Inert agricultural spray adjuvants may increase the adverse effects of selected insecticides on honey bees (*Apis mellifera* L.) under laboratory conditions

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Abstract

Currently, more than 350 spray adjuvants are registered in Germany (January 2021). Unlike plant protection products (PPPs), adjuvants are not subjected to regulatory risk assessment. In practice, numerous combinations of PPPs and adjuvants are therefore possible. Thus, tank mixtures containing insecticides that are classified as nonhazardous to bees and approved for use in bee attractive crops may raise pollinator safety concerns when mixed with efficacy increasing adjuvants. This study analyzes whether selected “PPP-adjuvant” combinations result in increased mortality and pose an elevated risk to honey bees. To answer this question, we chose six common spray adjuvants of different classes for laboratory screening. These were then tested in a total of 30 tank mixtures, each with a neonicotinoid (acetamiprid), pyrethroid (lambda-cyhalothrin), diamide (chlorantraniliprole), carbamate (pirimicarb), and butenolide (flupyradifurone) formulation. We followed OECD test guideline 214 (acute contact test) but adopted the use of a professional spray chamber for more realistic exposures. Our results showed that, in total, 50% of all combinations significantly reduced the lifespan of caged honey bees in comparison to individual application of insecticides. In contrast, none of the adjuvants alone affected bee mortality (Cox proportional hazard model, $p > 0.05$). With four of the five insecticide formulations, the organosilicone surfactant Break-Thru® S 301 significantly increased bee mortality within 72 h (for all insecticides except chlorantraniliprole). Furthermore, acetamiprid yielded the highest and second-highest mortality increases from a tank mixture with the crop oil surfactants LI-700 (hazard ratio = 28.84, $p < 0.05$) and Break-Thru® S 301 (hazard ratio = 14.66, $p < 0.05$), respectively. In the next step, field trials should be performed to provide a more realistic exposure scenario under colony conditions to verify these findings.

1 Introduction

Multiple factors, such as pathogens, parasites, loss of habitat, malnutrition, and the use of plant protection products (PPPs), are currently suspected to be causes of pollinator decline, which is being discussed globally. Scientific evidence suggests that this decline is not the result of an individual but the interaction of many different stressors (vanEngelsdorp & Meixner 2010). Honey bees in particular are relatively resilient, and not just because of their ability as a superorganism to mitigate various stresses by means of social buffering (Straub et al. 2015). Bee colonies are usually cared for by a beekeeper and safeguarded against outside adverse effects. Thus, only strong acute and chronic toxic effects of PPPs are visible in bees under field conditions. Sublethal effects, however, can remain unnoticed due to social buffering (Odemer et al. 2018).

When investigating these sublethal effects, it became increasingly apparent that co-formulants and adjuvants believed to be "inert" (EPA 2021) can also have potentially toxic effects on bees (Mullin 2015; Mullin et al. 2015; Chen et al. 2019; reviewed in Iwasaki & Hogendoorn 2021). Even harmless herbicides without insecticidal activity have demonstrated lethal and sublethal effects on bumblebees and honey bees (Straw et al. 2021; Odemer et al. 2020). Hence, there has recently been an emerging interest not only on the synergistic effects of the tank mixtures of different PPPs but also on their "inert" adjuvants.

Tank mixes can increase total mortality in honey bees in predictable (Pilling & Jepson 1993; Iwasa et al. 2004; Wernecke et al. 2019) and unpredictable ways (Johnson et al. 2013; Zhu et al. 2014; Wade et al. 2019). Despite their relevance under normal field conditions, these aspects of pesticide toxicology are often overlooked (Chmiel et al. 2020). Most emphasis has focused on formulations and their active ingredients rather than on the adjuvants that can be added to the spray solution. However, foragers are evidently confronted with numerous spray adjuvants in the field (Ciarlo et al. 2012, Mullin et al. 2015; reviewed in Iwasaki & Hogendoorn 2021).

By January 2021, over 350 such adjuvants were authorized in Germany. They may be added to the spray solution as wetting agents, adhesives, or water conditioners and are frequently used in bee-attractive crops such as orchards or

oilseed rape during flowering (BVL 2019a) to ensure the full efficacy of PPPs even under unfavorable conditions. Since they do not contain active ingredients with biological activities, only an application for approval is required for commercialization (dlz agrarmagazin 2006; BVL 2019b; Mullin et al. 2016).

Adjuvants in some EU countries, such as France and Italy, routinely undergo risk assessment (personal communication, N. Kurlemann). In Germany, unlike the assessment of PPPs, no data are generally submitted for adjuvants as a basis for assessing risks and effectiveness in the approval process. Therefore, it is currently unclear whether the environmental impact of adjuvants on pollinators is realistically classified or over- or underestimated. In contrast to formulated PPPs, the effects of which on pollinators are being intensively investigated, the level of knowledge about tank mixtures, especially with adjuvants and their possible risks (Fine et al. 2017), and knowledge on the effects on the physiology, behavior, and immune competence of honey bees are low (Zuh et al. 2014). The literature provides evidence that the toxicity of formulated products, including adjuvants, can increase the adverse effects of insecticides compared to that of their active ingredient (Mullin et al. 2016; reviewed in Iwasaki & Hogendoorn 2021). As a result, adjuvants can partly reduce the effective PPP application rate by as much as 10-fold (Green & Green 1993).

Negative effects of adjuvants in combination with insecticides (including lethal and sublethal effects, e.g., on the ability to learn or immune defense) have already been identified in scientific studies for adult honey bees (e.g., Ciarlo et al. 2012; Chen et al. 2018; Wernecke et al. 2018) and honey bee larvae (Fine et al. 2017; Zuh et al. 2014). The questions on whether and to what extent tank mixtures from PPPs and adjuvants actually present a realistic risk to honey bees and other nontarget organisms have far been controversial. Hence, their contribution to the decline in biodiversity in agricultural landscapes has not been adequately investigated. Closing these knowledge gaps is therefore a basic requirement for pollinator-friendly, sustainable agriculture to preserve biological diversity and protect pollinators.

This study aimed to address uncertainties for a sound, technically well-founded assessment of adjuvants. Moreover, we wanted to identify the further need for action in the approval of adjuvants by the Federal Office for Consumer Protection and Food Safety (BVL) concerning bee protection. It should be clarified whether and to what extent adjuvants mixed with PPPs cause an increase in bee toxicity and consequently sublethal and lethal effects, with the aim of detection and reducing risks for honey bees.

Adjuvants can be added to herbicides, fungicides and insecticides. For our screening, we placed our focus on insecticide formulations, since a possible increase in efficacy there can presumably lead to higher damage than with the other two groups. In addition, when selecting products, we ensured that they were all registered for use in flowering crops. This allows field-realistic mixtures to be simulated in the laboratory. Based on the evidence provided by other studies, we suspect that when applied as a tank mixture, spray adjuvants may increase the toxicity of insecticidal formulations.

