Research Article

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A major forest insect pest, the pine weevil *Hylobius abietis*, is more susceptible to Diptera- than Coleoptera-targeted *Bacillus thuringiensis* strains

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Abstract

BACKGROUND: The pine weevil (*Hylobius abietis*) is a major forest regeneration pest causing high levels of seedling mortality and economic losses. Current management relies on silviculture, stem coatings and insecticides. Here we evaluated for the first time the effects of *Bacillus thuringiensis* (*Bt*) strains on *H. abietis* adults: two producing the Coleoptera-targeted toxins *Cry3Aa* (*Bt tenebrionis* NB-176) and *Cry8Da* (*Bt galleriae* SDS-502), and one producing the Diptera-targeted *Cry10A* (*Bt israelensis* AM65-52). Choice and nonchoice assays using individual and mixtures of *Bt* formulations, containing these strains respectively, were conducted.

RESULTS: We found that *Bt* had toxic and lethal effects on *H. abietis*, but effects varied with strain and formulation concentration. The Diptera-targeted *Bt israelensis* had the most negative effects on weevil weight, feeding and mortality (70–82% feeding reduction, 65–82% greater mortality than control), whereas the effect was lower for the Coleoptera-specific *Bt tenebrionis* (38–42%; 37–42%) and *Bt galleriae* (11–30%; 15–32%). Reduced weevil feeding was observed after 3 days, and the highest mortality occurred 7–14 days following *Bt* exposure. However, we found no synergistic toxic effects, and no formulation combination was better than *Bt israelensis* alone at reducing consumption and survival. Also, pine weevils were not deterred by *Bt*, feeding equally on *Bt*-treated and non-*Bt* treated food.

CONCLUSION: There is potential to develop forest pest management measures against *H. abietis* that include *Bt*, but only the Diptera-targeted *Bt israelensis* would provide effective seedling protection. Its Diptera-specificity may need reconsideration, and evaluation of other *Bt* strains/toxins against *H. abietis* would be of interest.
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Keywords: biopesticides; forest pest; forest regeneration; insecticidal toxins; pest management; plant protection

1 INTRODUCTION

The pine weevil, *Hylobius abietis* (L.), is one of the major regeneration pests of conifer forests in Europe. Adult weevils cause high levels of seedling mortality by feeding on the stem bark. They often consume an entire ring of bark around the stem circumference, which hinders nutrient transport and eventually results in plant death. Thus, preventive measures are necessary for successful conifer forest regeneration.[1] Insecticide-treated seedlings have been commonly used, but for legislation, biodiversity and human health reasons there are ongoing efforts to phase them out.[2] In Sweden and other north European countries, different silvicultural practices as well as stem physical coatings or barriers are used to decrease pine weevil damage and maximize seedling survival.[3–5] Yet, these measures cannot be applied at all regeneration sites and vary in efficacy depending on conditions.[6] In other countries, biological control of the pine weevil with entomopathogenic

nematodes has been attempted, but its success is dependent on several factors.[7,8] Relative to other control measures, effects of biopesticides on the pine weevil, such as those containing the entomopathogenic bacteria *Bacillus thuringiensis*, have remained unconsidered.

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Bacillus thuringiensis Berliner (*Bt*) is a gram-positive entomo-pathogenic bacteria that is naturally found in soils, aquatic environments, dead insects and leaf surfaces, among others.[9] *Bt* strains can synthesize different crystal (*Cry*) and cytolitic (*Cyt*) proteins, which are toxic to insects when ingested. Ingestion of these toxins can result in midgut degradation, feeding reduction or cessation, and eventually death of susceptible individuals.[10] *Bt* has been extensively used against agricultural pests and vectors of human diseases, especially those in the insect orders Lepidoptera and Diptera. Both transgenic plants (expressing specific *Bt* toxic proteins) as well as treatment with *Bt* spores/toxins in the form of different formulation products have been successfully used as methods of protection.[9,11] Several end-use products are available in the market and these can contain toxins or spores stemming from one specific *Bt* strain, for example Trident® (*Bt* ssp. *tenebrionis* SA-10) or DiPel® (*Bt* ssp. *kurstaki* ABTS-351). They can also contain combinations of toxins from different strains, such as Crymax® (*Bt* ssp. *kurstaki* and *Bt* ssp. *aizawa* EG7841). These formulations have been developed for direct usage by spray treatment of plant tissue consumed by the insect, and are effective against pests affecting, for example, turf management, vegetable production and forestry.[11,12] Examples of such pests include the Colorado potato beetle (*Leptinotarsa decemlineata*) and so-called white grubs (Scarab insect pests, e.g., *Cyclocephala* spp.) in turfgrass. However, since larval stages are those that usually cause most plant damage, less focus has been placed on the toxic effects of *Bt* on adult insects.

In forestry, *Bt* has been mostly used to control Lepidopteran pests such as the gypsy moth *Lymantria dispar* and the spruce budworm *Choristoneura fumiferana*.[13,14] Although Coleopteran pests are numerous in forest ecosystems, relatively few have been tested for their susceptibility to *Bt* toxins. Moreover, among studies examining *Bt* effectiveness against Coleopteran insects, beetles in the family Chrysomelidae are overrepresented while Curculionidae (weevil) species have been less examined.[15,16] A moderate proportion of those Curculionidae species investigated have shown susceptibility to *Bt*, but few toxins have been tested.[15,16] The most commonly tested *Cry* proteins include Cry3Aa (*Bt* ssp. *tenebrionis*) and Cry8Da (*Bt* ssp. *galleriae*). More recently, however, there have been reports of certain Curculionidae species being susceptible to Cry10A (*Bt* ssp. *israelensis*) which stems from a *Bt* strain used against Dipteran species.[17–20] To our knowledge, no tests of *Bt* toxicity specifically against *Hylobius* spp. have been previously conducted.

In this study, we evaluated the susceptibility of adults of the forest insect pest *H. abietis* to three *Bt* strains that produce Coleoptera- and Diptera-targeted toxins: *Bt tenebrionis* NB-176, *Bt galleriae* SDS-502 and *Bt israelensis* AM65-52. To assess the ingestion toxicity, we selected three *Bt* formulations that contain these respective *Bt* strains: Novodor® (*Bt tenebrionis*), BeetleGONE tlc® (*Bt galleriae*) and VectoBac WDG® (*Bt israelensis*). To evaluate the toxicity of these strain formulations individually and in mixtures, and if *H. abietis* adults can distinguish between *Bt*-treated and nontreated food, we addressed the following questions using laboratory bioassays:

(1) How are the mortality, feeding and weight of *H. abietis* adults affected by consumption of *Bt*-treated Norway spruce (*Picea abies*) stem pieces using different strain formulations and concentrations, compared to those consuming water-treated stem pieces (control)?

(2) Do the toxic effects of *Bt* on *H. abietis* differ depending on whether stem pieces are treated with one single formulation or a combination of products?

(3) Do *H. abietis* adults avoid feeding on *Bt*-treated food or do they feed equally on *Bt*-treated and control stem pieces when exposed to them simultaneously?

2 MATERIALS AND METHODS

2.1 Plant material, insects and *Bt* formulations

The plant material used to evaluate the effects of *Bt* on *H. abietis* were stem pieces from 1.5 year-old seedlings (20–30 cm in height) of Norway spruce, *P. abies* (L.) H. Karst, which were purchased from a nursery (Stora Enso Plantor AB, Nässja, Sweden). On receipt, seedlings were planted in plastic pots (diameter = 9 cm) and kept in a greenhouse (16 h light:8 h dark, 18 °C) for a few weeks before the experiments began. The day of experimental tests, a piece from the lower part of the stem was freshly harvested from these plants (approximate length 8 cm, mean diameter of all pieces ± standard error 2.04 ± 0.06 mm). Needles were carefully removed from the bark, and the *Bt* product was applied by dipping the piece carefully in the mixture (or in water for controls; see description below).

