposed to a low dose of 7.5 ng/cm², growth inhibition after 24 h was reduced, decreasing from 45% to 34% compared to the control. In contrast, when larvae were exposed to the highest dose of 750 ng/cm² the effect was reversed, with growth inhibition increasing from 88% to 93%. These results suggest the existence of a threshold at which some benefit can be observed; further increases in protein exposure led to detrimental outcomes, rather than improvement. When the descendant L4 larvae were treated with Cry1Ca for 24 h, growth inhibition was similar to that of the control group (Figure 5B). A similar pattern was observed for Vip3Aa, except at the specific concentration of 10 ng/cm², where growth inhibition was higher in larvae derived from treated parents (83%) compared to the control group (74%).

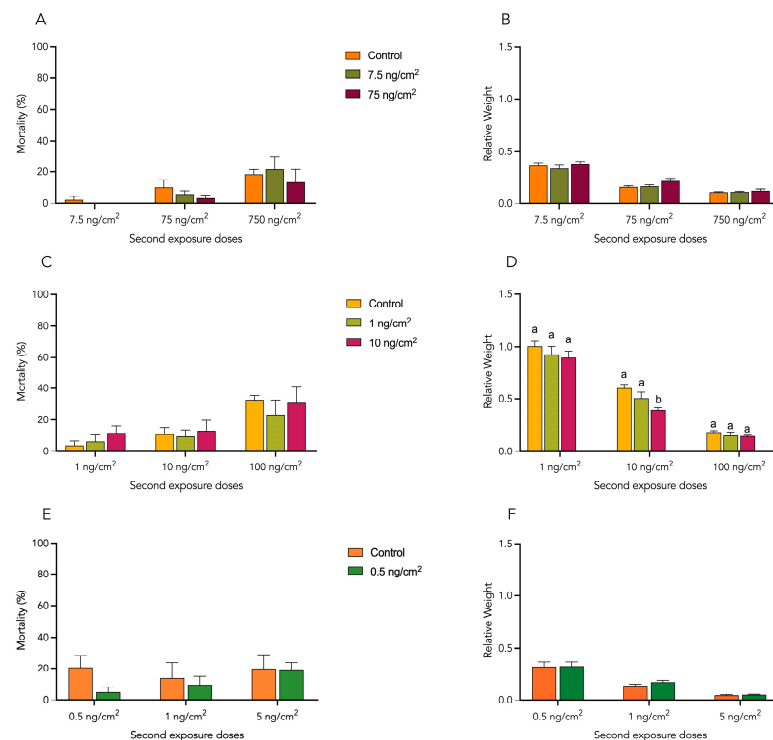


Figure 4. Effect of second Cry1Ab (A,B), Cry1Ca (C,D), or Vip3Aa (E,F) exposure on larval weight and mortality in descendants of *S. exigua*. Larval mortality (A,C,E) and relative weight (B,D,F) of L1 descendants after exposure. Each bar represents the treatment administered to the parental L1 larvae, while each group corresponds to the concentration used to expose the descendant L1 larvae for 5 days. Control: L1 larvae exposed to the buffer used for protein solubilization. Data are expressed as the mean $\pm$ SE. Lowercase letters indicate significant differences ($p < 0.05$) between the control and different concentrations of the protein, according to Kruskal–Wallis and Dunn’s tests for (panels A–D), and the Mann–Whitney U test for (panel E,F).

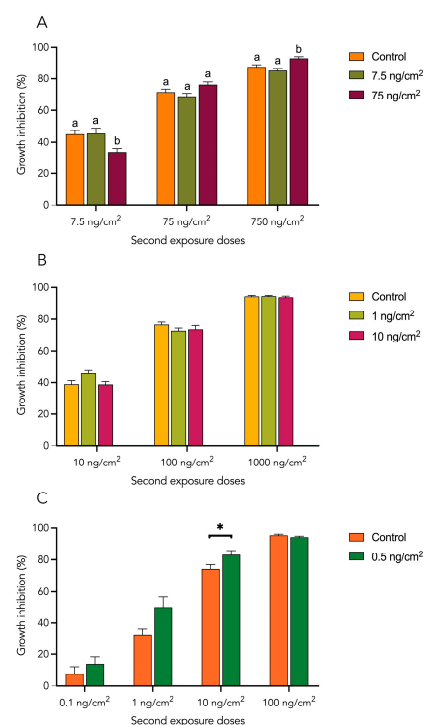


Figure 5. Growth inhibition (mean $\pm$ SE) of the 4th instar *S. exigua* larvae descendants following parental exposure to different concentrations of Cry1Ab (A), Cry1Ca (B) and Vip3Aa (C). Each bar

represents the treatment administered to the parental L1 larvae, while each group corresponds to the concentrations used to expose the descendant L4 larvae for 24 h. Control: L1 larvae exposed to the buffer used for protein solubilization. Lowercase letters indicate significant differences ($p < 0.05$) between the control and different concentrations of the protein, according to Kruskal–Wallis and Dunn’s tests (panels A,B). Asterisk (*) indicates significant differences ($p < 0.05$) between the control and treated group, according to the Mann–Whitney U test (panel C).