To investigate the magnitude of this increase and determine the class with the highest and lowest effects for both the insecticide formulation and the spray adjuvant, we investigated these issues with a series of acute laboratory contact tests in 2019 and 2020. This involved testing selected insecticide formulations (Mospilan® SG, Coragen®, Karate® Zeon, Sivanto® Prime, Pirimor® Granulat) and spray adjuvants (Break-Thru ® S 301, Acxcess®, LI 700®, Hasten® TM, Break-Thru® SP 133, Kantor®) alone and in combination. Conventional application rates were used based on the methods and guidelines established in the regulatory risk assessment. The products tested in the screening were systematically combined into a total of 30 different mixtures. All resulting combinations are theoretically allowed in practice and thus represent realistic exposure.

2 Material And Methods

2.1 Honey bees

A total of four honey bee colonies (*Apis mellifera* L.), healthy and queen-right, from the institute's beekeeping in Braunschweig (Germany) were used. The queens were sisters from a breeding line reared in the same year at the test facility. For the trials in the spray chamber, two colonies each were selected, and adult workers were sampled near the brood nest to standardize the age of the bees. The last treatment of the colonies against *Varroa destructor* was at least six weeks previous. No clinical symptoms of adult bee or brood diseases were visible during inspection.

2.2 Experimental design

The experiment consisted of five individual trials conducted between November 2019 and August 2020. For the laboratory studies, worker bees were taken near the brood chamber of each of the two colonies and subjected to CO_2 treatment for approximately 30 seconds. Anesthetized bees were then counted in standard stainless steel experimental cages (10 cm x 8.5 cm x 5.5 cm) with filter paper inserts at 10 bees per cage and randomly placed in a climate chamber (24°C ± 1°C, 60% RH ± 10%, no light, and overnight acclimation). Six replicates per treatment and control group were used in each experiment, yielding a total number of 60 bees per treatment. Feeding was *ad libitum* with 50% sugar solutions (w/v) via a 5 ml disposable syringe with the tip removed. The syringes were replaced daily for sanitary reasons. For all experiments, a pre-examination was performed on the application day to ensure that 100% of the experimental bees were vital and undamaged. If necessary, the cages were replaced with spare cages. To minimize segregation of the spray solution, exposure was started as soon as possible after preparation.

The acute contact tests were conducted following Test Guideline 214 (OECD, 1998) with certain adaptations. To simulate a more realistic field contact exposure, the usual application of a single droplet to the thorax of the bees was omitted. Instead, whole-body exposure was performed in a professional spray chamber (custom-built by Christan Schachtner Gerätetechnik, Ludwigsburg, Germany) following the method of Wernecke et al. (2019) with minor modifications.

Briefly, bees were immobilized at 4°C prior to application, transferred cagewise to Petri dishes, and sprayed in the chamber at room temperature with the respective spray solution (spray speed: 2.5 km/h; nozzle pressure: 2.9 bar; system pressure 7–8 bar; spray height: 42 cm; setting 300 l water/ha). The spray chamber was equipped with commercially available application nozzles allowing for the bees to be completely wetted with a fine spray mist (flat spray nozzle Teejet 9503 EVS). The bees were then retransferred in cages and brought back to the climate chamber. The effects of each mixture were evaluated by visual inspection of bees after 2, 4, 24, 48, and 72 h intervals directly in the climate chamber.

2.3 Chemical treatment

All test substances used for this study were approved in Germany at the start of the trial. To achieve systematic screening with a broad spectrum, insecticides were selected on the basis of active ingredient classes. Therefore, one representative formulation from each of the classes of neonicotinoids (Mospilan® SG), diamides (Coragen®), pyrethroids (Karate® Zeon), butenolides (Sivanto® Prime), and carbamates (Pirimor® Granulat) was selected. All insecticides are classified as nonhazardous to bees (rated as B4 in Germany) when they are used at their maximum application rate and when applied separately. Six representative, best-selling spray adjuvants were also tested (Table 1). All were approved for mixing with insecticides. They can be broadly classified into different categories (e.g., superspreaders, penetration agents, or multifunctional agents) depending on their properties. However, there is no clear classification definition, so some adjuvants may serve multiple purposes.

Table 1

Test substances (TS)

					Application rate		*LD_{50}contact*
Abbreviation	*Trade name*	*Type*[a]	*Active substance and co-formulants*	*Formulation type*[b]	*Product/ha*	*µg a.s./ bee*[c]	*µg a.s./ honey bee (TS = a.s.)*[d]
Mos	Mospilan® SG	I	200 g/kg acetamiprid	SG	0.325 kg	0.3348	8.09
Cor	Coragen®	I	200 g/l chlorantraniliprole	SC	0.125 l	0.1291	> 100
Kar	Karate® Zeon	I	100 g/l lambda-cyhalothrin	CS	0.0375 l	0.0192	0.038
Siv	Sivanto® Prime	I	200 g/l flupyradifurone	SL	0.75 l	0.6603	> 200
Pir	Pirimor® Granulat	I	500 g/kg pirimicarb	WG	0.45 kg	1.1588	17.8
301	Break-Thru® S 301	A	1030 g/l polyether-polymethylsiloxane-copolymer (100% w/w)	SL	0.25 l	n.a.	n.a.
Acx	Acxcess®	A	polyether-polymethyltrisiloxane-copolymer	n.a.	0.2 l	n.a.	n.a.
LI	LI 700®	A	350 g/l modified soy lecithin(35%), 350 g/l propionic acid (35%), 94 g/l alcohol ethoxylate, 15 g/l fatty acids	SL	1.5 l	n.a.	n.a.
Has	Hasten® TM	A	716 g/l rapeseed oil ethyl and methyl esters, 179 g/l nonionic surfactants	EC	0.75 l	n.a.	n.a.
133	Break-Thru® SP 133	A	80% fatty acid esters, 20% polyglycerol ester	SL	0.75 l	n.a.	n.a.

[a] I = insecticide; A = adjuvant

[b] SG = Water soluble granule; SC = Suspension concentrate; CS = Capsule suspension; SL = Soluble concentrate; WG = Water dispersible granule; EC = Emulsifiable concentrate; n.a. = not applicable

[c] see Eq. (1) for calculation details

[d] Data from the Pesticide Properties DataBase (PPDB 2021)

					Application rate		*LD_{50}contact*
Kan	Kantor®	A	79% alkoxylated soy oil, 12% fatty acid of tall oil, 6% alkyl polyglycosides, 3% acetic acid	EC	0.45 l	n.a.	n.a.
[a] I = insecticide; A = adjuvant							
[b] SG = Water soluble granule; SC = Suspension concentrate; CS = Capsule suspension; SL = Soluble concentrate; WG = Water dispersible granule; EC = Emulsifiable concentrate; n.a. = not applicable							
[c] see Eq. (1) for calculation details							
[d] Data from the Pesticide Properties DataBase (PPDB 2021)							

In the contact test, all adjuvants were applied at the maximum application rate permitted in Germany. The application rate of the insecticides was determined independently of the crop, i.e., irrespective of bee attractiveness and BBCH stage and the specified water application rate. For Karate® Zeon and Pirimor® Granulat, up to 50% less than the maximum application rate was used in the laboratory to reduce mortality and to determine increased efficacy.