Adult pine weevils were used to assess the toxicity of *Bt* strains. Insects were collected during their spring migration in May 2019 at a sawmill located in central Sweden (Balungstrands Sågverk AB; Enviken, Sweden). After collection, they were placed in wooden rearing boxes under constant darkness at 10 °C. In each box, pieces of stems/branches from young Scots pine trees (*Pinus sylvestris* L.) for weevils to feed on and access to water were provided; these were replaced every 4 weeks. Under these light and temperature conditions, the reproductive development of the pine weevil is interrupted. However, once they are transferred to warmer temperatures and exposed to light, oviposition and the period of high feeding activity associated with it can resume.[21] Pine weevils were placed under laboratory conditions (16 h light:8 h dark, 20 °C) for acclimatization, 10 days prior to experimental start.

All experiments were conducted between September and December 2019 at the laboratory facilities of the Department of Ecology, Swedish University of Agricultural Sciences (Uppsala, Sweden). The Swedish Chemicals Agency granted us a permit to purchase, import and use the three commercial *Bt* products (Table 1) for research purposes (permit 5.1.2.b-H19-06122, granted 2019-2108-05). Remains of *Bt* mixtures from experiments were properly disposed of as chemical waste through the Department of Ecology's waste management routines.

2.2 Testing toxicity of individual *Bt* formulations

Nonchoice feeding tests were conducted to test the toxicity of each *Bt* strain, exposing pine weevils to *Bt*-treated and non-*Bt* treated food (Norway spruce stem pieces) in separate experimental Petri dishes. Two different concentrations (low and high) of each formulation, in terms of liquid (ml) or grams (g) per volume depending on the product, were tested (Table 1). These two concentrations were based on a pilot study conducted prior to experimental tests. In this pilot study, we tested concentrations suggested by product labels and previous literature examining the effect of these formulations (not transgenic plants) on Coleopteran late-instar larvae or adults (if available), and in Diptera for *Bt*.[22–26] Concentrations (percentage of product in treatment solution; mass or liquid to volume ratios, g:vol or vol:vol) ranged from

Table 1. *Bacillus thuringiensis* (*Bt*) strains and formulations (strain, product, manufacturer, colony-forming units (CFU): no. of bacteria spores or toxin in product), and the concentrations (low and high; grams or milliliters of product per liter of water (g or ml L^{-1}); percentage of product and of water in the total mixture, % vol:vol ratio) tested for toxicity against the pine weevil, *Hylobius abietis*

Strain/formulation	Manufacturer	CFU	Low concentration (g or mL L^{-1}; % vol:vol)	High concentration (g or mL L^{-1}; % vol:vol)
Bt galleriae SDS-502 (BeetleGONE tlc®)	Phyllom BioProducts Corporation, USA	0.85×10^{10} CFU g^{-1}	100 g L^{-1} (10%:90%)	200 g L^{-1} (20%:80%)
Bt israelensis AM65-52 (VectoBac WDG®)	Valent BioSciences LLC, USA	0.30×10^{7} CFU g^{-1}	200 g L^{-1} (20%:80%)	400 g L^{-1} (40%:60%)
Bt tenebrionis NB-176 (Novodor®)	Biocont Magyarország Kft, Hungary	0.10×10^{5} CFU g^{-1}	400 mL L^{-1} (40%:60%)	800 mL L^{-1} (80%:20%)

0.1% to 25% in these studies and instructions, depending on the strain. As in previous studies, we took into account the number of colony-forming units (CFU; no. of bacteria spores/toxins) per formulation, and used greater amounts for those with lower CFU levels. Relative to larval stages, fewer studies had examined effects on adult insects. Older insects often have guts that contain more epithelial cells than those of younger ones, thus more toxin is needed to cause feeding cessation and ultimately death.[27] Given that our target stage were adults, we included treatments with concentrations above those suggested (>20%) for earlier insect stages.

The *Bt* mixture (product + distilled water) for each strain formulation was prepared on the day of the experiment. The corresponding amount of product (Table 1) was mixed thoroughly with distilled water to produce a homogenous solution. Once the mixture was prepared, it was poured into a container that allowed stem pieces to be easily submerged into the solution (total volume 150 mL). Each stem piece was submerged for about 2 min to allow the entire surface to be covered by the product, and then placed (with tweezers) on filter paper to air dry for an hour. The high concentration mixture of the *Bt israelensis* formulation had a thicker consistency relative to the others, thus stem pieces of this treatment only were quickly re-dipped in water to remove excess product. Two treated stem pieces were placed in each Petri dish on a round filter paper. For the control group, stem pieces were submerged in distilled water and the same procedure as for *Bt*-treated pieces was followed. One pine weevil was placed in each Petri dish (previously starved for 48 h) with access to water and allowed to feed for 3 days. After 3 days, fresh *Bt*-treated (or water-treated) food was replaced in each Petri dish and the pine weevils were allowed to feed for an additional 4 days. Pine weevils were weighed before and after the total 7 days of exposure to treated or untreated stem pieces. Mortality and amount of bark consumed on the two stem pieces (area debarked, mm^2) were recorded after 3 and 7 days of feeding. The area debarked was calculated by measuring the length and width of each feeding scar using millimeter paper, and adding the areas of all scars together to obtain the total area debarked for each stem piece. The area debarked for the two stem pieces in each Petri dish were added together, i.e. for each weevil placed in the Petri dish there was one corresponding value of total area debarked.

Subsequently, pine weevils were moved to a new Petri dish containing fresh non-*Bt* treated food and followed for an additional 7 days to monitor debarked area and eventual mortality. Weevils were also weighed after these 7 days. Once this experimental period (14 days) concluded, we grouped individuals from each treatment and concentration, and placed each group separately in ventilated plastic buckets with access to non-*Bt* treated food and water. We noted mortality in these boxes after 7 days (i.e. weevils were followed for a total of 3 weeks). A total of 40 replicates (Petri dishes) per *Bt* formulation and concentration were included (*n* per product = 80, *n* for controls = 40). Due to laboratory space limitations, the experiment was replicated in time. A total of four rounds were conducted, with 10 replicates per product and concentration in each round.

2.3 Testing toxicity of combinations of *Bt* formulations

To test if a combination rather than individual *Bt* formulations yielded higher toxic and lethal effects on pine weevils, we conducted nonchoice feeding tests with stem pieces treated with mixtures of more than one product. We followed the same experimental procedure as described above, but tested all possible combinations of the three *Bt* strains (Table 2). High concentrations of each formulation were used as a control group for comparisons. For the combination treatments, each individual product was first prepared at the highest dose separately. These were then combined and mixed thoroughly in one container (total volume 150 mL) according to each treatment's volume proportions (Table 2). Stem pieces were then submerged in this mixture as described for previous experiments. Pine weevils were exposed to 7 days of *Bt*-treated food, but unlike individual product tests no fresh treated *Bt* food was replaced after 3 days (i.e. they fed

Table 2. Concentrations used in tests aimed at evaluating the toxicity of combinations *vs* individual *Bacillus thuringiensis* (*Bt*) formulations, against the pine weevil, *Hylobius abietis*

Treatment (abbreviation)	Concentration (g or mL L^{-1}; vol:vol)
Bt galleriae (BeetleGONE; B)	200 g L^{-1}
Bt israelensis (VectoBac; V)	400 g L^{-1}
Bt tenebrionis (Novodor; N)	800 mL L^{-1}
Bt galleriae + *Bt israelensis* (BV)	(1:1)
Bt galleriae + *Bt tenebrionis* (BN)	(1:1)
Bt israelensis + *Bt tenebrionis* (VN)	(1:1)
Bt galleriae + *Bt israelensis* + *Bt tenebrionis* (BVN)	(1:1:1)

Treatments represent the individual formulations tested in the control group (high concentration of each strain; grams or milliliters of product per liter of water, g or mL L^{-1}) and all possible formulation combinations (volume ratio when mixing formulations, vol:vol).

on the same stems for 7 days). Mortality and debarked area were recorded on these same stems at 3, 5 and 7 days since the start of the experiment. Individuals were subsequently grouped per treatment, and placed with access to non *Bt*-treated food and water, in ventilated plastic buckets. Mortality was recorded after 7 days in the buckets (i.e. weevils were followed for a total of 2 weeks). A total of 17 replicates per treatment were included. The experiment was replicated in time, in two different rounds (round 1, *n* = 5 per treatment; round 2, *n* = 12 per treatment).

