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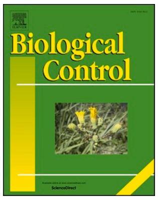

Entomopathogenic fungi and chemical insecticides have synergistic action against adult large pine weevil, *Hylobius abietis*

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HIGHLIGHTS

- The large pine weevil (*Hylobius abietis*) is the most serious insect pest of forestry in Europe.
- We show synergy among a range of entomopathogenic fungi and pesticides.
- Field trials support the conclusions of laboratory assays.
- This provides an effective future strategy to manage this important pest.

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ABSTRACT

The large pine weevil, *Hylobius abietis*, is a serious pest of reforestation across much of northern Europe. Larvae develop in the stumps and roots of recently felled conifers, and adults feed on the bark of young trees used to replant the site. Entomopathogenic fungi and chemical insecticides, alone and in combination, were tested against adult pine weevil in laboratory and field experiments. In a broad screening experiment, weevils were dipped in conidial suspension of *Beauveria bassiana* (Bb Naturalis and Bb SC4), *Beauveria caledonica* (Bc SG61) and *Metarhizium anisopliae* (Ma F52 and Ma SG6) and/or provided with twigs treated with chemical insecticides (cypermethrin, acetamiprid and thiacloprid). When assessed after 14 or 17 days, there was higher weevil mortality in combined treatments than expected based on individual treatments, with synergistic effects demonstrated in most cases. In a second laboratory experiment, Bb Naturalis, Bc SG61 and Ma F52 applied in each of two ways, either by dipping weevils or contaminating the substrate, gave synergistic effects in combination with cypermethrin, and mainly additive effects with acetamiprid. Weevil cadavers in all fungus-insecticide treatments tested developed mycosis, which would facilitate recycling of fungi in the field. In a field trial, a commercial formulation of Ma F52 (Met52) and cypermethrin were tested alone and in combination for protection of young Sitka spruce trees. Weevil feeding damage on trees treated with Met52 and half the recommended rate of cypermethrin did not differ significantly from that of trees that received full rate cypermethrin, but damage to trees that received half rate cypermethrin was higher. This study demonstrates the potential of entomopathogenic fungi to reduce the amount of chemical insecticide used to protect trees against pine weevil damage as part of an integrated pest management approach to pine weevil.

1. Introduction

The large pine weevil *Hylobius abietis* L. is the most important pest of reforestation across northern Europe (Lalik et al., 2021a; Längström & Day, 2004; Leather et al., 1999). Adult pine weevils are attracted onto clear-felled coniferous forest sites by volatile organic chemicals released by freshly cut timber. The roots and stumps of these newly felled trees provide ideal breeding material for the weevils. Adult weevils feed on

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the bark of newly planted trees, resulting in serious injury and death and providing a significant obstacle to restocking. The standard means of protection is by dipping, spraying or electrodyne treatment of seedlings or young trees with chemical insecticides. Both cypermethrin and acetamiprid are highly effective in protecting against pine weevil (Hardy et al., 2020; Moore et al., 2021; Nordlander et al., 2011; Willoughby et al., 2020), and thiacloprid has also proved promising in field trials (Olenici et al., 2018; Willoughby et al., 2020). Non-chemical alternatives include physical barriers or coatings applied to the stems of young trees to prevent weevils accessing them and these, together with silvicultural practices, have greatly reduced reliance on chemicals in Sweden (Nordlander et al., 2009). Increasing pressure to reduce the use of chemical pesticides including EU legislation (Sustainable Use of Pesticides Directive) and requirements of Sustainable Forest Management certification bodies has led to an increasing body of research on alternative synthetic and non-synthetic insecticides and physical methods of plant protection (Galko et al., 2022; Hardy et al., 2020; Moore et al., 2021; Willoughby et al., 2020).

Biological control agents with potential against pine weevil include entomopathogenic nematodes and entomopathogenic fungi. Entomopathogenic nematodes (*Heterorhabditis* and *Steinernema*) applied to tree stumps efficiently find and infect the developing weevils and can lead to population reduction (Dillon et al., 2006; Kapranas et al., 2017). Hypocrealan fungi, particularly of the genera *Beauveria* and *Metarhizium*, are widely used for control of several insect pests (Khun et al., 2020; Vega et al., 2012), but have received less attention than nematodes for use against pine weevil. In laboratory assays, all stages of *H. abietis* were susceptible, with variation between species and strains of fungus (Ansari & Butt, 2012; Barta et al., 2019; Wegensteiner & Führer, 1988). In field trials, *M. anisopliae* and *Beauveria* spp. applied to soil around stumps infected weevil larvae at depths of up to 23 cm in soil, and when applied together with entomopathogenic nematodes there was a small additive effect in suppressing the emergence of adult weevils (Mc Namara et al., 2018; Williams et al., 2013). In a recent study, Quinzo-Ortega et al. (2024) also showed additive interactions between wild entomopathogenic fungi and commercial entomopathogenic nematodes and in one of their field trials, demonstrated a significant reduction in adult pine weevil emergence relative to control. Although pine weevil adults are less susceptible than larvae and pupae (Ansari & Butt, 2012), their greater accessibility at and above the soil surface make them a more promising target for entomopathogenic fungi than the cryptic larval and pupal stages which occur within tree roots at depths of up to 50 cm below soil surface. In field trials, *M. anisopliae* applied to restocked plants across six forest sites in Britain gave generally poor levels of protection against pine weevil damage (Willoughby et al., 2020). However, a strain of *Beauveria bassiana* isolated from pine weevils in Slovakia showed promise both when applied to saplings as conidial suspension (Barta et al., 2019) and when deployed in forest plots on carriers with attractants (Lalik et al., 2021b), showing potential for entomopathogenic fungi to reduce numbers of adult weevils in a lure-and-kill strategy.