To determine the dose of active substance per bee, six bulk samples of 10 live bees each were weighed before and after spraying with water and the weights were averaged (laboratory analytical balance DENVER INSTRUMENT, type SI-234). It was determined that there was an average application quantity of 1.545 mg of water per bee. The formula for calculating the nominal amount of active ingredient per bee is as follows (1):

$$\frac{a.s.\,(\mu g)}{bee} = \left[\frac{weight\ of\ product\ (g) \times \left[\frac{weight\ percent\ a.s.}{100}\right]}{amount\ of\ dissolved\ water\ (ml)}\right] \times application\ quantity\ per\ bee\ (g) \quad (1)$$

Honey bees were acutely exposed to the test substances. The use of a toxic reference substance was omitted in favor of increasing the number of replicates. Further details on the test substances can be found in Table 1.

2.4 Statistical analysis

All statistical analyses were performed using the software 'R' version 4.0.4 (R Core Team 2021) and a significance level α of 0.05. Plots were generated using the package 'tidyverse' version 1.3.0 (Wickham et al. 2019) and the package 'survminer' version 0.4.9 (Kassambara et al. 2021). Cox proportional hazards models from the package 'survival' version 3.2.7 (Therneau 2020) were used for mortality analysis. Parameter estimates and 95% confidence intervals are reported. To examine the effects of all test substances (including their combinations) with the control as a reference, the following model was fitted: (Survival ~ Treatment + Replicate). There was no correlation between variables. To measure the magnitude of the impact of the spray adjuvants on the insecticide, the control was omitted, and the insecticide was used as a reference. Consequently, the final model was (Survival ~ Treatment), with only one insecticide of the five tested at a time with all combinations of spray adjuvants. The proportional hazards were checked for each experiment to validate the Cox proportional hazards assumption. The Cox proportional hazards model yields a type of hazard ratio (HR). Its simplified interpretation is as follows: HR = 1 means equal or no effect of treatment (T) vs. control/reference (C). If the

treatment was worse than the control, then HR was > 1 and vice versa. We chose to use HR as a relative measure versus median survival and time point estimate (absolute measure) because it summarizes the treatment effect over the entire study period (72 h) and uses all the information in the entire Kaplan-Meier (KM) curve. Assuming HR (T vs. C) = 1.25, this can be either interpreted as an average of approximately 25% higher risk of death (25% as 1–1.25 = -0.25) or an average of approximately 20% decrease in survival time (20% as 1/1.25 = 0.8) from any point in the trial (Barraclough et al. 2011). Thus, unlike comparing survival curves for treatment and control, e.g., by a log-rank test, which gives only binary discrimination, an HR tells the magnitude and direction of this difference (Emmert-Streib & Dehmer 2019).

3 Results

For a clearer presentation, the five selected insecticides were used as a reference in each case instead of the controls to measure the magnitude of mortality increase associated with the spray adjuvant (see section Statistical analysis). The overall results of all five trials in reference to the control are included in the supplementary information (Figs S1-S5; Tabs S1-S5).

In total, Break-Thru® S 301 significantly increased bee mortality in four out of the five trials (for all insecticides except Coragen®). Break-Thru® SP 133 was effective in three of the five trials. The spray adjuvants Acxcess®, LI 700®, Hasten® TM, and Kantor® each increased mortality in two of five trials (Figs. 1–5). Of all 30 combinations of spray adjuvants and insecticides evaluated, 15 significantly increased bee mortality.

Bee mortality of the six tested adjuvants alone did not differ significantly relative to the control group (Figs S1-S5).

Coragen® (chlorantraniliprole)

Bee mortality of the insecticide Coragen® did not increase significantly over a 72 h period with any of the spray adjuvants tested (Fig. 1, Cox proportional hazard model, $p > 0.05$).

Karate® Zeon (lambda-cyhalothrin)

Karate® Zeon, on the other hand, showed a significantly higher risk of death between 67–142% and a significant decrease in survival time between 40.1–58.7% for all adjuvants tested within 72 h. Bees treated with Kar + Acx had a 67% higher risk of death and a 40.1% decrease in survival time. Treatment group Kar + Has had a 73% higher risk of death and a 42.2% decrease in survival time, similar to the combination Kar + Kan with 142% and 58.7% and the mixture of Kar + LI with 116% and 53.7%, respectively. Groups Kar + 133 and Kar + 301 had a likewise higher risk of death, with 94% and 91% or a decrease in survival time of 48.5% and 47.6%, respectively (Fig. 2, Cox proportional hazard model, $p < 0.05$).

Mospilan® SG (acetamiprid)

Half of the Mospilan® SG adjuvant tank mixtures resulted in statistically significant efficacy increases relative to the single insecticide application. Mospilan® SG showed a 741% significant increase in the risk of death and a significant 88.1% decrease in 72 h survival time in the Mos + Kan combination. Mos + LI showed the highest increase in the risk of death by 2784% and the largest reduction in survival time by 96.5% of all trials. The Mos + 301 combination had a 1366% higher risk of death and a 93.2% decrease in survival time and was the second-highest increase of all trials. All other tested combinations with Mospilan® SG did not significantly increase bee mortality over a 72 h period (Fig. 3, Cox proportional hazard model, $p < 0.05$).

Pirimor® Granulat(pirimicarb)

Pirimor® Granulat showed an overall increase in efficacy with four of the six adjuvants. The Pir + Acx combination had a 278% increased risk of death and a 73.5% shortened survival time. Pir + Has increased the risk of death by 369% and shortened survival by 78.7%. Pir + 133 and Pir + 301 unevenly increased the risk of death by 248% and 601% and shortened survival by 71.3% and 85.7%, respectively. The combination of Pir + Kan and Pir + LI showed no statistically significant increase in bee mortality (Fig. 4, Cox proportional hazard model, $p < 0.05$).

Sivanto® Prime (flupyradifurone)

Sivanto® Prime showed a significant increase in effect only for the combinations Siv + 133 and Siv + 301. Here, the risk of death was 1097% and 1003% higher, respectively, and the survival time was 91.6% and 90.9% shorter when compared to the single test substance (Fig. 5, Cox proportional hazard model, $p < 0.05$).

4 Discussion

Agricultural spray adjuvants improve the ability of PPP to spread or better adhere to the leaves of the crop or the surface of the target insect. These adjuvants are currently not regulated in Europe, the United States, or Canada (Durant et al. 2021). That is, they can be mixed with any insecticide class without prior risk assessment. Therefore, especially in agricultural practice, we find situations where bees may be exposed to a mixture of insecticides classified as nonhazardous to bees (B4-rated) and these specific adjuvants, which may enhance the toxicity of the insecticides (reviewed in Iwasaki & Hogendoorn 2021).