2.4 Testing avoidance of *Bt* formulations
To test if pine weevils could distinguish between *Bt* and non-*Bt* treated food, a choice feeding test using a paired design was conducted. One control and one *Bt*-treated stem piece were placed in the same petri dish. Each stem piece was treated with *Bt* (or water for controls) as described above for the individual toxicity tests. However, only high concentrations of each formulation were used, and a total of three paired treatments were included: control and *Bt galleriae*, control and *Bt israelensis*, control and *Bt tenebrionis*. Each treatment was replicated 10 times, but unlike the other experiments, no replication in time occurred. One pine weevil was placed in each Petri dish, with access to water, and allowed to feed for 7 days in total without replacing the food. Debarked area and mortality were scored at 3, 5 and 7 days after the start of the experiment. After this time, pine weevils were not followed for the subsequent 7 days since the objective here was to assess if they could differentiate between the two types of food.

2.5 Statistical analyses
All analyses were conducted in R version 3.6.3 using R studio 1.2.5033.[28,29] For experiments testing individual strain toxicity, the fixed effects of treatment [control (C), *Bt galleriae* formulation low and high (BL, BH), *Bt israelensis* (VL, VH), *Bt tenebrionis* (NL, NH)] on the response variable of interest were examined using linear mixed effects models with the package *lme4* (version 1.1-23).[30] Since the experiment was replicated in time, round was included as a random effect in models. Response variables included total area debarked per treatment, area debarked per weevil per treatment, mortality and pine weevil weight. For models examining effects on mortality, a binomial distribution was used. The effect of treatment on each variable was tested independently for each time period examined (i.e. at 3, 7 days, etc.). For area debarked and weevil weight, significance of main effects was tested using the *anova* command in *lme4*; for weevil mortality, analysis of deviance using the *car::Anova* command (*car* package, version 3.0-8)[31] was conducted. Initial weight (before *Bt* exposure) was included as a covariate in models examining differences in weight after 7 days of *Bt*-treated and the subsequent 7 days of non-*Bt* treated food. If main effects were found to be significant, comparisons among the control and treatments were conducted with the *emmeans* package (version 1.4.6),[32] using a Dunnett adjustment. No transformations of response variables were necessary, except for area debarked per weevil, which was square-root transformed at one time point (after 7 days of *Bt*-treated food).

For experiments testing toxicity of combinations of *Bt* formulations, linear models were used to examine the effects of treatment [*Bt galleriae* formulation alone (B), *Bt israelensis* (V), *Bt tenebrionis* (N), combination of formulations BN, BV, VN, and BVN] on the response variable of interest. Response variables included total area debarked per treatment and mortality. For models examining effects on mortality, a binomial distribution was used. Models

were fitted using the *glmmTMB* package (version 1.0.1).[33] Since the experiment was replicated in time, round was included as a fixed effect in all models (round = 2; hence not as a random effect). No transformations of response variables were necessary. The effect of treatment on each variable was tested independently for each time period examined using analysis of deviance (*car::Anova* command). If main effects were found to be significant, comparisons among treatments were conducted with Tukey's multiple comparisons using the *emmeans* package.

For experiments testing avoidance of *Bt* formulations, linear mixed effects models (*lme4* package) were used to examine the effect of treatment [control and *Bt galleriae* treated food (BC, BH), control and *Bt israelensis* (VC, VH), control and *Bt tenebrionis* (NC, NH)] on total area debarked. Since each Petri dish contained both *Bt*-treated and non-*Bt* treated food, Petri dish number was included as a random factor in the models. The effect of treatment was tested independently for each time period examined using the *anova* command. Pairwise comparisons among treatments were conducted with Tukey's multiple comparisons using the *emmeans* package. No transformation of the response variable was necessary.

3 RESULTS

3.1 Toxicity of individual *Bt* strains
We found that all three *Bt* strains were toxic to adult pine weevils, but these strains differentially affected their weight, mortality and feeding. With regards to mean total area debarked, all strains (except for *Bt galleriae* low concentration, BL) showed that consumption was reduced during the 3- and 7-day consecutive period of exposure to *Bt*-treated food compared to the control group (Fig. S1a and Table S1). However, the average reduction in consumption (across both concentration levels) during these 7 days varied among treatments with a 30% decrease for the *Bt galleriae* formulation, 42% for *Bt tenebrionis* and 70% for *Bt israelensis*. This reduction in feeding remained even during the subsequent 7 days of feeding on non-*Bt* treated food (Fig. S1b and Table S1). Again, the highest decrease during this subsequent period was observed for *Bt israelensis* followed by *Bt tenebrionis* and *Bt galleriae* (82%, 38% and 11% reduction, respectively; averaged across concentrations for each strain, and compared to the control group). These differences were statistically significant only for *Bt israelensis* and *Bt tenebrionis* (Table S1).

Since weevils died during the course of the experiment, we also examined area debarked per weevil based on the average number of weevils alive during the first 3- and 7-day periods of exposure to *Bt*-treated food. We found that consumption per weevil followed a similar pattern to that of total area debarked (Fig. 1 vs Fig. S1). Consumption per individual was significantly lower for all *Bt* treatments compared to control (except for *Bt galleriae* low concentration, BL) during both time periods (Table 3), but especially for those exposed to the *Bt israelensis* formulation (Fig. 1(a),(b)). For those weevils surviving the initial experimental stage of 7 days and exposed to non-*Bt* treated food for an additional 7 days, the feeding eventually increased for some of the treatments (Fig. 1(c)). Weevils exposed to *Bt tenebrionis* and *Bt galleriae* fed to a similar extent to those exposed only to nontreated control food, while those exposed to *Bt israelensis* continued to show decreased feeding (Table 3 and Fig. 1(c)).