Beauveria and *Metarhizium* infect insects through the cuticle, following adhesion of spores (conidia). When the insect has been consumed, hyphae exit the cadaver through the cuticle and sporulate outside (Shah & Pell, 2003). These fungi are easily mass produced, and numerous species and strains have been developed for commercial use against a variety of pests, including many weevil species (Khun et al., 2020). However, entomopathogenic fungi have several drawbacks that can limit efficacy, including susceptibility to environmental factors and slow rate of kill (Lacey et al., 2015). There is considerable evidence that certain combinations of entomopathogenic fungi and chemical insecticides can have synergistic effects, resulting in increased or accelerated mortality of the target insect over that expected from each agent on its own (Nawaz et al., 2022). Evidence is particularly strong for combinations involving imidacloprid, a neonicotinoid (e.g. Farooq et al., 2018; Fisher et al., 2017; Kamiya et al., 2025; Halder & Soni, 2025), but there are also reports of synergy with the synthetic pyrethroids cypermethrin or alpha-cypermethrin (Chen et al., 2021; Meyling et al., 2018), insecticides used against pine weevil.

The objective of our study was to test the potential for synergy against adult pine weevil of entomopathogenic fungi combined with chemical insecticides. This objective was addressed in two laboratory experiments; the first including a broad range of fungus/insecticide treatments while the second, with a reduced range of agents, included a comparison between two methods of delivery of the fungus, by dipping and by the more realistic method of contaminating the substrate. One fungus-insecticide combination (*M. anisopliae* and cypermethrin) was also tested in the field under commercial forestry conditions.

2. Material and methods

2.1. Fungi, insecticides and insects

Five strains of entomopathogenic fungus were used in experiments, two commercial strains (*Beauveria bassiana* ATCC 74040, the basis of the Naturalis® product and *Metarhizium anisopliae (= M. brunneum* or *M. robertsii)* F52, the basis of the Met52® product) and three strains from clear-fell forest sites in Ireland: *B. bassiana* SC4, *B. caledonica* SG61 and *M. anisopliae* SA6, isolated from bark of a pine stump, forest soil and a *H. abietis* larva, respectively. For laboratory experiments, all strains were cultured on potato dextrose agar (PDA). Conidia were harvested by flooding 15-day old cultures grown at 20 °C on PDA with 20 mL of PBS and 0.03 % Tween80 and gently scraping the agar surface with a spatula. The suspensions were filtered through 250 µm mesh and the filter was then rinsed with 10 mL of PBS/0.03 % Tween80. Spore suspensions were prepared and adjusted to 10^8 spores/mL using 0.03 % Tween80. Germination assays were carried out by spreading 100 µL of the suspension on PDA medium and checking the proportion of spores (N = approx. 100) germinated after incubation in the dark at 20 °C. The germination assay was conducted twice. Germination rates were always > 89 % (89 % to 100 %).

The chemical insecticides used were formulations of cypermethrin (Forester™, Agriphar S.A.), acetamiprid (Gazelle®, Nisso Chemical Europe GmbH) and thiacloprid (Calypso®, Bayer Cropscience AG). For each experiment, the day 15 LC_{50} was calculated using a subpopulation of the insects that were to be used in the experiment and insecticides were applied at that concentration.

Adult pine weevils for use in experiments were collected from emergence traps erected over pine stumps on clear-felled forest sites. Weevils were maintained in 4.5 L plastic boxes with ventilation holes and lined with moistened tissue paper and were provided with fresh pine twigs as food.

2.2. Laboratory experiment 1

Fungus was applied by immersing each insect individually in spore suspension (5 mL, 10^8 spores/mL in 0.03 % Tween80) for 5 s. Control insects were dipped in Tween. Fresh cut pine twigs (8 cm long, diam. 4–12 mm) were provided as food. Both ends (0.5 cm) of the twig were dipped in melted paraffin wax (Sigma) to keep them fresh. Insecticide was applied by dipping the twigs for 10 s in a solution of cypermethrin (0.035 % a.i), acetamiprid (0.003 % a.i.), thiacloprid (0.006 % a.i.), or water for controls. Twigs were let air dry in a fume cabinet. Assays were carried out in 300 mL clear plastic pots (diam. 6 cm) with a 1.5 cm layer of saturated vermiculite in the bottom. The medium was kept moist during the experiments by regular spraying with tap water. The top of the pot was covered with 250 µm mesh. Twigs were positioned vertically, two per pot, with combined diameter of 16 mm and a total surface area of bark of about 40 cm^2 before the insects (5 pine weevil adults per tub) were added. Pots were placed in clear plastic boxes with ventilated lids and arranged in randomized block design with one pot of each treatment in a block. Boxes were placed in a growth room with 16:8 L:D at 20 °C.

This experiment included all three insecticides and all five fungi, both alone and in each possible fungi-insecticide combination, giving a total of 8 single-agent treatments and 15 combinations. There were 4 replicate pots (total 20 insects) per treatment. The state of the insects (dead, knockdown) was assessed on days 3, 6, 11, 14, 17, 20 and 27. Knockdown was assessed by the response of the insect to sustained and varied mechanical stimulation. A healthy insect reacted immediately while a knockdown insect reacted after some handling but at a very slow pace and with very small amplitude of movement. Knockdown insects were left in the pot as they could recover from the intoxication. Dead insects were removed, rinsed with tap water, and incubated individually with moistened paper at 20 °C in the dark for up to 14 days to record mycosis (Expt. 1 only).

2.3. Laboratory experiment 2

Fungi were applied in each of two ways: either by dipping the insects (as above) or by spraying the surface of the vermiculite with 1 mL of spore suspension (10^8 spores/mL in 0.03 % Tween80; or Tween only, for the controls). In this experiment there were two insecticides (0.12 % a.i cypermethrin and 0.002 % acetamiprid), three fungi (*B. bassiana* Naturalis, *B. caledonica* SG61 and *M. anisopliae* F52), and all six fungus-insecticide combinations. The experiment was conducted as described above, with five replicate pots (total 25 insects) per treatment. Mortality was assessed every 2–3 days for 26 days.

2.4. Field trial

A field trial was set up in a commercial forest plantation, using cypermethrin and Ma F52, alone and in combination. These agents were chosen as a synergistic effect was recorded in both lab experiments, and both agents were registered for field use in Ireland. The trial was set up in the spring of 2016 in a clearfell forest site near Loughrea, Co. Galway, Ireland (53.0926382,-8.6957023). The site was previously planted with Sitka spruce (*Picea sitchensis* Bong.) and was felled in 2014. The soil was mineral (gley) overlain by a very thin layer of organic matter (mountain blanket peat Teagasc soil map – https://teagasc.ie/crops/soil-soil-fertility/county-soil-maps/). Size 1 (40–70 cm) Sitka spruce were planted mid-March with 2 m between rows.