In this study, we mixed five representative insecticides of different classes, all B4-rated, with six different spray adjuvants and investigated bee longevity. To systematically screen as many combinations as possible, this experiment was conducted in the laboratory in a professional spray chamber. We found that in 50% of the different combinations, there was an effect of increasing mortality. The agent Break-Thru® S 301, an organosilicone surfactant (OSS), showed this increase in four out of five insecticide classes – the highest ratio in the trial.

The highest and second highest mortality increases totaled in this study were for Mospilan® SG in a tank mixture with LI 700® (Mos + LI) and Mospilan® SG mixed with Break-Thru® S 301 (Mos + 301) (HR = 28.84 and 14.66, respectively). These increases thus fall into the class of neonicotinoids, which also includes a third increasing adjuvant, the combination of Mospilan® SG and Kantor® (Mos + Kan) (HR = 8.41). Similar results were reported by Chen et al. (2019). Here, three laboratory-tested adjuvants (N-methyl-2-pyrrolidone (NMP), Silwet L-77, Triton X-100) in combination with acetamiprid resulted in significantly higher acute contact toxicity to honey bees than with the insecticide alone. Under semifield conditions, the Silwet L-77 (OSS)-acetamiprid combination also negatively affected colony strength and brood development (Chen et al. 2019).

Previous studies have already pointed out the effect-increasing property of OSSs (Mullin et al. 2016; Chen et al. 2019) but it was also highlighted that OSSs are toxic even when applied alone (Goodwin & McBrydie 2000; Mullin 2015). All tested spray adjuvants, including organosilicones, nonionic adjuvants, and crop oil concentrates alone, did not affect honey bees in their lifespan in our trials. However, the effect-increasing property could be confirmed for pyrethroids, neonicotinoids, butenolides, and carbamates but not for diamides.

OSSs cause a larger reduction in surface tension than other nonionic surfactants and crop oil concentrates. This makes them extreme surfactants and superpenetrants that can lead to learning disorders in adult bees (Ciarlo et al. 2012). Such potent surfactant activity can allow the uptake of even bacteria-sized mineral particles through the stomata of leaves (Kaiser 2014). May et al. (2015) consequently suggest that OSS may be more likely to penetrate the cuticle of honey bees (and other nontarget arthropods) to increase uptake of the insecticide and support delivery of active ingredients into bee

tissue. This would explain why all of the tested adjuvants alone did not cause mortality in our experiments, as they did not contain any active ingredients.

Our result is consistent with that of Donovan and Elliot (2001), who also did not observe any mortality effects with topical application of four different adjuvants (including LI 700®, which was also used in our study) at field-realistic application rates (OSS trisiloxane, crop oil concentrate, synthetic latex + alcohol ethoxylate, and OSS + synthetic latex) and orally. Even when fed to nurse bees and transferred by royal jelly to queen larvae, OSS did not affect the survival or development of honey bee queens (Johnson & Percel 2013). In a semifield study, further evidence was provided by Chen et al. (2019), who tested a solvent (NMP), a surfactant (octylphenol ethoxylates), and an OSS (trisiloxane) without effects when applied alone. This, however, contrasts with the findings of Goodwin and McBrydie (2000). They assessed negative effects on survival when bees were oversprayed with a nonionic wetting agent (ethoxylated octylphenol), a surfactant (polyethoxylated tallow amines), and two OSS (one siloxane and one trisiloxane). These varying results indicate that the mode of action of various spray adjuvants on bees needs to be studied in more detail to understand why there are differences and where they come from.

A recent study by Straw et al. (2021) suggests co-formulants cause lethal effects that directly come with the formulation but have similar properties to spray adjuvants. The authors tested glyphosate, which is nontoxic to bees, as a pure substance with various commercial formulations for toxicity to sprayed bumblebees (*Bombus terrestris audax*). It was found that some of the formulations increased the mortality of the bees but not the pure substance. From this, the authors conclude that it is not the active ingredient but the co-formulants that must be responsible for the reduced lifespan. The hairs of the bees matted and perhaps the respiratory openings were covered. The narrow sections in the respiratory system may have also been blocked by the coating of the surface, which could be associated with the suffocation of the bees. In addition, Straw et al. (2021) noted that Stevens (1993) found insect stigmas to be similar in size to plant stomata. He pointed out that the surfactants could allow water to enter the tracheal system, which in turn could lead to the drowning of the sprayed animal. However, these are still hypothetical conclusions that need to be further substantiated by experiments.

Adjuvants are advertised to increase the efficacy of PPPs (Alzchem 2021; Adama 2021), and combined effects that increase the overall effectiveness are therefore to be expected from the manufacturer. As already suggested, they can be partly explained by their physicochemical properties. For example, adhesion agents not only improve the adhesion of the spray solution to the plant (Stevens 1993) but may also increase the adhesion of the active ingredients to the bee. Wetting agents, on the other hand, lower the surface tension of the spray solution so that the PPP is distributed evenly on the leaf, which prevents spillage and increases wetting and active ingredient uptake (Miller & Westra 1998; Stevens 1993; reviewed in Jibrin et al. 2021).

Goodwin and McBrydie (2000) described such observations in their studies of contact exposure of *A. mellifera* to adjuvants. While single droplets formed on the hair coat of bees when water was applied alone, bees that died as a result of adjuvant application were completely soaked or matted, which supports the drowning hypothesis. In addition, adjuvants can increase leaf surface permeability (reviewed in Jibrin et al. 2021). This occurs particularly with lipophilic adjuvants such as crop oil concentrates such as Hasten® TM (Dubovic et al. 2020). In combination, such increased penetration of insecticides through the cuticle can lead to higher toxicity of the active ingredient (quasi-synergism) (Sun & Johnson 1972). This could explain the large toxicity increase for the Mos + LI combination. Furthermore, Sims and Appel (2007) found an increased binding ability of surfactants with small HLB (hydrophilic-lipophilic-balance), which penetrate the fatty wax coating of cockroaches and thus enter the body via the epicuticle, corroborating the earlier hypothesis for the OSS mode of action (May et al. 2015). In addition, there is evidence that cytochrome P450 monooxygenases are involved in the metabolism of adjuvants (e.g., ethoxylated alcohol surfactants) (Sims & Appel

2007). Thus, the increased toxicity of tank mixtures may also be explained by competition for access to the same P450 enzyme.

Adjuvants that can influence the toxicity of PPPs are evidenced by a variety of formulations. For identical active ingredients, some formulations are thousands of times more toxic than the active ingredient. This has been demonstrated for herbicides as well as fungicides and insecticides (Mesnage et al. 2014). It is thus clear that it is not only the dose of a particular active ingredient alone that makes the poison but also the formulation composition, i.e., the formulation aids and co-formulants contained therein (Mullin 2015; Mullin et al. 2015; Straw et al. 2021).