With regards to mortality, all treatments (except for *Bt galleriae* low concentration, BL) resulted in higher deaths compared to the control group over the entire experimental and observational
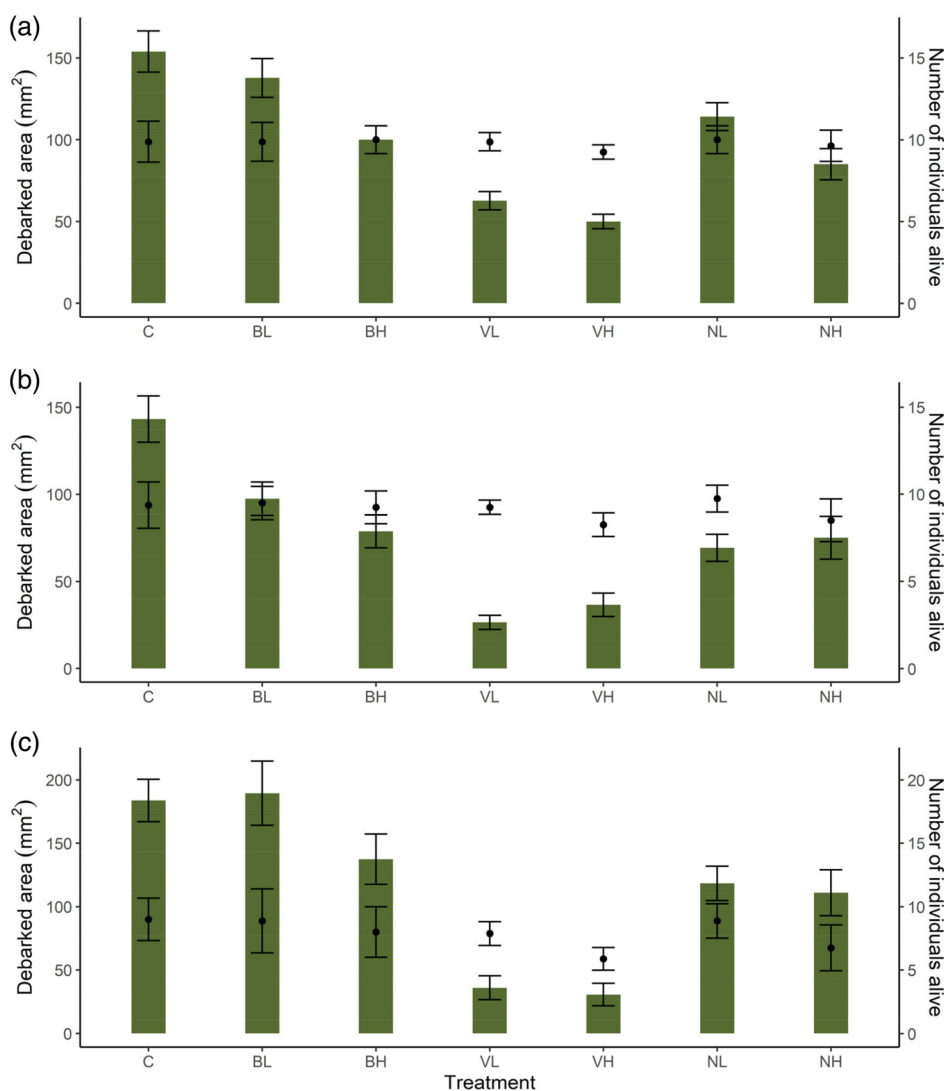
Figure 1. Mean area debarked (mm²) per surviving weevil ± standard error (green bars), per treatment when *Hylobius abietis* was exposed to (a) 3 days and (b) 7 days of *Bt*-treated food and (c) an additional 7 days of non-*Bt* treated food (stem pieces of *Picea abies*). Mean number of individuals (± standard error) that were alive at that time period for each treatment (averaged across 4 rounds, *n* per round = 10), is also shown (black dots). Total area debarked per treatment (Fig. S1) was divided by the mean number of individuals alive at each time point to obtain area debarked per weevil per treatment. Treatments are abbreviated as follows: control (C), *Bt galleriae* formulation (low and high concentration: BL, BH), *Bt israelensis* (VL, VH), *Bt tenebrionis* (NL, NH).

period (7 days of *Bt*-treated food + 7 subsequent days of non-*Bt* treated food + 7 subsequent days in boxes with non-*Bt* treated food; Fig. 2 and Table 4). The increase in mortality per treatment and concentration differed, with the highest increase observed for the *Bt israelensis* formulation (VH = 82%, VL = 65%), second least increase for *Bt tenebrionis* (NH = 42%, NL = 37%) and least increase for *Bt galleriae* (BH = 32%, BL = 15%) compared to the control group. Mortality increased with time across all *Bt* treatments when examining the experimental (7 + 7 days) and additional observation period in boxes (7 days, for a total of 3 weeks), and the increase was especially prominent for the *Bt israelensis* formulation (Fig. 2). Likewise, pine weevil weight decreased with experimental time (7 days of *Bt*-treated food + 7 subsequent days of non-*Bt* treated food) for all treatments, but this decrease was statistically significant only for the *Bt israelensis* formulation (Fig. 3 and Table 4). This is in line with the pattern observed for reduced feeding per weevil during this same period (Fig. 1(b),(c)).

3.2 Toxicity of combinations of *Bt* formulations

When mixing different *Bt* formulations, we found that *H. abietis* was not more susceptible to combinations relative to formulations alone. Treatments containing *Bt israelensis* were most toxic and lethal compared to the other two individual strains (Figs 4 and S2). When combinations of formulations contained *Bt israelensis*, the magnitude of effects observed was that of this formulation when examined alone. For example, the reduction in weevil feeding for *Bt galleriae* and *Bt israelensis* together, *Bt tenebrionis* and *Bt israelensis* together, and the combination of all three strains was comparable to that when *Bt israelensis* occurred alone (Fig. S2, compare V *vs* BV, V *vs* VN, V. *vs* BVN). Hence, these treatments (BV, VN and BVN) did not differ significantly in weevil consumption from the *Bt israelensis* formulation (V), but were significantly different from *Bt galleriae* (B) and *Bt tenebrionis* (N) treatments (Table 5). On the other hand, the mortality pattern was different than that of weevil consumption. Compared to the *Bt israelensis* formulation alone, a greater number of dead pine weevils occurred for the

Table 3. Analysis of variance (ANOVA) results (degrees of freedom, df; *F* value statistic, *F*; *P* value, *P*) from linear mixed models

	Consumption per weevil (mm²) during 3 days of *Bt*-treated food			Consumption per weevil (mm²) during 7 days of *Bt*-treated food			Consumption per weevil (mm²) during subsequent 7 days of non-*Bt* food		
	df	F	P	df	F	P	df	F	P
Treatment	6, 270	16.98	**<0.0001**	6, 270	20.46	**<0.0001**	6, 270	11.95	**<0.0001**
Contrasts	*df*	*t*	*P*	*df*	*t*	*P*	*df*	*t*	*P*
C *vs* BL	11.3	−1.20	0.66	6.73	−2.18	0.23	10.2	0.57	0.94
C *vs* BH	11.3	−4.31	**0.005**	6.73	−4.13	**0.02**	10.2	−1.18	0.68
C *vs* VL	11.3	−7.18	**0.0001**	6.73	−7.54	**0.0007**	10.2	−5.56	**0.001**
C *vs* VH	11.3	−7.88	**<0.0001**	6.73	−7.42	**0.0008**	10.2	−5.48	**0.001**
C *vs* NL	11.3	−3.21	**0.03**	6.73	−4.25	**0.01**	10.2	−2.38	0.15
C *vs* NH	11.3	−5.16	**0.001**	6.73	−5.57	**0.004**	10.2	−1.76	0.36

These models examined the effect of treatment [control (C); *Bt galleriae* formulation low and high concentration (BL, BH); *Bt israelensis* (VL, VH); *Bt tenebrionis* (NL, NH)] on individual *Hylobius abietis* consumption (mean stem area debarked per surviving weevil, mm²) during 3 and 7 cumulative days of *Bt*-treated food and an additional 7 days exposed to non-*Bt* treated food (stem pieces of *Picea abies*). Pairwise comparisons (using Dunnett adjustment; *t* ratio, *t*) examine differences between each treatment and the control group. Significant effects (*P* < 0.05) are shown in bold.
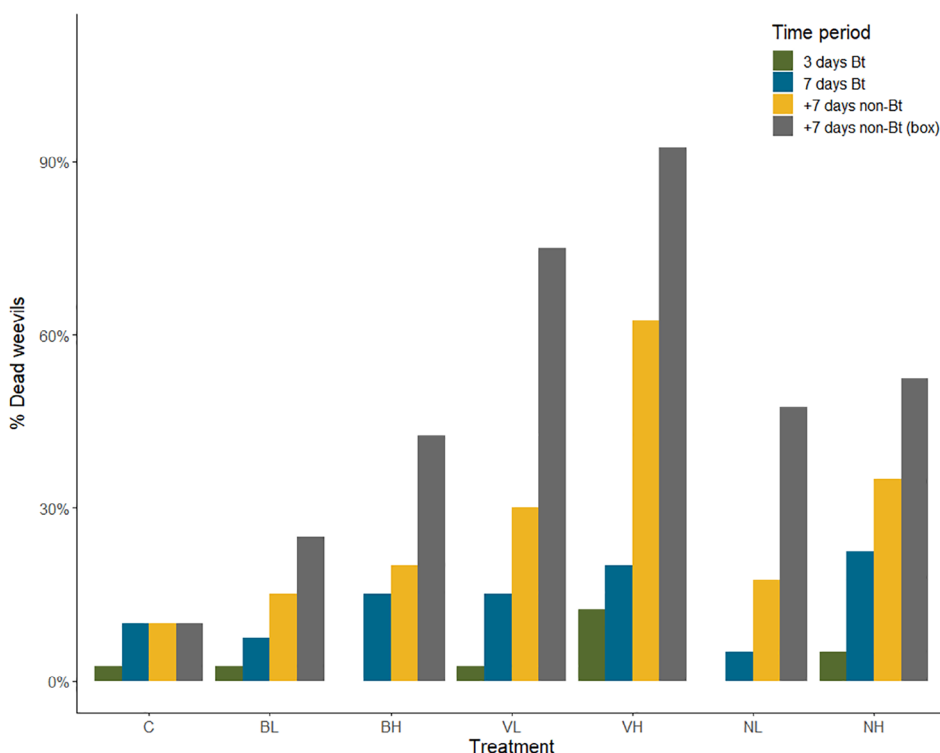