Chemical treatment consisted of spraying the entire plant following standard protocol of the cypermethrin-based insecticide Forester™ at a concentration of 0.2 % and 0.1 % a.i., the former being the standard application rate. *Metarhizium anisopliae* was applied at 55 x 10^8 spores per tree (5.8 g granular formulation of Met52 (Fargro Ltd, UK) mixed in 200 mL moss peat, spread around the base of the tree in a ring 10–12 cm wide. A liquid top-up application was carried out a month later. The same quantity of spores (55 x 10^8) suspended in 40 mL 0.03 % Tween 80 was applied around the base of each tree. This split method of application of EPF in dry spores around the base of the tree, followed by top-up to the same site, is an innovative experimental approach developed with industry input.

There were five treatments: (1) Reduced (half) rate (0.1 %) cypermethrin; (2) Reduced (half) rate (0.1 %) cypermethrin with Met52; (3) Met52; (4) Full rate (0.2 %) cypermethrin (5) no protection (control). The experiment was set out in randomized block design with one plot of each treatment per block and ten blocks. Each block was contained within a bay separated from neighbouring bays by windrows – branches and other debris from the felling operation piled in rows. One block was contained in two short bays. Within a block, plots were arranged linearly, and were separated from each other by a buffer zone of untreated plants which ran the width of the bay and was 2–3 plants wide. Plots contained 36 plants (6 x 6) except for one which contained 24 plants (4 x 6) per plot, giving a total of 348 plants per treatment.

Cypermethrin was applied on 26th April 2016. Met52 was applied two days later (28th April) and the liquid top-up application on 31st May. Damage on every tree in each plot was assessed twice, one and five months after treatment (31st May and 7th September), using the scale shown below. Trees that were dead due to causes other than weevils (category 5) accounted for < 1 % of trees at each assessment and were not included in analysis.. The temperature and precipitation during the trial ranged from 7 °C (April) to 15 °C (August) and 50.6 mm rain (April) to 121.01 mm rain (August). These are typical values for the region, though it was a little cooler and wetter than normal. The 30-year mean annual temperature (1990–2020) for April – September for the area was 10 °C (April) to 16 °C (July and August) and mean annual precipitation ranged from 40 mm (June) to 100 mm (August and September) (Coonan et al., 2024).

Score and description of recorded damage on plants after treatments:

Score	Designation	Description
0	None	No weevil damage
1	Light	A few small feeding punctures expected to occlude rapidly
2	Medium	One or a few larger feeding areas up to approx. 20 mm in length which are so distributed as not to seriously threaten the vigour and future growth of the plant
3	Heavy	One or a few larger feeding areas up to approx. 20 mm in length which are so distributed as to seriously threaten the vigour and future growth of the plant, though plant not yet dead
4	Death	Category 3, with death
5	Death (non-weevil)	Death without weevil damage or with damage level not exceeding category 2

2.5. Statistical analysis

Survival curves for each treatment were produced using Oasis survival analysis (Han et al., 2016) and significant differences were ascertained by a log-rank test with a Bonferroni correction for multiple comparisons.

Mortality in laboratory experiments was corrected for natural mortality in the controls (no insecticide and no fungus) using Abbott's formula. Synergistic, additive or antagonist interactions between agents in the combination treatments were determined using a chi-square test (Morales-Rodriguez & Peck, 2009). The expected interaction mortality value M_E for combined agents was calculated using the formula $M_E = M_I + M_F(1 - M_I/100)$ where M_I and M_F are the observed percentage mortality caused by the insecticide and fungus treatment, respectively. Results from the chi-square tests were compared to the X^2 table value for 1 df, using the formula $X^2 = (M_{IF} - M_E)^2/M_E$ where M_{IF} = observed mortality in the combined treatment. Where the expected value for M_E was 0, we used $M_{(E+1)}$.

Kruskal Wallis tests were used to explore whether fungal treatment influenced the amount of feeding damage, with a separate test for each insecticide treatment. To ascertain whether insecticides differentially impacted mycosis, the proportion of dead insects developing mycosis in each of the three insecticides was compared using chi-square. The test was performed for each fungus separately.

In the field trial, the mean damage rating for each plot was subjected to ANOVA ($P < 0.05$) and Tukey's test was used for separation of means.

3. Results

3.1. Laboratory experiment 1

Survival curves for entomopathogenic fungi alone versus control and for each of the three insecticides alone and in combination with various entomopathogenic fungi are shown in Fig. 1. The associated summary statistics are given in Table 1. Amongst the fungi, the two *B. bassiana* strains gave highest mortality, reaching ~ 75 % and ~ 40 % on day 27 for Bb Naturalis and Bb SC4 respectively (Fig. 1A), though only Bb Naturalis differed significantly to the control (Table 1). Mortality due to