The present experiment shows that the efficacy-enhancing potential of adjuvants can vary considerably and that the observed effects are mostly invariably dependent on the combined insecticide. Sims and Appel (2007) describe a relationship between the observed effects and the molecular characteristics of adjuvants. For example, alkyl chain length, HLB (hydrophilic-lipophilic balance), degree of ethoxylation (Sims & Appel 2007), and molecular weight (Verge et al. 2001) can modify the toxicity of adjuvants.

The effect of all adjuvants in combination with the diamide Coragen® (chlorantraniliprole) failed, which can be attributed to the low contact toxicity in adult bees. Interestingly, Demkovich et al. (2018) showed that the tank mixture of Altacor® (chlorantraniliprole) + Dyne-Amic® (OSS siloxane) increased the mortality of sprayed adult navel orange worms, *Amyelois transitella* (approximately 41%), compared to that of Altacor® alone (approximately 24%). However, the results were inconsistent in eggs and larvae when high and low concentrations of the OSS adjuvant were used. The low concentration mixture resulted in more killed eggs, and the high concentration mixture resulted in more dead larvae. Conversely, however, no significant effects were measurable compared to Altacor®.

The same adjuvant (Dyne-Amic®) was fed to bee larvae by Kordecki (2019) in the laboratory and provided reduced hatching rates. However, the tank mixture of Altacor®+ Dyne-Amic®, similar to Demkovich et al. (2018), did not increase efficacy. This suggests that mortality was induced by OSS rather than insecticide in bee broods. Moreover, Zhu et al. (2014) showed that the commonly used solvent N-methyl-2-pyrrolidone (NMP), which is utilized in PPP formulations, is highly toxic to honey bee larvae upon chronic oral exposure. Thus, bee broods seem to appear significantly more sensitive to certain adjuvants and co-formulants, such as NMP, than adult bees (Zuh et al. 2014; Mullin et al. 2015; Chen et al. 2018). Moreover, chlorantraniliprole (Coragen®) is considered a highly selective insecticide (de Sousa Pereira et al. 2019) and, similar to Sivanto® Prime, has a very high LD50 in contact exposure (Table 1). This may explain the lower activity of the two insecticides in combination with the adjuvants in our study.

Another finding we were able to demonstrate was that both formulations, Karate® Zeon and Pirimor® Granulat, showed clear bee toxicity under laboratory conditions. Karate® Zeon also showed an increased effect with all adjuvants, and Pirimor® Granulat showed an increased effect with four of the six adjuvants. In this context, Barnett et al. (2007) indicate that lambda-cyhalothrin (Karate® Zeon) and other pyrethroid compounds are acutely toxic to bees under laboratory conditions. However, they are not considered a poisoning risk for honey bee colonies when applied to bee attractive crops at their maximum authorized application rate in the field. The authors suggest that this could be due to a repellent effect, leading to a reduction in exposure. However, in our study, contact exposure was given so that a lack of repellent effects alone cannot explain the higher mortality of the respective tank mixtures in this study. Moreover, in the field, it could be conceivable that this protective effect is modified or even neutralized by the chemical properties of the spray adjuvants tested, such as ergosterol-biosynthesis-inhibiting fungicides (Thompson & Wilkins 2003), so damage to bee colonies cannot be excluded.

Lastly, unlike Coragen® and Sivanto® Prime, both Karate® Zeon and Pirimor® Granulat have a rather low LD50, with Karate® Zeon having the lowest LD50 of all insecticides tested in this study (Table 1). This suggests that the acute toxicity of the active ingredient also largely determines the effect enhancement by the adjuvant. Consequently, the

toxicities of adjuvants are highly dependent not only on the particular target organism or PPP (class) combination but also on how they were administered (i.e., route of exposure) and what developmental stage they reach (i.e., egg, larva, adult) (Demkovich et al. 2018; Lie et al. 2019; de Sousa Pereira et al. 2019). Whether the adjuvants examined in this study can have negative effects on honey bees after oral administration or under field conditions must be answered in further experiments.

This study was conducted with bees in an artificial laboratory environment using hoarding cages, which is a limitation. The actual concentrations to which bees are exposed during foraging may depend on weather, temperature, time of day, and the time difference between application and foraging. These factors are all field-dependent, and future studies are needed to further translate what is known in the laboratory to field exposure studies. In addition, pesticide exposure is exacerbated by the transfer of nectar within the colony as foragers return to the hive and pass the collected food to the hive bees. The freshly collected nectar is then first ripened and possibly later fed to larvae. Therefore, future studies should investigate whether bioaccumulation of the active ingredients or adjuvants occurs during trophallaxis and ripening (Kordecki 2019).

To perform the experiments, we used two different colonies with sister queens in each trial. In trials C and E (see Figs S3 and S5), we found that the origin of the bees (replicate) was a significant covariate that may have influenced the respective reported HR. However, this may have been a minimal influence, as we did not have this difference in trials B, D, and E with the same colonies used. In addition, the test substances in question were replicated at least once in the other trials mentioned, which confirmed their negative or neutral effects compared to the control. Nevertheless, in future studies, care should be taken to randomize or mix the bees of both colonies before placing them in the cages. A third colony could also be added to increase the variance between replicates to avoid bias from the genetic background of the bees.

As previously discussed, it is now immensely important to understand the mode of action of adjuvants only, as well as that of co-formulants in PPP formulations (Straw et al. 2021). However, the ingredients of many formulations are legally protected, and their composition is usually not accessible to the user or the scientific community (EC 2009; Cox & Surgan 2006). This makes it difficult to understand the mode of action of these substances and hinders the ecotoxicological testing of potentially hazardous substances. Straw et al. (2021) urges that all components should be disclosed in the product's safety data sheet to allow for individual testing. This is already possible for most solo adjuvants. Mullin (2015) critically commented that 100% of the co-formulants were searched for in bee matrices, while only 70% of the PPP active ingredients were detected (Mullin et al. 2015). Documentation of the formulation and adjuvants used would make it easier to trace potential bee poisoning to a specific active ingredient or co-formulants. This knowledge would help to better protect pollinators from pesticide hazards (Mullin 2015).

To maintain the health of bee pollinators, May et al. (2015) suggested that labeling requirements should be changed to include sublethal and synergistic bee-toxic agrochemicals. In addition, ecotoxicological risk assessments should include adjuvants such as pesticides (Mesnage & Antoniou 2018) that require larval and chronic toxicity testing as part of this registration (May et al. 2015).