Figure 2. Cumulative pine weevil mortality (percentage of dead weevils: number of dead pine weevils out of total *n* = 40) per treatment after *Hylobius abietis* was exposed to 3 and 7 consecutive days of *Bt*-treated food (green and blue bars respectively), 7 subsequent days of non-*Bt* treated food (stem pieces of *Picea abies*) (yellow bars) and an additional 7 days of observation in boxes (i.e. after 3 weeks; grey bars). Treatments are abbreviated as follows: control (C), *Bt galleriae* formulation (low and high concentration: BL, BH), *Bt israelensis* (VL, VH), *Bt tenebrionis* (NL, NH).

Bt israelensis and *Bt galleriae* together, and least for the combination of all three strains (Fig. 4, compare V *vs* BV, V *vs* VN, V. *vs* BVN), but these differences were not statistically significant (Table 5). For the remaining combination, *Bt galleriae* and *Bt tenebrionis* together, the effect on weevil consumption was somewhere in between each of these strains alone (Fig. S2, compare N *vs* BN, B *vs* BN; Table 5), but mortality was comparable to that of *Bt galleriae* treatment (Fig. 4). Similar to the previous results, toxic effects could be observed after 3 days of feeding on *Bt*-treated food,

but mortality was more prominent in the period following *Bt* exposure.

3.3 Avoidance of *Bt* formulations
When providing pine weevils with a choice of food, we found that they did not distinguish between *Bt*-treated and non-*Bt* food overall, but there were some differences among products at certain time points. Twigs treated with the *Bt galleriae* formulation (B) were considerably less eaten compared to control twigs after

Table 4. Analysis of variance (ANOVA) (degrees of freedom, df; *F* value statistic, *F*; *P* value, *P*) and analysis of deviance (degrees of freedom, df; chi-square statistic, χ^2; *P* value, *P*) results from linear mixed models

	Mortality after 7 days of Bt-treated + 7 subsequent days of non-*Bt* food + 7 days box			Weight after 7 days of *Bt*-treated food			Weight after 7 subsequent days of non-*Bt* food		
	df	χ^2	P	df	F	P	df	F	P
Treatment	6	54.52	**<0.0001**	6, 269	3.18	**0.004**	6, 269	12.95	**<0.0001**
Contrasts	df	z	P	df	t	P	df	t	P
C *vs* BL	NA	1.75	0.31	10.2	0.36	0.98	8.19	0.51	0.96
C *vs* BH	NA	3.34	**0.004**	10.2	−0.20	0.99	8.19	−0.07	0.99
C *vs* VL	NA	7.82	**<0.0001**	10.2	−1.63	0.43	8.19	−3.47	**0.03**
C *vs* VH	NA	13.87	**<0.0001**	10.2	−2.66	0.09	8.19	−6.57	**0.0008**
C *vs* NL	NA	3.82	**0.0008**	10.2	0.83	0.85	8.19	−0.36	0.98
C *vs* NH	NA	4.35	**0.0001**	10.2	−1.55	0.47	8.19	−2.19	0.22

These models examined the effect of treatment [control (C); *Bt galleriae* formulation low and high concentration (BL, BH); *Bt israelensis* (VL, VH); *Bt tenebrionis* (NL, NH)] on total mortality (14 experimental days + 7 days exposed to non-*Bt* treated food in boxes) and pine weevil weight (grams) after *Hylobius abietis* was exposed to 7 days of *Bt*-treated food and 7 subsequent days to non-*Bt* treated food (stem pieces of *Picea abies*). Pairwise comparisons (using Dunnett adjustment; *t* ratio, *t* or *z* ratio, *z*) examine differences between each treatment and the control group. For contrasts examining differences in mortality, no degrees of freedom can be calculated (NA in table). Significant effects (*P* < 0.05) are shown in bold.
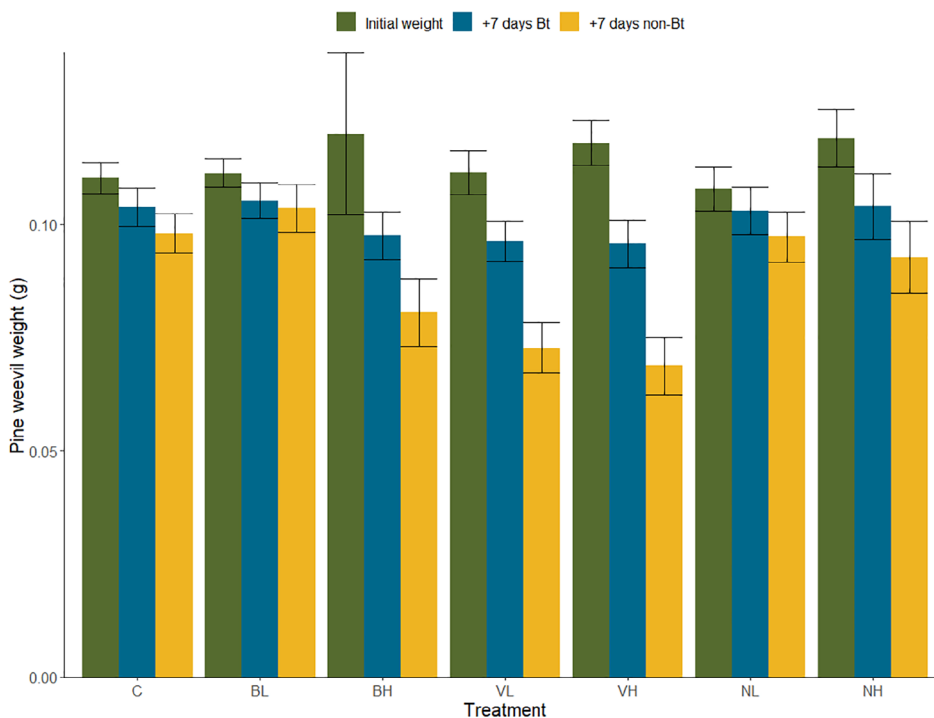

Figure 3. Mean pine weevil weight ± standard error per treatment at the start of the experiment (green bars) and after *Hylobius abietis* was exposed to 7 consecutive days of *Bt*-treated food (blue bars) and 7 subsequent days of non-*Bt* treated food (stem pieces of *Picea abies*) (yellow bars). Treatments are abbreviated as follows: control (C), *Bt galleriae* (low and high concentration: BL, BH), *Bt israelensis* (VL, VH), *Bt tenebrionis* (NL, NH).

3 days, but these difference became nonsignificant and less pronounced with time (Fig. 5 and Table 6). Twigs treated with *Bt israelensis* (V) and water-treated twigs were eaten to the same extent across all time points (Fig. 5(a) and Table 6). While the *Bt tenebrionis* formulation (N) and water-treated twigs were similarly eaten initially, with time more feeding was observed on the *Bt*-treated twigs (Fig. 5). Similar to previous experiments, the treatment including the *Bt israelensis* formulation resulted in the highest mortality compared to the others (data not shown), even if this was not the main purpose of this test.