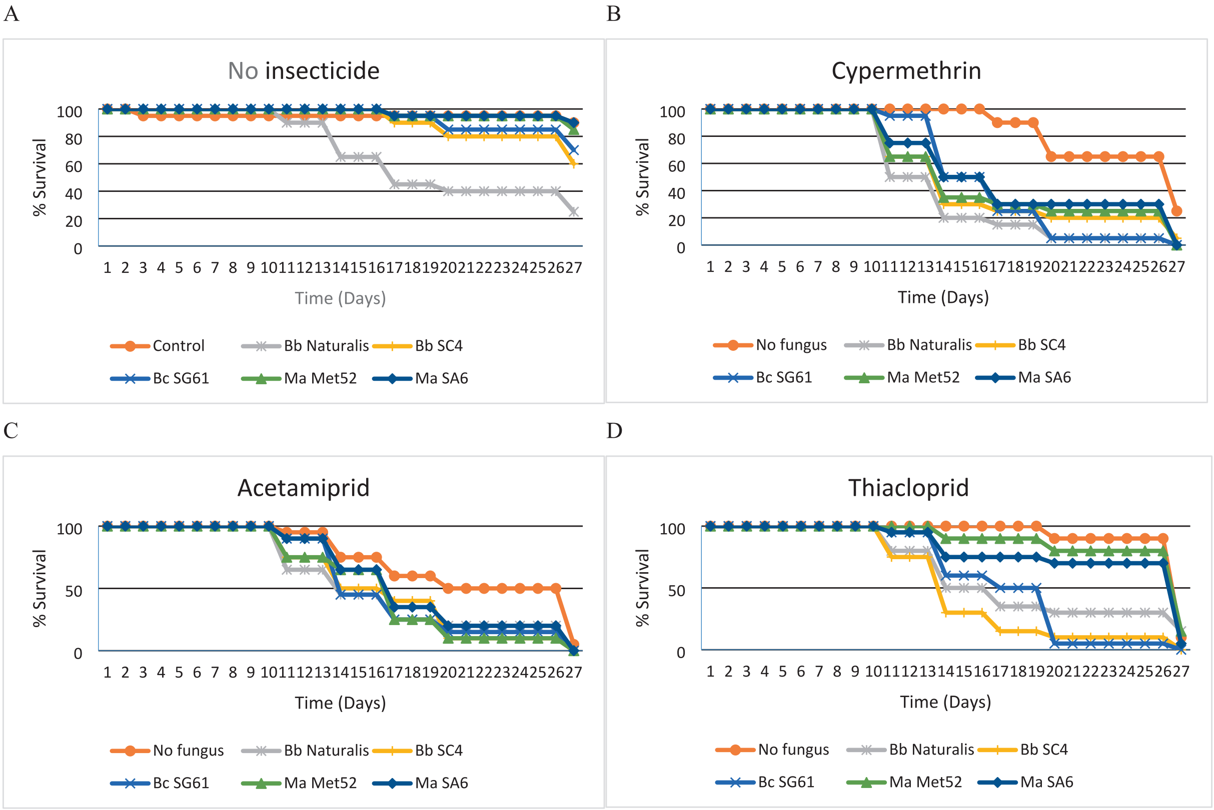

Fig. 1. Survival curves for *Hylobius abietis* adults (N = 20) exposed to single and combined control agents, either insecticides (applied to twigs at LC_{50} concentration) or entomopathogenic fungi (weevils dipped in 10^8 spores/ml). Lab experiment 1. For log-rank test results among curves in each panel see Table 1. Bb = *Beauveria bassiana*, Bc = *Beauveria caledonica,* Ma = *Metarhizium anisopliae.*

either strain of *M. anisopliae* (Ma F52 and Ma SA6) alone was minimal by this time (Fig. 1A). Mortality in the insecticide-only treatments reached 75–95 % (Fig. 1B-D). The rate of mortality was significantly improved when combined with some (for acetamiprid and thiacloprid) or all (for cypermethrin) of the fungal strains (Table 1). Interaction between the two agents was tested for on days 14 and 17 after treatment, when mortality for all single-agent treatments was less than 50 % (with one exception on Day 17) (Fig. 1). On both Day 14 and Day 17 after treatment, most of the combinations of chemical insecticide and fungus resulted in higher mortality than expected based on the results of two agents alone, and the observed mortality was significantly different to the expected (chi-square test, $P < 0.05$), providing evidence of a synergistic interaction (Table 2).

A proportion (21–100 %) of the insects that died in each fungus treatment developed mycosis by the end of the experiment (Fig. 2). To see whether there was a difference due to insecticide, we compared the incidence of mycosis for each of the fungal species separately. There was no difference due to insecticide for either of the two *B. bassiana* strains (chi square, 2 DF, $P > 0.05$ in each case), and at least 88 % of the dead insects were mycosed in each combination that included these species. For the other three strains of fungus, there was a significant difference due to insecticide, with $P = 0.02$, $P = 0.001$ and $P = 0.007$ for Bc SG61, Ma F52 and Ma SA6, respectively (chi Square, 2 d.f). For these three strains, there was a tendency for a lower incidence of mycosis in combinations involving thiacloprid and acetamiprid than those involving cypermethrin or no insecticide (Fig. 2).

The chemical insecticides resulted in knockdown – behavioural incapacity – as well as (and prior to) death of weevils (Fig. 3). Knockdown was observed in all three insecticide treatments by day 3, and at least 50 % of the weevils were affected by day 6. Knockdown was the major cause of incapacity in the insecticide treatments for the first two weeks of the experiment, after which mortality took over (Fig. 3). There was no knockdown in the controls (no fungus, no insecticide). A few weevils were recorded as being knocked down in fungus-only treatments (data not shown), shortly before they died.

3.2. Laboratory experiment 2

The highest weevil mortality on day 26 in fungus-only treatments was for weevils dipped in a suspension of Bc SG61 (75 %), followed by Bb Naturalis (55 %) and then Ma F52 (20 %). Where conidia were sprayed on the medium, mortality was negligible and did not differ from control mortality. Mortality of weevils exposed to insecticide alone reached 100 % by day 19 (acetamiprid) or 24 (cypermethrin). When insecticides were combined with fungus, mortality occurred more quickly than without fungus. The interaction of cypermethrin with all three fungal species was synergistic on both day 10 and day 12, whether fungus was applied as dip or spray, while in the case of acetamiprid, synergy was detected in only one instance (with Bb Naturalis [dipped] on day 10), being otherwise additive (Table 3).

3.3. Field trial

At the first assessment in May, 5 weeks after application, there was relatively little weevil damage (mostly category 1 and 2) and the percentage of plants that were undamaged (category 0) ranged from 70 % in the control to 92 % in the full dose of cypermethrin (Fig. 4A). There was a significant difference between treatments in the damage rating (ANOVA, $F_{4,45} = 9.05$, $P < 0.001$). The fungus-only treatment did not differ from the control, but each of the three treatments that included cypermethrin did (Table 4).

By the second assessment date in September, 5 months after application, most trees had at least some level of weevil damage, including category 4 (death). Even in the full dose cypermethrin, only 19 % of

Table 1

Results of survival analysis of *Hylobius abietis* adults with LT_{50} and 95 % confidence interval for each insecticide and fungus treatment, alone and in combination. Weevils were dipped in conidial suspension (10^8 spores/ml) of entomopathogenic fungus and provided with twigs treated with chemical insecticide (see methods section for details). Letters show significant differences among survival curves as tested with a log-rank test with Bonferroni correction ($P < 0.05$). Comparisons are made within each insecticide treatment (or among fungi and controls where no insecticide was included) and Bonferroni corrections were set for each.