4. Discussion

The use of Bt-derived products, whether through formulations or transgenic plants, represents a significant advance in pest control technology. However, inappropriate use of these tools threatens their long-term efficacy and sustainability. One of the risks is the potential reduction in insect susceptibility to Bt products. Numerous studies have demonstrated that repeated exposure to Bt vegetative cells, spores and crystals or heat-killed bacteria preparations can enable insects to develop mechanisms that enhance their tolerance [33–39]. However, little is known about this phenomenon in *S. exigua*, a key agricultural pest. In this context, our study aimed to explore the impact of repeated oral exposure to purified Bt Cry1 and Vip3Aa proteins, an unexplored approach given the scarcity of studies isolating the impact of these proteins from other Bt components. To closely simulate field conditions, we used an oral challenge model, in contrast to the more commonly used direct hemocoel injection method. This approach mimics natural exposure scenarios, where the protein needs to overcome various barriers and insect defense mechanisms to reach its target site [30]. In contrast, hemocoelic injection bypasses these defenses and introduces the toxin directly into the hemocoel, which may be useful for studying systemic immune responses but does not accurately reflect field exposure.

Using this exposure model, we investigated whether there was an increase in tolerance within the same generation after repeated challenges. Rather than using mortality assays, we employed growth inhibition assays, as the larvae were in their fourth instar at the time of the second exposure. At this developmental stage, growth inhibition is a more reliable indicator of toxin impact than mortality, as older instars are less susceptible, requiring notably higher toxin concentrations to induce lethal effects. Growth inhibition, therefore, serves as an alternative measure of toxin susceptibility and has been used in previous studies [55,56,58,59].

Our findings reveal that prior exposure to Cry1Ab or Vip3Aa did not result in significant changes in tolerance to subsequent Cry1Ab/Vip3Aa challenges. This suggests that *S. exigua* does not develop an increased tolerance to these proteins within the same generation. In contrast, exposure to Cry1Ca led to a significant decrease in growth inhibition, indicating an enhanced tolerance. However, this response was not consistent across all tested concentrations, suggesting a dose-dependent effect. Similar patterns have been reported in other studies, where minimal changes occur at low initial doses, while higher doses may overwhelm the system, causing physiological damage and impairing the insect’s defense against subsequent exposures [40,60].

The differing responses to the proteins could be related to their distinct effects on *S. exigua*. Specifically, Cry1Ab shows moderate insecticidal activity, while Cry1Ca exhibits more potent toxicity, which can lead to higher mortality rates [56]. Furthermore, previous studies have shown that sublethal exposure to Cry1Ca induces significant physiological changes, including alterations in immune gene expression [48]. These changes could facilitate short-term adaptive responses in the insect, potentially contributing to the increased tolerance observed upon subsequent exposure. Nevertheless, this response cannot be attributed uniquely to immune modulation, as Vip3Aa also induces high toxicity with a delayed effect and changes in immune genes, yet no reduction in growth inhibition was observed

following sublethal exposure. These findings suggest that physiological or environmental factors could also be involved [61].

It has been shown that *S. exigua* can improve survival against several pathogens after prior exposure, via hemocoelic injection, to bacterial components. However, unlike the findings of Haraji et al. [47] in this case, prior oral exposure to isolated proteins only confers a temporary advantage in specific scenarios. Both the nature of the exposure agent, whether a mixture of molecules or a single component, and the inoculation method are crucial in determining the response. Länger et al. [62] demonstrated that exposure to the Cry3Aa toxin could induce increased tolerance in *T. molitor* but observed that this response was less pronounced compared to exposure in combination with other molecules. The use of whole bacterial cells or complete pathogen formulations may then provide greater protection, as insects have evolved over time to detect and respond to such conserved elements. In general, the insect's immune system can recognize pathogens often by surface protein recognition. In *G. mellonella*, direct hemolymph injection of purified LPS induces protective responses against lethal doses of *Photobacterium luminescens* TT01. However, the injection of PirA2B2 toxin did not enhance resistance to bacterial infection [63]. Our findings support the idea that exposure to isolated proteins alone is insufficient to elicit robust protective responses; instead, the insect probably requires additional molecular signals to recognize the pathogen and mount a priming defense.