4 DISCUSSION

We found that *Bt* has toxic and lethal effects on adults of the forest insect pest *Hylobius abietis*, but these effects varied with *Bt* strain. Across the three strains examined, *Bt israelensis* (Cry10Aa toxin) had the most negative effects on the weevil variables examined (weight, feeding and mortality). *Bt tenebrionis* (Cry3Aa toxin) and *Bt galleriae* (Cry8Da toxin) were less detrimental to weevils, and their effects were similar in extent. No combination of *Bt* formulations caused significantly higher toxicity or mortality compared to
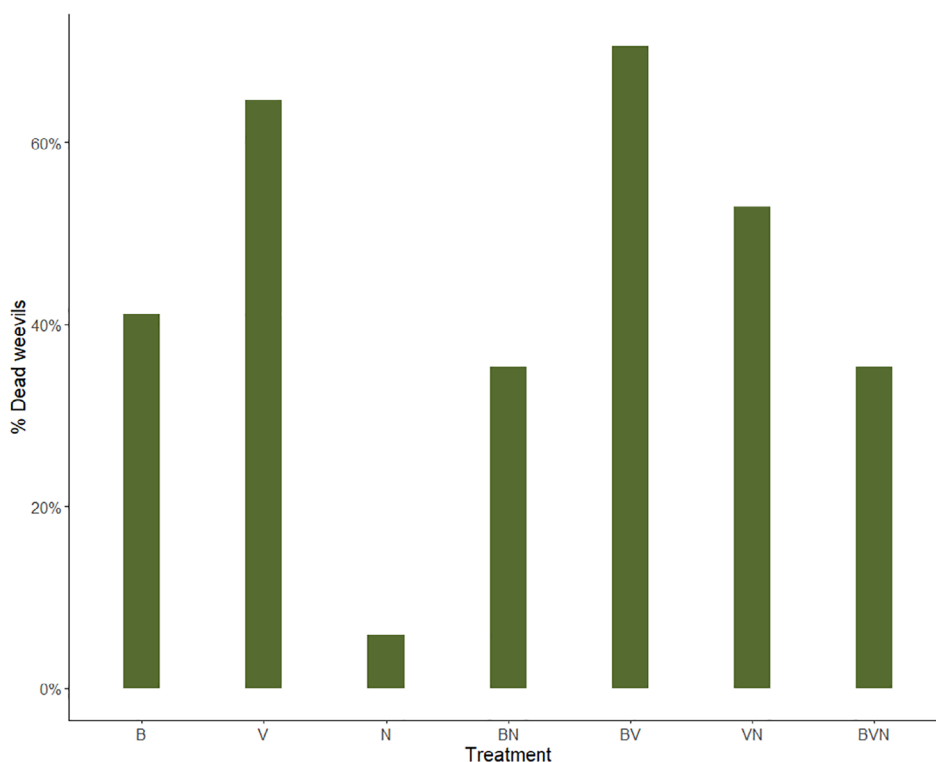
Figure 4. Total pine weevil mortality (percentage of dead weevils: number of dead pine weevils out of total $n = 17$) per treatment after *Hylobius abietis* was exposed to a total 7 days of *Bt*-treated food and an additional 7 days of non-*Bt* treated food (stem pieces of *Picea abies*) in an experiment comparing individual and combination of *Bt* formulations. Treatments are abbreviated as follows: *Bt galleriae* formulation (B); *Bt israelensis* (V); *Bt tenebrionis* (N); combination of formulations (BN, BV, VN and BVN).

Table 5. Analysis of deviance (degrees of freedom, df; chi-square statistic, χ^2; P value, P) results from linear models

	Area debarked (mm²) during 3 days of *Bt*-treated food			Area debarked (mm²) during 5 days of *Bt*-treated food			Area debarked (mm²) during 7 days of *Bt*-treated food			Mortality after 7 days of *Bt* food + 7 days non-*Bt* food in box		
	df	χ^2	P	df	χ^2	P	df	χ^2	P	df	χ^2	P
Treatment	6	55.59	**<0.0001**	6	61.93	**<0.0001**	6	75.36	**<0.0001**	6	15.4	**0.02**
Contrasts	df	t	P	df	t	P	df	t	P	df	z	P
B *vs* BN	104	−1.23	0.88	104	−1.04	0.94	104	−0.83	0.98	NA	0.37	0.99
B *vs* BV	104	5.51	**<0.0001**	104	4.30	**0.0007**	104	3.65	**0.007**	NA	−1.87	0.50
B *vs* BVN	104	4.02	**0.002**	104	4.37	**0.0006**	104	4.29	**0.0008**	NA	0.37	0.99
V *vs* BV	104	−0.17	1.00	104	0.65	0.99	104	0.47	0.99	NA	0.38	0.99
V *vs* VN	104	−0.17	1.00	104	−0.08	1.00	104	0.85	0.97	NA	0.73	0.99
V *vs* BVN	104	1.55	0.71	104	1.37	0.81	104	0.70	0.99	NA	−1.88	0.49
N *vs* BN	104	1.08	0.93	104	0.005	1.00	104	−0.93	0.97	NA	2.39	0.21
N *vs* VN	104	3.29	**0.02**	104	4.57	**0.0003**	104	5.78	**<0.0001**	NA	−3.87	**0.003**
N *vs* BVN	104	−2.60	0.14	104	−4.03	**0.002**	104	−4.93	**0.0001**	NA	2.39	0.21

These models examined the effect of treatment (*Bt galleriae* formulation (B); *Bt israelensis* (V); *Bt tenebrionis* (N); combination of formulations BN, BV, VN, and BVN) on total *Hylobius abietis* consumption (mean area debarked, mm²) during 3, 5 and 7 cumulative days of feeding on *Bt*-treated food (stem pieces of *Picea abies*) and total mortality (number of dead weevils) after these 7 days and an additional 7 days exposed to non-*Bt* treated food in boxes. Pairwise comparisons (Tukey's multiple comparisons) examine differences among combinations and individual *Bt* formulations. Significant effects (*P* < 0.05) are shown in bold.

Bt israelensis alone, indicating a possible lack of synergistic effects among strains. All strains affected consumption per individual in line with the known mechanisms of *Bt* toxicity after ingestion. Once ingested, toxins bind to epithelial cells in the insect gut, causing cell swelling and rupture, and eventually starvation.[9]