Insecticide	Fungus	LT_{50}	95% CI	Log-rank test
Cypermethrin	–	27	20.0–26.0	a
Cypermethrin	*B. bassiana* Naturalis	12	11.0–13.0	b
Cypermethrin	*B. bassiana* SC4	14	11.0–16.0	b
Cypermethrin	*B. caledonica* SG61	15	14.0–16.0	b
Cypermethrin	*M. anisopliae* Met52	14	11.0–19.0	b
Cypermethrin	*M. anisopliae* SA6	14	14.0–26.0	b
Acetamiprid	–	27	17.0–26.0	a
Acetamiprid	*B. bassiana* Naturalis	14	11.0–16.0	ac
Acetamiprid	*B. bassiana* SC4	14	14.0–19.0	bcd
Acetamiprid	*B. caledonica* SG61	15	14.0–16.0	ade
Acetamiprid	*M. anisopliae* Met52	17	14.0–16.0	bcef
Acetamiprid	*M. anisopliae* SA6	17	14.0–19.0	adf
Thiacloprid	–	–	–	a
Thiacloprid	*B. bassiana* Naturalis	15	14.0–26.0	bc
Thiacloprid	*B. bassiana* SC4	14	14.0–16.0	b
Thiacloprid	*B. caledonica* SG61	17	14.0–19.0	b
Thiacloprid	*M. anisopliae* Met52	–	–	ac
Thiacloprid	*M. anisopliae* SA6	27	20.0–26.0	ac
Control –	–	–	–	a
–	*B. bassiana* Naturalis	17	14.0–26.0	b
–	*B. bassiana* SC4	–	27.0–27.0	a
–	*B. caledonica* SG61	–	27.0–27.0	a
–	*M. anisopliae* Met52	–	–	a
–	*M. anisopliae* SA6	–	–	a

plants had no damage (Fig. 4 B). Average damage ratings ranged from 1.58 for full cypermethrin to 2.64 for untreated control and fungus-only treatment (Table 4). There was a significant difference between treatments (ANOVA, $F_{4, 45} = 17.31$, $P < 0.001$). Each of the three treatments containing cypermethrin differed significantly to the control and fungus-only. Reduced dose cypermethrin differed significantly to full dose cypermethrin, but the combination treatment did not, and was intermediate in damage between these two (Table 4). Inspection of Fig. 4B suggests that the combination (reduced cypermethrin plus fungus) differed from reduced dose cypermethrin alone in having a lower percentage of plants in category 3 (heavy damage), and a higher proportion in category 1 (light damage).

4. Discussion

This is the first report of synergy between chemical insecticides with entomopathogenic fungi for pine weevil, showing potential for development of reduced-insecticide strategies for this pest. Synergistic interactions involving *Beauveria* or *Metarhizium* and the neonicotinoid imidacloprid have been extensively demonstrated for diverse pests (e.g. (Farooq et al., 2018; Ge et al., 2020; Morales-Rodriguez & Peck, 2009; Quintela & McCoy, 1997; Russell et al., 2010), but other neonicotinoids, including the two tested here, acetamiprid and thiacloprid, have received little attention in this regard (Farooq & Freed, 2016a, 2016b). We also found synergy with cypermethrin; previous reports for this pyrethroid show effects ranging from antagonism (Shapiro-Ilan et al., 2011) to synergy (Chen et al., 2021; Meyling et al., 2018).

Various mechanisms have been proposed to explain the synergistic effects of synthetic insecticides and fungi, including altered behaviour, starvation and effects on the insect immune system. Many insecticides, including those used in our study, are neurotoxic, inhibiting insect movement before causing death; in our experiments we recorded high levels of knockdown or incapacitation shortly after exposure to the insecticides. Impaired activity should reduce the rate at which insects encounter conidia on contaminated substrate, resulting in lower rather than higher mortality (Furlong & Groden, 2001), but synergy was detected both when the weevils themselves were directly contaminated with conidia and when weevils acquired conidia from the substrate, so reduced activity alone is unlikely to be an important factor in explaining synergy in our experiments. Impairment of movement may reduce the rate at which conidia are removed from the cuticle either by deliberate grooming (Boucias, 1996) or by shearing as the insect moves through a substrate (Quintela & McCoy, 1998a, 1998b). Adult pine weevils groom in response to entomopathogenic nematodes on their cuticle (Ennis

Table 2

Observed mortality on days 14 and 17 of *Hylobius abietis* adults that were dipped in conidial suspension (10^8 spores/ml) of entomopathogenic fungus and provided with twigs treated with chemical insecticide, together with the mortality expected if the effects of fungus and insecticide are additive, and the results of the chi-square test with the null hypothesis of additivity. Where the observed value exceeds the expected, the effect is synergistic (Syn) where $P < 0.05$ and additive (Add) where $P > 0.05$. (Laboratory expt. 1).

Treatment*	Day 14					Day 17				
	Obs	Exp	X^2	P		Obs	Exp	X^2	P	Effect
Cypermethrin										
Bb Naturalis	78.9	31.6	68.7	0.001	Syn	84.2	55.1	15.4	0.001	Syn
Bb SC4	68.4	0.0	4542.8	0.001	Syn	73.7	10.2	392.8	0.001	Syn
Bc SG61	47.4	0.0	2153.0	0.001	Syn	73.7	5.3	890.1	0.001	Syn
Ma Met52	63.2	0.0	3868.8	0.001	Syn	68.4	5.3	758.4	0.001	Syn
Ma SA6	47.4	0.0	2153.0	0.001	Syn	68.4	5.3	758.4	0.001	Syn
Thiacloprid										
Bb Naturalis	50.1	31.6	10.2	0.01	Syn	66.8	52.6	3.8	> 0.05	Add
Bb SC4	68.4	0.0	4542.8	0.001	Syn	84.2	5.3	1184.2	0.001	Syn
Bc SG61	36.8	0.0	1281.6	0.001	Syn	47.4	0.0	2150.0	0.001	Syn
Ma Met52	5.3	0.0	18.5	0.001	Syn	5.3	0.0	18.2	0.001	Syn
Ma SA6	21.1	0.0	404.0	0.001	Syn	21.1	0.0	402.1	0.001	Syn
Acetamiprid										
Bb Naturalis	52.6	46.0	0.86	> 0.05	Add	73.7	70.1	0.19	> 0.05	Add
Bb SC4	47.4	21.1	33.2	0.001	Syn	57.9	40.2	7.8	0.01	Syn
Bc SG61	52.6	21.1	47.6	0.001	Syn	73.7	36.8	36.8	0.001	Syn
Ma Met52	31.6	21.1	5.4	0.05	Syn	73.7	36.8	36.8	0.001	Syn
Ma SA6	31.6	21.1	5.4	0.05	Syn	63.2	36.8	18.8	0.001	Syn

[*] Bb = *Beauveria bassiana*, Bc = *Beauveria caledonica*, Ma = *Metarhizium anisopliae*.