The results of the present study confirm the toxicity-increasing potential of adjuvants in combination with several different insecticide classes under laboratory conditions. We were able to show that B4-rated products, which are actually declared nonhazardous to bees, in the laboratory significantly shortened the lifespan of sprayed bees due to some of the adjuvants tested. Among these, organosilicon surfactants (OSS) showed particularly prevalent and particularly strong effects. In light of our and other work, the classification of adjuvants as "inactive" or "inert" should be reconsidered, since adverse effects on bees in practice cannot be ruled out at the current state of research. Because data are insufficient to realistically assess the risk of adjuvants to pollinators without over- or underestimating environmental effects at present, further work, especially under field conditions, is essential. Until then, it has been doubtful that current data requirements

in risk assessment and regulatory practices for adjuvants can prevent adverse effects on bees. Another important step is the declaration and labeling of ingredients and adjuvants, which is not required at the moment for adjuvant formulations, unlike PPPs. Addressing this problem and regulatory gaps is crucial for pollinator-friendly and more sustainable agriculture to maintain biodiversity and protect pollinators.

Declarations

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Authors' contributions:

Conceptualization: A.W., J.H.E., R.F., and N.K.; Data curation: A.W. and R.O.; Formal analysis: A.W. and R.O.; Funding acquisition: R.F.; Investigation: A.W. and J.H.E.; Methodology: A.W. and J.H.E.; Project administration: A.W.; Software: R.O.; Visualization: A.W. and R.O.; Writing – original draft: A.W. and R.O.; Writing - review & editing: J.H.E., R.F. and N.K.. All authors read and approved the final manuscript.

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Figures

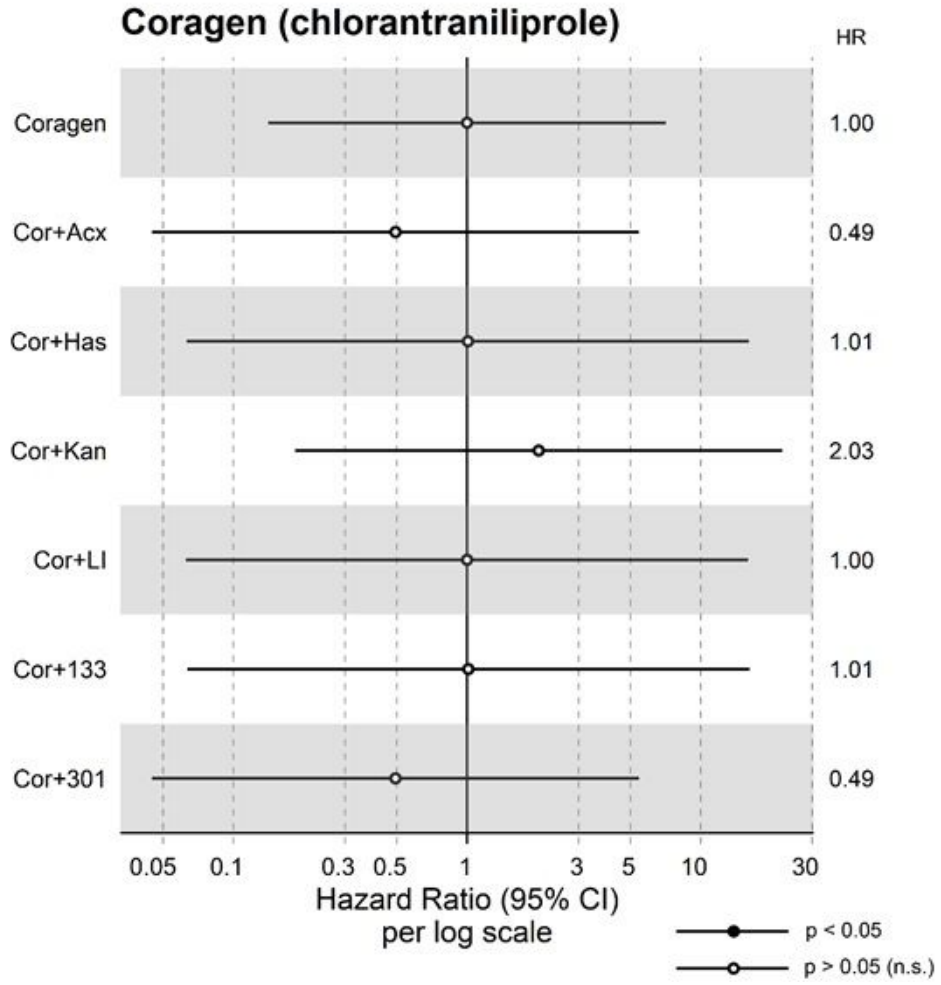

Figure 1

Hazard ratios (HRs) for the six spray adjuvants mixed with Coragen® relative to the single insecticide are shown for a 72-hour period. A total of 60 (n) bees per treatment were used. The dots represent the mean, and the horizontal lines represent the lower and upper limits of the 95% confidence interval (CI) of the estimates. Data to the left of the vertical dividing line (no-effect line) indicate a higher risk of mortality for the reference (insecticide only); data to the right of the vertical dividing line indicate a higher risk of mortality for the treatment (tank mix). If the confidence interval crosses the no-effect line, there is no (statistically) significant difference between the treatment (insecticide-adjuvant mixture) and reference (insecticide solo), represented by hollow dots. Overall, no increased risk of death was observed for any of the adjuvants in the mixture with Coragen® (Cox proportional hazard model, $p > 0.05$).