The first negative effects on consumption were observed after 3 days, but lethal effects became more evident under the period following initial *Bt* exposure (7–14 days after). Overall, pine weevils fed similarly on *Bt*-treated and non-*Bt* treated stem pieces when offered both simultaneously, indicating that *Bt*-treated
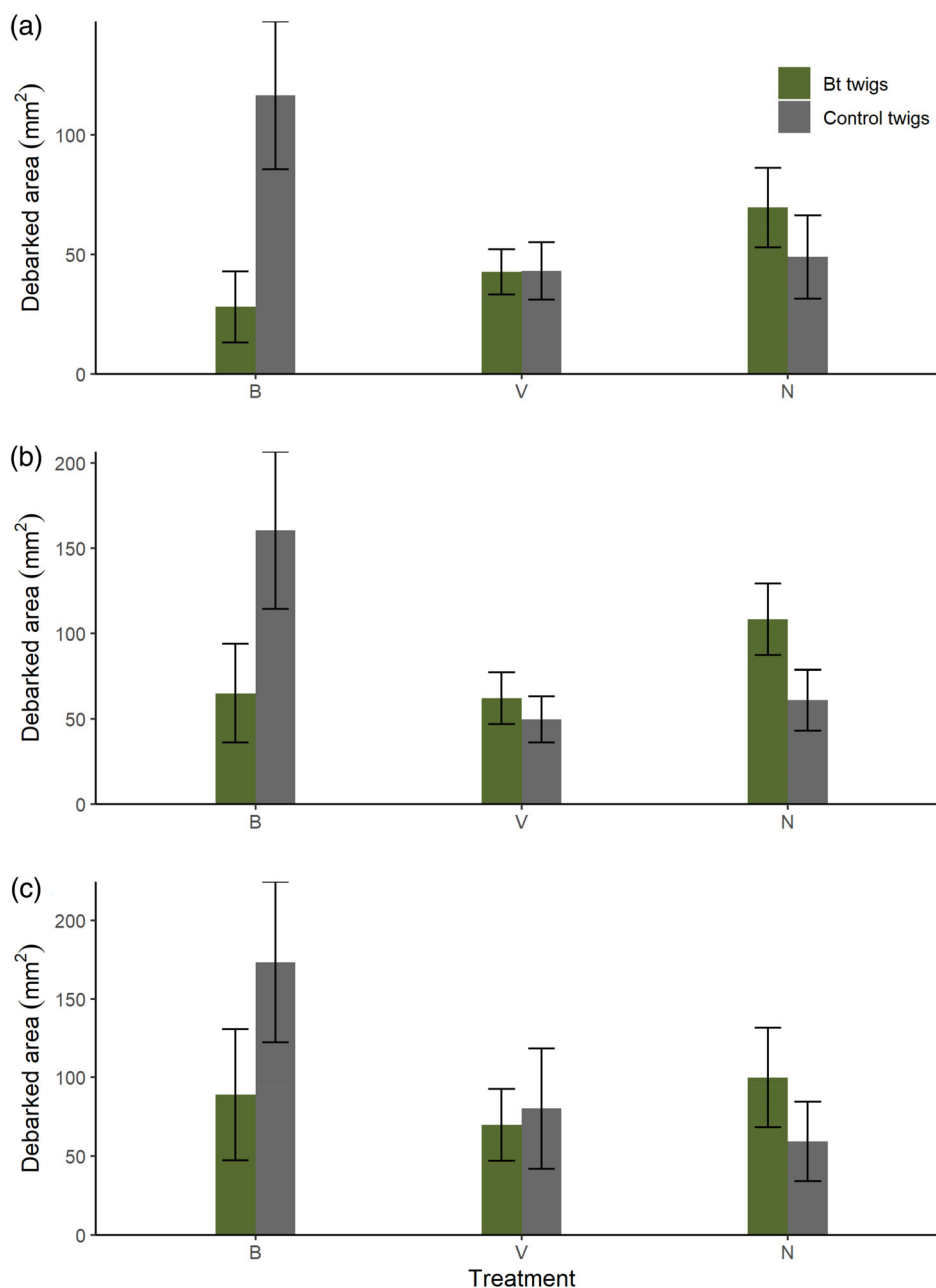
Figure 5. Mean area debarked (mm^2) ± standard error per treatment while *Hylobius abietis* was cumulatively exposed to (a) 3 days, (b) 5 days and (c) 7 days of *Bt*-treated (green bars) and control (water-treated; grey bars) food (stem pieces of *Picea abies*) in a choice experiment. Treatments are abbreviated as follows: control and *Bt galleriae* treated food (B); control and *Bt israelensis* treated food (V); control and *Bt tenebrionis* treated food (N).

twigs were not less palatable. We discuss our results in detail below.

4.1 Toxicity of individual *Bt* strains

The strain expected to be most effective against Diptera, *Bt israelensis* AM65-52, was most toxic to the pine weevil compared to the other two strains, *Bt tenebrionis* NB-176 and *Bt galleriae* SDS-502, which are expected to target Coleopteran insect pests. A few other studies have also reported toxicity of *Bt israelensis* against Coleopterans. For example, bioassays with spore suspensions from *Bt israelensis* in an artificial diet yielded up to 100% mortality of Cotton ball weevil third-instar larvae (*Anthonomus grandis*, Coleoptera: Curculionidae).[34] Moreover, similar bioassays have

resulted in 80–100% mortality of Coffee berry borer first- to fifth-instar larvae (*Hypothenemus hampei*, Coleoptera: Curculionidae).[17,35] In our study, *Bt israelensis* caused 65–82% greater mortality and a 70–82% feeding reduction relative to the control group, in adult weevils, thus suggesting that this strain has the potential to be used for management of the forest pest *H. abietis*. Also, ours and previous results suggest that Coleopteran larvae and adults may be more susceptible to *Bt israelensis* toxins than expected, and its Diptera-specificity may need reconsideration.

Bt strains are expected to have targeted insecticidal activity for the pest of interest, but cross-order activity is being increasingly reported.[36] For example, Redmond *et al.*[26] found negative effects

Table 6. Contrasts among types of food (degrees of freedom, df; *t* statistic, *t*; *P* value, *P*) to examine the effects of *Bt* on the choice of food by *Hylobius abietis*

Contrasts	Area debarked (mm²) during 3 days of feeding			Area debarked (mm²) during 5 days of feeding			Area debarked (mm²) during 7 days of feeding		
	df	t	P	df	t	P	df	t	P
BC *vs* BH	54	3.43	**0.01**	54	2.57	0.12	54	1.61	0.59
VC *vs* VH	54	0.01	1.00	54	−0.33	0.99	54	0.17	1.00
NC *vs* NH	54	−0.80	0.96	54	−1.27	0.79	54	−1.33	0.76

Total consumption (mean area debarked, mm²) during 3, 5 and 7 cumulative days of feeding were compared among control (water-treated) and *Bt*-treated *Picea abies* stem pieces (high concentration of the individual formulation). Treatments are abbreviated as follows: control and *Bt galleriae* treated food (BC, BH); control and *Bt israelensis* (VC, VH); control and *Bt tenebrionis* (NC, NH). Significant effects (*P* < 0.05) are shown in bold.

of the Coleopteran-targeted formulation of *Bt galleriae* SDS-502 on Lepidopteran species present in their field trials, such as the monarch butterfly (*Danaus plexippus*) and fall armyworm (*Spodoptera frugiperda*). Likewise, Lepidopteran-targeted strains have been found in some cases to be more toxic to Coleoptera species than Coleopteran-targeted ones. For instance, the Lepidopteran-specific strain *Bt kurstaki* (Raven® formulation; *Cry1Ac* toxin) has been shown to yield higher mortality of a leaf beetle than the Coleopteran-specific *Bt tenebrionis* (*Cry3Aa* toxin).[22] However, these same *Cry1* and *Cry3* toxins have little toxic effects on the Coffee berry borer.[35] Hence, results from our study are a valuable addition to our knowledge on the nonspecificity of *Bt* strains, which certainly deserves further consideration.

We also found that the strain *Bt galleriae* SDS-502, thought to be effective against Coleoptera and specifically Curculionidae pests, was least detrimental to pine weevils. This strain caused a feeding reduction of 11–30% and increased mortality between 15–32%, which was not statistically different from the control group for most time points examined (Tables 3 and S1). These results are in contrast to those found for the toxicity of the same *Bt galleriae* formulation on the Rice water weevil (*Lissorhoptrus oryzophilus*, Coleoptera: Curculionidae) and the Alfalfa weevil (*Hypera postica*, Coleoptera: Curculionidae). Larval mortality ranged between 50–80% and 27–60%, respectively, for *L. oryzophilus* and *H. postica*.[20,25] For adults, a 50–90% reduction in defoliation and 50–80% mortality were recently reported for the Japanese beetle (*Popillia japonica*, Coleoptera: Scarabaeidae)[26] and 100% mortality for the bean weevil *Acanthoscelides obtectus* (Coleoptera: Chrysomelidae).[37] In contrast, adult mortality of the Emerald ash borer (*Agrilus planipennis*, Coleoptera: Buprestidae) was reported to be somewhat lower and varied between 20% and 50%, with 4 days until first death after exposure to *Bt galleriae* SDS-502.[23] Thus, it appears that *Bt galleriae* can have variable effects on Coleopteran pests and, in our case, it does not provide adequate protection against the pine weevil.