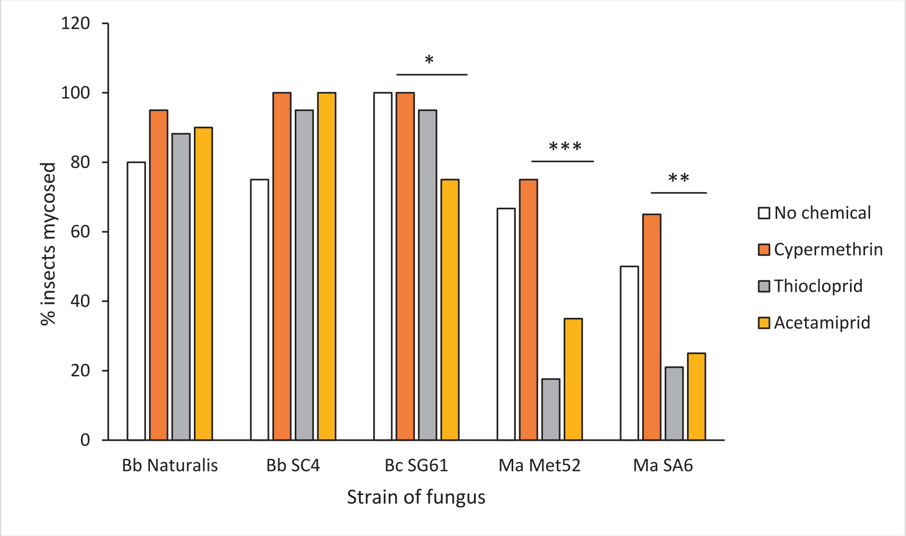

Fig. 2. Percentage of dead *Hylobius abietis* adults that developed mycosis by Day 27 in Lab Experiment 1. Asterisks indicate a difference between chemical treatments in the incidence of mycosis for a strain of fungus (*P < 0.05; **P < 0.01; ***P < 0.001). Bb = *Beauveria bassiana*, Bc = *Beauveria caledonica,* Ma = *Metarhizium anisopliae.*

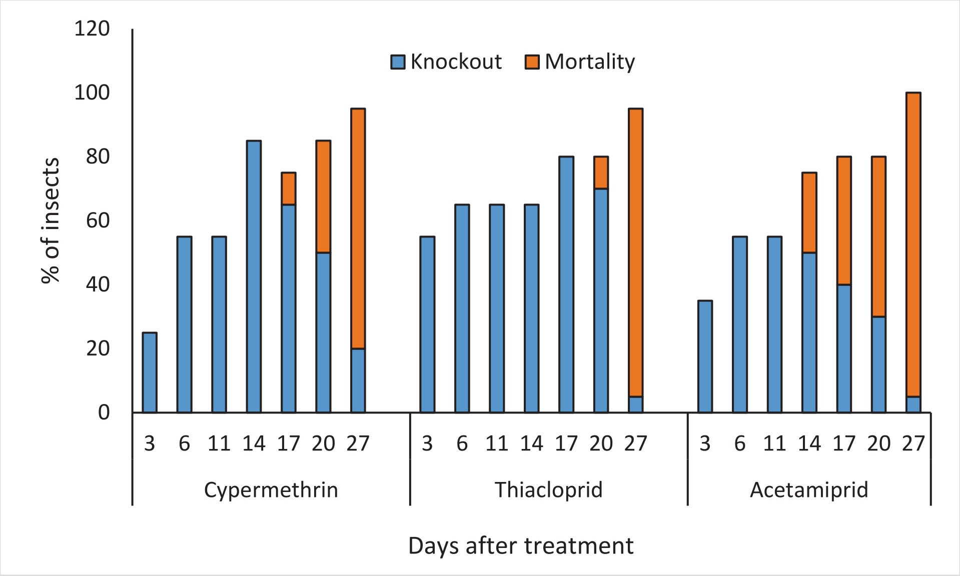

Fig. 3. Cumulative percentage of adult *Hylobius abietis* (N = 20) showing an effect (knockdown or mortality) at various times after exposure to insecticide-treated twigs in Lab Experiment 1.

et al., 2010). The activity and large size of nematodes would make them easier to detect than fungal spores, but baseline grooming that occurs at a low level (Ennis et al., 2010) could still help remove conidia, and so impairment of this could contribute to synergy. However, this is likely to play a minor role compared to other effects. Weevils subjected to neurotoxins feed less than normal, and it has been suggested that starvation may act as a stressor, impairing the insect’s ability to deal with pathogens (Furlong & Groden, 2001; Russell et al., 2010). Starvation had a similar effect to imidacloprid on survival of *M. brunneum*-inoculated Asian longhorn beetles (Fisher et al., 2017), but insecticides including imidacloprid may also directly affect insects’ cellular and humoral immune response (Fisher et al., 2017; Jia et al., 2016; Lin et al., 2018; Tomilova et al., 2016).

Synergy between entomopathogenic fungi and chemical insecticides may result in a faster or higher overall mortality (Chen et al., 2021; Farooq et al., 2018; Fisher et al., 2017; Furlong & Groden, 2001; Meyling et al., 2018; Russell et al., 2010). At the concentrations used in our laboratory experiments, the insecticides alone eventually resulted in high mortality, so the addition of fungus mainly decreased the time to death. The ideal strategy for integrated pest management is to use reduced or sublethal concentrations of insecticide along with the pathogen. Our laboratory assays are “proof of concept” that combinations of insecticides and fungi can have a synergistic mode of action, while the field trial, conducted under commercial forestry conditions, indicates that such combinations might allow use of a reduced rate of insecticide in practice. In our trial, the protection of trees from weevil damage due to half-rate application of cypermethrin was reduced and differed significantly to full rate alone, but not when combined with Met52. Despite demonstrations of efficacy in the lab (Ansari & Butt, 2012; Wegensteiner & Führer, 1988), entomopathogenic fungi have rarely been tested against adult pine weevil in the field. Conidial suspension of Met52 sprayed on trees did not give any additional protection over the

Table 3
Observed mortality on days 10 and 12 of *Hylobius abietis* adults exposed to entomopathogenic fungus and provided with twigs treated with chemical insecticide, together with the mortality expected if the effects of fungus and insecticide are additive, and the results of the chi-square test with the null hypothesis of additivity. Where the observed value exceeds the expected, the effect is synergistic (Syn) where $P < 0.05$ and additive (Add) where $P > 0.05$. Fungus was applied either by dipping adults or by spraying conidial suspension on the substrate (Laboratory expt. 2).