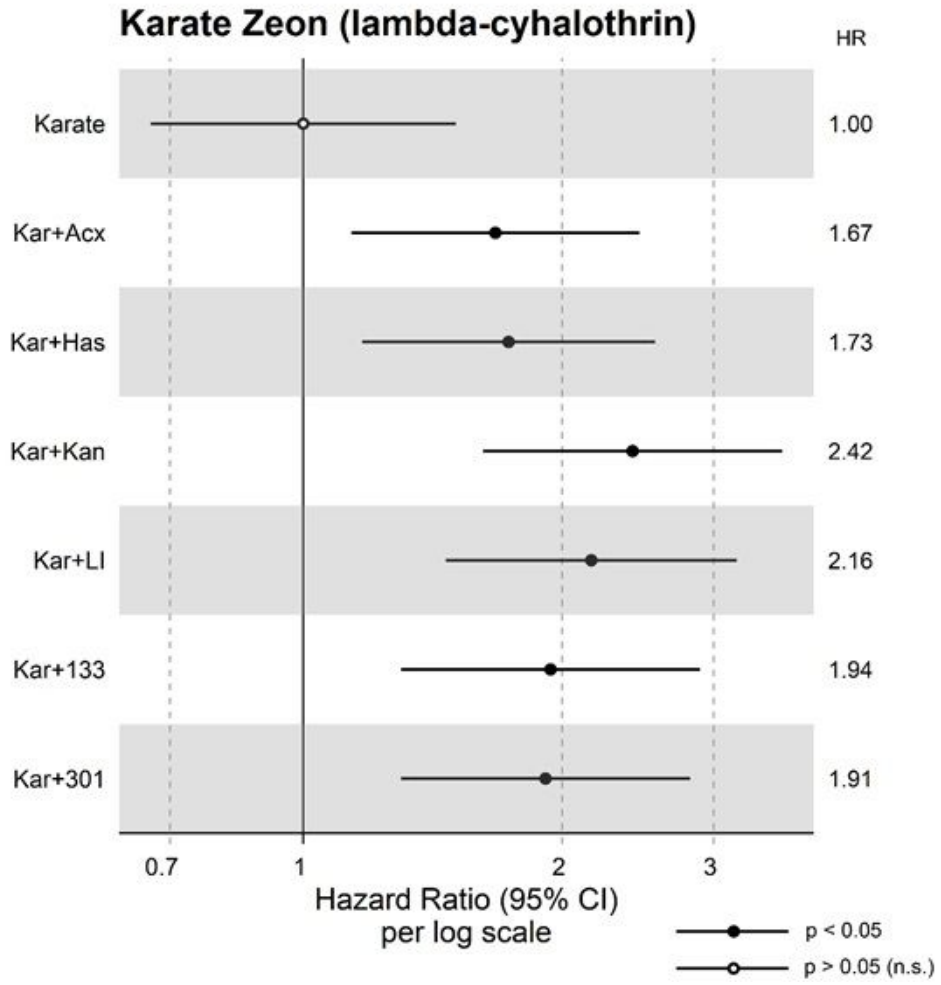

Figure 2

Hazard ratios (HRs) for the six spray adjuvants mixed with Karate® Zeon relative to the single insecticide are shown for a 72-hour period. A total of 60 (n) bees per treatment were used. The dots represent the mean, and the horizontal lines represent the lower and upper limits of the 95% confidence interval (CI) of the estimates. Data to the right of the vertical dividing line (no-effect line) indicate a higher risk of mortality for the treatment (tank mix). If the confidence interval does not cross the no-effect line, there is a (statistically) significant difference between the treatment (insecticide-adjuvant mixture) and reference (insecticide solo), represented by filled dots. Overall, an increased risk of death was observed for all adjuvants tested in mixture with Karate® Zeon (Cox proportional hazard model, $p < 0.05$).

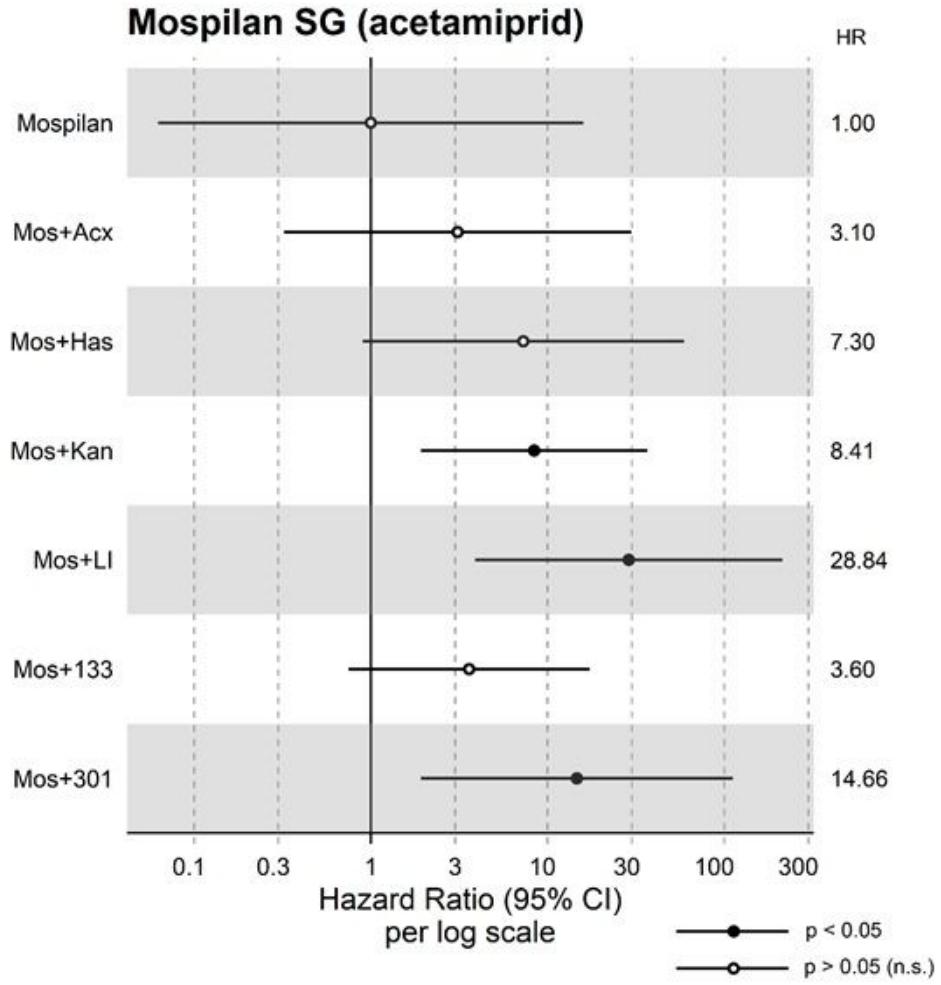

Figure 3

Hazard ratios (HRs) for the six spray adjuvants mixed with Mospilan® SG relative to the single insecticide are shown for a 72-hour period. A total of 60 (n) bees per treatment were used. The dots represent the mean, and the horizontal lines represent the lower and upper limits of the 95% confidence interval (CI) of the estimates. Data to the right of the vertical dividing line (no-effect line) indicate a higher risk of mortality for the treatment (tank mix). If the confidence interval crosses the no-effect line, there is no (statistically) significant difference between the treatment (insecticide-adjuvant mixture) and reference (insecticide solo), represented by hollow dots. In contrast, filled dots indicate a significant difference from the reference. Overall, an increased risk of death was observed for the adjuvants Kantor®, LI-700® and Break-Thru® S 301 in mixture with Mospilan® SG (Cox proportional hazard model, $p < 0.05$).