When transgenerational effects were examined, we observed a global scenario in which previous exposures generally did not have an impact on offspring, with few exceptions. In the case of Cry1Ab, assays were extended for a third generation which revealed no changes in the offspring; consequently, analyses presented here were limited to only two generations for all proteins. In contrast to what has been observed in other species, where parental exposure to Bt resulted in increased survival or tolerance in the offspring [41–43,45], the tolerance induced by Cry1Ca in this study was not transmitted to the next generation. A difference in our study is that we exposed larvae at early developmental stages, while many of the previously mentioned studies focused on exposing adult insects. Similar results were found by Schulz et al. [64], where oral exposure of *T. castaneum* larvae to Bt did not confer any transgenerational benefits in terms of tolerance, contrasting with the effects of adult injection. This suggests that the developmental stage at which exposure occurs plays a critical role in influencing transgenerational effects.

In addition, the development of tolerance mechanisms can often incur fitness costs in the parental generation, which may negatively affect offspring development. For example, in *T. molitor*, offspring exhibited prolonged developmental times or reduced weight at the pupal stage following exposure [44]. Similarly, studies in *T. castaneum* have reported reduced fecundity and prolonged development [42,64]. In the case of *S. exigua* exposed to Cry1Ca, while there was some evidence of increased tolerance in the parental generation at certain concentrations, the subsequent generation showed a reduced tolerance at the higher sublethal concentrations. For Cry1Ab and Vip3Aa, parental exposure to higher concentrations resulted in delayed developmental time and, in specific cases, greater growth inhibition in the offspring, suggesting that sublethal exposure not only failed to increase tolerance but may have compromised the fitness and resistance in the next generation. Interestingly, for Cry1Ab, a reduction in growth inhibition was observed when the second exposure involved a lower concentration, demonstrating the dose-dependent response involved in this process. These results highlight the complexity of transgenerational tolerance mechanisms. While initial exposures may provide short-term benefits, they may also lead to increased susceptibility in subsequent generations. In *S. exigua*, the number of generations per year can vary from two to six depending on environmental conditions [65];

however, no positive effect on larval survival is expected, given the biological costs detected in this study.

The differences observed here were found under highly controlled laboratory conditions, which may not fully capture the complexity of field environments where multiple factors interact, such as plant-insect interactions, exposure duration, toxin concentrations, exposure to different combinations of proteins, degradation factors, etc [66]. Although statistical differences were found, they may not be biologically significant, as the effects were minimal and the fact that these responses were neither generalized nor sustained over time. Therefore, based on the current data, the risk of reduced efficacy associated with the use of low doses of purified Cry and Vip3Aa proteins for controlling *S. exigua* appears to be minimal.

5. Conclusions

We orally exposed *S. exigua* to the purified proteins of Bt (Cry1Ab, Cry1Ca and Vip3Aa) and analyzed changes following a second exposure within the same generation and in the subsequent generation. The intragenerational analysis showed that pre-exposure to Cry1Ab or Vip3Aa did not improve tolerance to subsequent challenges. However, Cry1Ca induced a slight increase in tolerance under certain conditions. Furthermore, transgenerational analysis revealed no significant increase in tolerance in offspring. These results suggest that while initial oral exposure to sublethal doses of purified Bt proteins may trigger minor physiological changes, such responses are insufficient to drive the development of long-term resistance, particularly in a field-relevant context. Future studies should investigate the interactions between crystals and spore preparations, as these may provide additional stimulation mechanisms. Understanding these interactions will help to refine Bt-based pest management strategies and ensure their long-term effectiveness.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/insects17020193/s1>. Figure S1: Protein quantification. Representative SDS-PAGE gel and bovine serum albumin (BSA) standards used to quantify activated Cry1Ca and Cry1Ab proteins and Vip3Aa protoxin. Figure S2: Mortality observed in control groups during initial exposure of L1 *S. exigua* larvae to Bt toxins; M, Blue Star protein marker. Protein bands were stained with Coomassie Blue; Table S1: Reported LC50 for Cry1Ab, Cry1Ca and Vip3Aa in *S. exigua*.

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Abbreviations

The following abbreviations are used in this manuscript:

Bt	<i>Bacillus thuringiensis</i>
L1	First-instar
L4	Fourth-instar
L3	Third-instar
SDPS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
IPTG	Isopropyl- β -D-thiogalactopyranoside
PBS	Phosphate-buffered saline
BSA	Bovine serum albumin
GI	Growth inhibition
IC ₅₀	Concentration causing 50% growth inhibition
LC ₅₀	Concentration causing 50% mortality

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