Treatment			D10		D12		D10		D12		Outcome	
Chem		Fungus	Obs	Exp	Obs	Exp	X2	p	X2	p	D10	D12
Cyp	dip	Bb Naturalis	91.6	25.4	100.0	40.6	172.63	0.001	86.71	0.001	Syn	Syn
		Bc SG61	83.1	1.8	100.0	28.1	3665.93	0.001	183.49	0.001	Syn	Syn
		Ma Met52	87.3	13.6	95.7	34.4	400.19	0.001	109.35	0.001	Syn	Syn
	spray	Bb Naturalis	62.0	5.7	82.9	25.0	551.95	0.001	133.73	0.001	Syn	Syn
		Bc SG61	74.7	9.7	95.7	28.1	437.29	0.001	162.26	0.001	Syn	Syn
		Ma Met52	53.5	9.7	65.7	28.1	199.19	0.001	50.17	0.001	Syn	Syn
Acet	dip	Bb Naturalis	87.3	79.6	95.7	86.1	0.75	> 0.05	1.08	> 0.05	Add	Add
		Bc SG61	83.1	73.2	95.7	83.1	1.35	> 0.05	1.90	> 0.05	Add	Add
		Ma Met52	87.3	76.4	100.0	84.6	1.57	> 0.05	2.80	> 0.05	Add	Add
	spray	Bb Naturalis	95.8	72.3	95.7	82.4	7.63	0.01	2.15	> 0.05	Syn	Add
		Bc SG61	70.4	75.8	82.9	83.1	0.38	> 0.05	0.00	> 0.05	Add	Add
		Ma Met52	87.3	73.4	95.7	83.1	2.62	> 0.05	1.90	> 0.05	Add	Add

*Cyp = Cypermethrin; Acet = Acetamiprid; Bb = Beauveria bassiana, Bc = Beauveria caledonica, Ma = Metarhizium anisopliae.

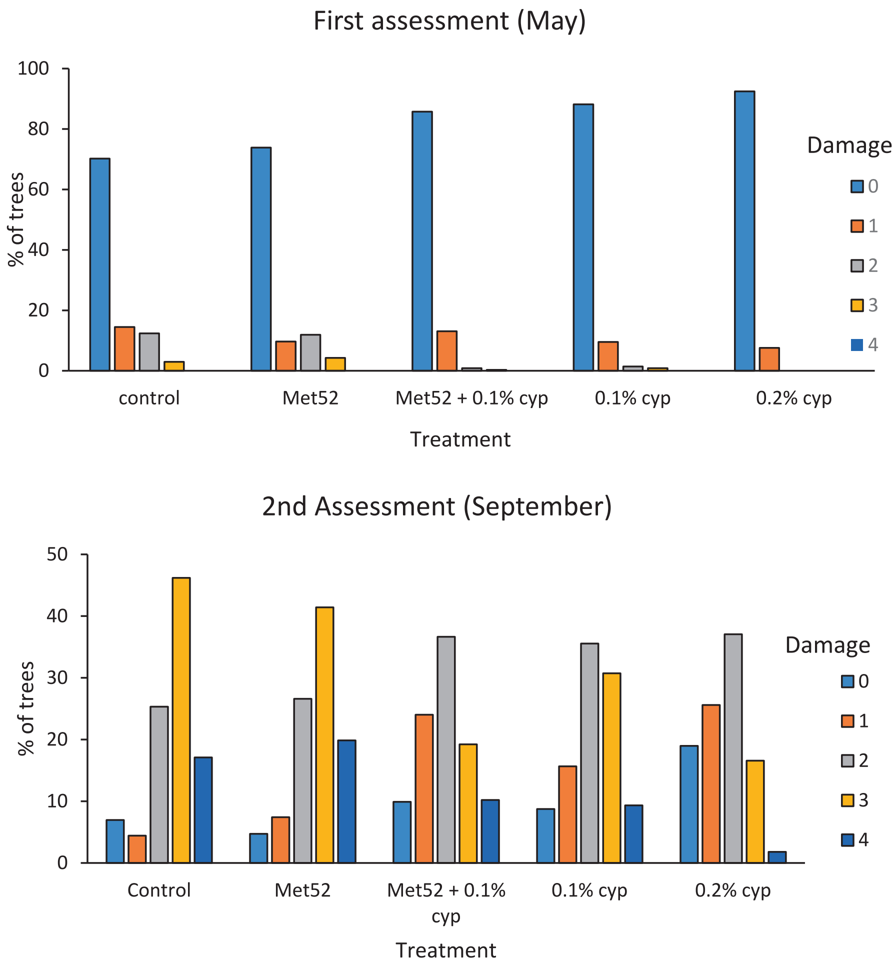

Fig. 4. Percentage of Sitka spruce trees in each weevil damage category (0–4) one (May) and five (September) months after treatment in a field trial. Treatments included *Metarhizium anisopliae* Met52 and cypermethrin, alone and in combination.

already good protection provided by the insecticides alpha-cypermethrin or acetamiprid alone (Willoughby et al., 2020), highlighting the importance of using a reduced application rate of insecticides for synergy to be demonstrated. In the same series of trials, Met52 was also tested in combination with a physical barrier (Flexcoat) and gave a significant but small amount of extra protection over barriers alone at some sites (presumably an additive rather than synergistic effect) (Willoughby et al., 2020). In an alternative approach, *B. bassiana*-

Table 4
Mean (and SE) damage rating to trees treated with cypermethrin and/or *Metarhizium anisopliae* Met52 in a field experiment one month (31st May 2016) and 5 months (Sept 2016) after treatment. Within a column, values that are accompanied by the same letter are not significantly different (Tukey's post hoc test following one-way ANOVA, $P < 0.05$).