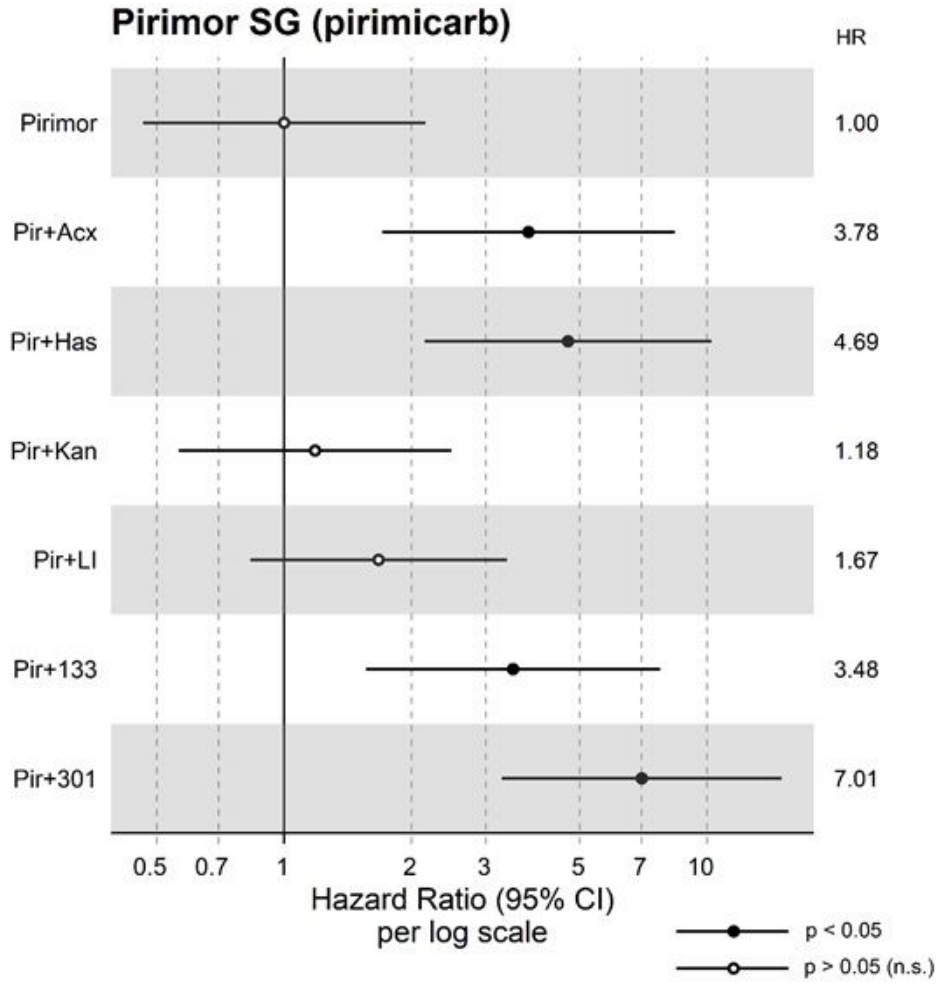

Figure 4

Hazard ratios (HRs) for the six spray adjuvants mixed with Pirimor® Granulat relative to the single insecticide are shown for a 72-hour period. A total of 60 (n) bees per treatment were used. The dots represent the mean, and the horizontal lines represent the lower and upper limits of the 95% confidence interval (CI) of the estimates. Data to the right of the vertical dividing line (no-effect line) indicate a higher risk of mortality for the treatment (tank mix). If the confidence interval crosses the no-effect line, there is no (statistically) significant difference between the treatment (insecticide-adjuvant mixture) and reference (insecticide solo), represented by hollow dots. In contrast, filled dots indicate a significant difference from the reference. Overall, an increased risk of death was observed for the adjuvants Acxcess®, Hasten® TM, Break-Thru® SP 133 and Break-Thru® S 301 mixed with Pirimor® Granulat (Cox proportional hazard model, $p < 0.05$).

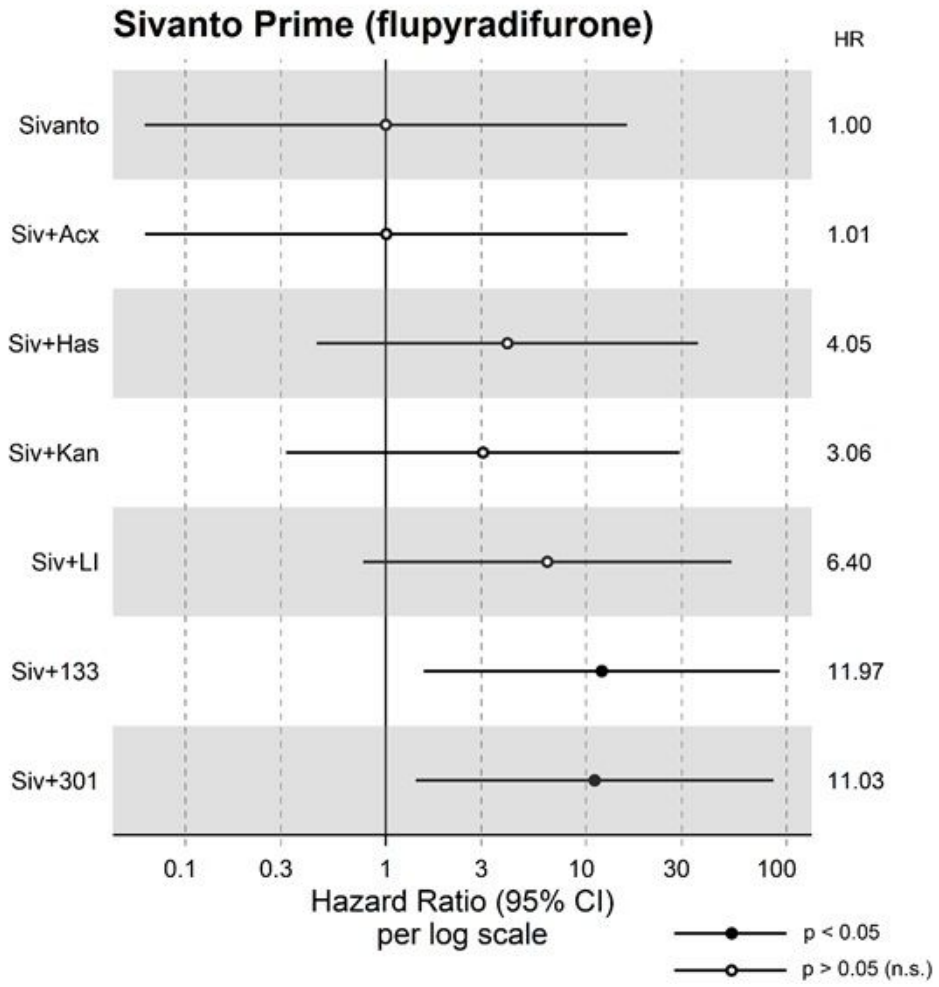

Figure 5

Hazard ratios (HRs) for the six spray adjuvants mixed with Sivanto® Prime relative to the single insecticide are shown for a 72-hour period. A total of 60 (n) bees per treatment were used. The dots represent the mean, and the horizontal lines represent the lower and upper limits of the 95% confidence interval (CI) of the estimates. Data to the right of the vertical dividing line (no-effect line) indicate a higher risk of mortality for the treatment (tank mix). If the confidence interval crosses the no-effect line, there is no (statistically) significant difference between the treatment (insecticide-adjuvant mixture) and reference (insecticide solo), which is represented by hollow dots. In contrast, filled dots indicate a significant difference from the reference. Overall, an increased risk of death was observed for the adjuvants Break-Thru® SP 133 and Break-Thru® S 301 in a mixture with Sivanto® Prime (Cox proportional hazard model, $p < 0.05$).

Supplementary Files

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- supplementalJPDAP.docx