Similar to *Bt galleriae*, many Coleopteran species have shown a high degree of susceptibility to *Bt tenebrionis* NB-176.[15,16] This strain was of intermediate toxicity to *H. abietis*, causing a 37–42% increase in weevil mortality and an average 38–42% feeding reduction after exposure compared to the control group. Mortality levels caused by *Bt tenebrionis* have been reported to be both lower and higher than those found in our study. For example, mortality for larvae of the Cigarette Beetle (*Lasioderma serricorne*, Coleoptera: Anobiidae) varied between 60–75%,[38] while adult mortality of the Cottonwood leaf beetle (*Chrysomela scripta*, Coleoptera: Chrysomelidae) using the same *Bt tenebrionis*

formulation as in our study ranged between 10% and 50% but with a much greater feeding reduction (40–80%).[22] For Curculionidae, Weathersbee *et al.*[39] tested the susceptibility of the root weevil *Diaprepes abbreviatus* to the same formulation and found strong reductions in weight and survival of neonate larvae, but only very delayed mortality for older larvae (<100 days until death). Hence, similar to *Bt galleriae*, the effects of *Bt tenebrionis* appear to be variable, and this strain did not result in sufficient lethal effects to provide effective control of the pine weevil.

For all the *Bt* strains examined, the toxic effects on *H. abietis* were evident on consumption per weevil after 3 days of exposure and became even stronger after 7 days of feeding on *Bt*-treated food (Fig. 1(a),(b)). However, this reduction in feeding remained only for *Bt israelensis* during the subsequent period of non-*Bt* treated food. Weevil feeding increased for those exposed to *Bt galleriae* and *Bt tenebrionis*, and did not differ from the control group (Fig. 1(c) and Table 4). These results suggest that the toxic effects of *Bt galleriae* and *Bt tenebrionis* subsided with time, and some of the weevils were able to recover. This is also reflected in the lower reduction in weevil weight across time for these two strains (Fig. 3). Recovery following *Bt* ingestion can occur and depends on factors such exposure time and subsequent access to non-*Bt* treated food.[40]

Mortality, on the other hand, was low in the initial exposure period and reached its peak 2 weeks after consumption of *Bt*-treated food (Fig. 2). This pattern was consistent among strains, but was more pronounced for *Bt israelensis*, especially for the high concentration formulation which yielded the highest total mortality (Fig. 2). The observed delay in lethal effects are in line with previous studies, and the *Bt* action mechanism of slow gut degradation led to a lag period between starvation and death.[23,37,41,42] In our case, it is important to take this delay into consideration if *Bt* is to be used for control of *H. abietis*. One of the main causes of seedling mortality due to pine weevil damage is the removal of an entire ring of bark around the stem (i.e. the seedling is girdled). If *Bt* ingestion does not reduce weevil consumption fast enough, the seedlings could become girdled and effective protection would not be achieved.

4.2 Toxicity of combinations of *Bt* formulations
We also explored the possibility of combining *Bt* strains to examine if stronger pest control could be achieved. However, we found no synergistic effects and in the different combinations examined the toxicity and mortality of one strain tended to dominate the others. In particular, treatments that included *Bt israelensis* reduced weevil consumption the most, in line with our first

findings that this strain is most detrimental to *H. abietis*. However, these combinations did not provide greater protection from pine weevil damage than the strain alone. These reductions in consumption also resulted in mortality levels that were similar and nonsignificantly different from *Bt israelensis* alone (Table 5). It is worth noting that the combination of *Bt israelensis* and *Bt tenebrionis* (VN) and all three strains (BVN) together yielded somewhat lower mortality than *Bt israelensis* and *Bt galleriae* (BV) together and *Bt israelensis* (V) alone (Fig. 4). Thus, it would be of interest to examine if certain strain combinations can exhibit antagonistic interactions and potentially hamper the pest control effectiveness of *Bt israelensis*. The two other strains, *Bt galleriae* and *Bt tenebrionis*, resulted in low toxicity and mortality of *H. abietis* relative to *Bt israelensis*, also in line with our first findings. Opposite to our first findings, *Bt galleriae* resulted in greater mortality than *Bt tenebrionis* (Fig. 2 *vs* Fig. 4), indicating that their effects on pine weevils can be variable. Hence, these two strains do not appear to provide reliable control of *H. abietis*.

Other studies examining strain or protein combinations have found both a lack of or evidence for synergism in toxicity.[43–46] For example, a combination of *Cry* and *Cyt* toxins had synergistic effects on the European crane fly *Tipula paludosa* (Diptera: Tipulidae) compared to when a single toxin from *Bt israelensis* was used.[47] Likewise, a cocktail of *Cry1A* and *Cry1C* proteins reduced the adult lifespan of the tobacco budworm *Heliothis virescens* (Lepidoptera: Noctuidae) to a greater extent compared to each protein alone.[48] On the other hand, antagonistic effects of *Cry1Ab* and *Cry2A* have been observed on larvae of *Ephestia kuehniella* and *Plodia interpunctella* (Lepidoptera: Pyralidae).[49] Most studies, however, have focused on combinations of Diptera- and Lepidoptera-targeted *Bt* toxins,[46] and little is known about interactions among Coleopteran proteins or those targeted for different insect orders. Our study presents a preliminary indication that combinations of *Bt israelensis*, *Bt galleriae* and *Bt tenebrionis* do not enhance control of *H. abietis*. Nevertheless, we used commercial *Bt* formulations and a test including combinations of the pure *Bt* toxins/proteins is required to confirm these results.

4.3 Avoidance of *Bt* formulations

In addition to determining the toxicity of *Bt* strains for effective pest control, it is important to establish if insects can distinguish between *Bt*-treated and non-*Bt* treated food. Previous studies with other pests have found evidence both for and against distinction between *Bt*-treated and non-*Bt* treated food, as well as changes in insect behavior due to *Bt*.[50–54] For *H. abietis*, our results suggest that weevils mostly do not differentiate between both types of food. In choice assays, pine weevils fed initially more on control than *Bt galleriae*-treated twigs, but this difference disappeared with time. By the end of the experiment, weevils fed to a similar extent on control and *Bt*-treated twigs for all strains. Hence, suggesting that the properties of the formulations themselves do not influence the amount of feeding. Treatment of seedlings with *Bt* formulations should thus not deter *H. abietis* from feeding, but field choice assays should also be conducted.

For all our three experiments it is important to keep in mind that *Bt* formulations were used to examine the susceptibility of *H. abietis* to *Bt*. Even though these formulations contain specific *Bt* strains, shown to be effective against the intended target pests, evaluation of pine weevil toxicity to the pure *Bt* toxins/proteins should follow. Quantification of the medial lethal concentration (LC50) for each *Bt* toxin should also be conducted. Moreover, examination of pine weevil susceptibility to other crystal toxins

or exotoxins produced by *Bt israelensis*, *Bt galleriae* and *Bt tenebrionis* also warrants further investigation.

5 CONCLUSION

Our study showed that *Bt* has toxic and lethal effects on a major forest pest, the pine weevil *H. abietis*. However, the susceptibility of this pest to *Bt* was greatest for a Diptera-targeted strain (*Bt israelensis* AM65-52, *Cry10Aa* toxin), while it was least susceptible to those especially targeted for Coleopteran insects (*Bt tenebrionis* NB-176, *Cry3Aa* toxin, and *Bt galleriae* SDS-502, *Cry8Da* toxin). Individually, only *Bt israelensis* shows potential to provide forest seedling protection through its toxic effect on *H. abietis*, while strain combinations did not seem to provide any greater effect. Moreover, pine weevils do not appear to be deterred from feeding on *Bt*-treated food. Altogether, we conclude that *Bt* could be prospectively developed as a strategy of management for this forest regeneration pest, and given the observed nonspecificity of *Bt* strains, evaluation of other strains/toxins against *H. abietis* and other Coleoptera would be of interest.

CONFLICT OF INTEREST DECLARATION

The authors disclose no conflicts of interest.

AUTHORS' CONTRIBUTIONS

AP, GN and AK conceived and designed experiments. AT conducted laboratory experiments and data analyses. AP lead the writing of the manuscript with input from AT and GN. All authors read and approved the manuscript.

SUPPORTING INFORMATION

Supporting information may be found in the online version of this article.

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