Treatment	1 month (May)	5 months (September)
Control	1.44 (0.073) A	2.64 (0.107) A
Met52 only	1.45 (0.103) A	2.64 (0.093) A
1/2 cypermethrin	1.14 (0.035) B	2.15 (0.083) B
full cypermethrin	1.06 (0.024) B	1.58 (0.115) C
Combination (reduced cypermethrin + Met52)	1.14 (0.033) B	1.95 (0.142) BC

colonized carriers placed in a felled forest site resulted in infection of *H. abietis* and showed potential as a strategy for reducing numbers of adults (Lalik et al., 2021b).

One of the reasons why entomopathogenic fungi that show promise in lab trials fail in the field is that the conidia are sensitive to environmental conditions including low moisture, high temperature and especially ultraviolet light (Jaronski, 2010). In our field trial, we applied the fungal inoculum mixed with peat around the base of the tree to afford it some degree of protection, and the cool, moist conditions prevailing during the trial would also have favoured survival of inoculum. While a weevil approaching a tree to feed may pass quickly over this treated area, weevils intoxicated by feeding on insecticide-treated trees are expected to fall onto it and become contaminated.

In our lab experiments, the combination of cypermethrin and substrate contaminated by Met52 had a synergistic outcome, suggesting that this may be the basis for any improved efficacy over insecticide alone. Applied conidia have limited persistence, especially when used above-ground (Jaronski, 2010), but sporulation on cadavers around the base of a tree could potentially renew the inoculum. Thus, mycosis (development of the fungus in the insect and production of conidia on the surface) is of interest in addition to pest mortality. Chemical insecticides differ in their compatibility with entomopathogenic fungi, with some inhibiting growth and spore production in vitro or on insects (Chen et al., 2021; Pelizza et al., 2018; Sain et al., 2019; Shah et al., 2009). Mycosis was recorded in all of our insecticide-fungus combinations, though with some evidence of differential effects of insecticides, with a lower percentage of dead insects developing mycosis in combinations involving *M. anisopliae* strains and either of the two neonicotinoids (but not cypermethrin). Inhibitory effects on spore production on insects have previously been demonstrated for the neonicotinoid imidacloprid (Chen et al., 2021; Russell et al., 2010) while beta-cypermethrin included in the same study was less inhibitory (Chen et al., 2021). Since inhibition levels depend not only on the identity and dose of the insecticide but also the strain of fungus (Pelizza et al., 2018), any inhibition of mycosis should be assessed for the particular combination of agents planned for field use.

When tested without chemical insecticides in our lab assays, mortality due to entomopathogenic fungi was slow, with little or no kill within the first week. Slow rate of kill is a feature of entomopathogenic fungi and has been noted in other studies on adult pine weevil (Ansari & Butt, 2012; Barta et al., 2019; Wegensteiner & Führer, 1988). In our assays, *Beauveria* spp. tended to perform better than *M. anisopliae*, which may be partly due to faster action, since our experiments were terminated after 26 or 27 days. However, the best strain against adult pine weevil identified by Ansari and Butt (2012) was a strain of *M. brunneum* which killed all weevils in 12 days. This is the first report of testing *B. caledonica* against adult pine weevils, and results for this species were promising. Similarly, *B. caledonica* was at least as effective as *B. bassiana* or Met 52 at infecting the earlier stages of pine weevil developing in stumps (Mc Namara et al., 2018). *Beauveria caledonica* is a naturally occurring pathogen of forest beetles, reported from *H. abietis* developing in tree stumps in Scotland and Ireland, and also from scolytid beetles in New Zealand where it is assumed to be introduced (Glare et al., 2008). In some cases, strains of fungus isolated from the target pest may perform better than those baited from soil, commercial strains or commercial formulated products, but in other cases they show limited capacity to control the species from which they were isolated; there is no consistent trend one way or the other, and so screening of strains from diverse origins is recommended (Khun et al., 2020). Although three of our strains were isolated from the forest environment, and one of them from a pine weevil larva, none was actually isolated from an adult pine weevil, the target of our study. Natural infection of adult pine weevils with entomopathogenic fungi appears to be relatively uncommon, which is not surprising, given their low susceptibility in lab assays (present study; Ansari & Butt, 2012; Barta et al., 2019; Wegensteiner & Führer, 1988). *B. bassiana* was recovered from adult pine weevils in Sweden, Poland and Austria, but rarely (Gerdin, 1977; Wegensteiner et al., 2015) and both *B. bassiana* and *B. caledonica* were occasionally found on adult weevils in emergence traps erected over conifer stumps in Ireland (van Vlaenderen, Meade and Griffin, unpublished). Presumably, these weevils were exposed to infection either in the stump or as they emerged from it through the soil. The only study in which prevalence was quantified was in Slovakia where 2 % of adult weevils were infected with either *B. bassiana* or *B. pseudoobscura* (Barta et al., 2019). We are unaware of any reports of adult pine weevil naturally infected by any species of *Metarhizium*.

5. Conclusion

Our work shows that using entomopathogenic fungi has potential to reduce the amount of chemical insecticide used to protect trees from attack by pine weevil. Synergy was demonstrated across a variety of species and strains of fungus, and with insecticides of two chemical classes. While entomopathogenic fungi on their own may have limited scope for effective protection of young trees against pine weevil damage, deployment along with chemical insecticides may be a viable option in integrated pest management for this pest, and so screening programmes of species or strains of fungus against pine weevil should include tests in combination with chemical insecticides currently in use or under consideration for pine weevil protection. In addition to screening for more effective strains, improved formulations or application techniques could increase the efficacy or persistence of the fungal inoculum. While the use of two agents could be more costly, in terms of product and labour, than chemical insecticide alone, employing such a strategy on suitable sites could be an important step towards complying with proposed ambitions in the EU to halve use of pesticides by 2030.

CRediT authorship contribution statement

Johan van Vlaenderen: Methodology, Experiments, Formal analysis, Writing (first draft), Writing – review & editing. **Niamh Hennessy:** Writing – review & editing, Methodology, Investigation. **Padraig O'Tuama:** Writing – review & editing, Supervision, Resources, Project administration. **Christopher D. Williams:** Writing – review & editing, Methodology, Formal analysis. **Christine T. Griffin:** Writing – review & editing, Writing – original draft, Supervision, Project administration, Methodology, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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