**Supplementary information:**

**Co-formulant in a commercial fungicide product causes lethal and sub-lethal effects in bumble bees**

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**Supplementary Methods**

Supplementary Table 1. Number of datapoints by treatment for each analysis done.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Treatment Abbreviation | Mortality *n*= | Weight Change *n*= | Sucrose Consumption *n*= | Area of Melanisation *n*= |
| Negative control | 35 | 35 | 35 | 35 |
| Positive control | 34 | 34 | 0 | 0 |
| Alcohol ethoxylates | 30 | 30 | 21 | 29 |
| Naphthalenesulfonic acid | 33 | 33 | 32 | 33 |
| Benzisothiazol | 36 | 36 | 36 | 36 |
| Co-formulant mixture | 25 | 25 | 17 | 23 |
| Amistar® | 31 | 31 | 24 | 30 |

All treatment groups started with 35-37 workers, but because some bees did not consume the whole treatment droplet, and some bees died prior to exposure, sample sizes per treatment group changed

**Statistical analysis**

The negative control treatment was the reference used for comparison of the remaining treatments (Amistar®, co-formulant mixture and alcohol ethoxylates) for mortality testing. Because the negative control experienced no mortality, which causes a failure of the model to converge, we changed the mortality data for a single randomly selected negative control bee who survived the full 120 hours to a death at the halfway mark, 60 hours. This allowed for a meaningful comparison with the remaining treatments while being an even more conservative estimate of the effect size. This manipulation would only serve to reduce the probability of finding a significant result and is an accepted practice in mortality analysis.

Supplementary Table 2. Listed ingredients in Amistar®, taken from the material safety data sheet (Amistar Material Safety Data Sheet)

|  |  |
| --- | --- |
| Substance(s) | Concentration in Pure Formulation (%) |
| Azoxystrobin | 20-25% |
| C16-18 alcohols, Ethoxylated | 10-20% |
| Naphthalenesulfonic acid, dime- thyl-, polymer with formaldehyde and methylnaphthalenesulfonic acid, sodium salt acid | 1-10% |
| 1,2-benzisothiazol-3(2H)-one | 0.025-0.05% |

The MSDS for Amistar® includes the information provided in Supplementary Table 2. The upper end of the concentration ranges was used to inform the doses chosen. All doses are proportionate to their concentrations in Amistar® relative to a 200µg dose of the active ingredient azoxystrobin, which is equivalent to 0.8µL of Amistar® pure formulation.

Supplementary Table 3. Full details for formulation, active ingredients and co-formulants used in the experiment.

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| Brand Name | Azoxystrobin concentrationtion Pure (g/L) | MAPP | Syngenta ID | Cas No | Producer | Purchased From |
| Amistar® | 250 | 18039 | A12705B | NA | Syngenta, Cambridge UK | Agrigem.co.uk, Lincoln, UK |
| C16-18 alcohols, Ethoxylated | 0 | NA | NA | 68439-49-6 500-212-8 | Making Cosmetics | Amazon, London UK |
| Naphthalenesulfonic acid | 0 | NA | NA | 9084-06-4 | Sigma Aldrich, Gillingham  UK | Sigma Aldrich, Gillingham  UK |
| 1,2-benzisothiazol-3(2H)-one | 0 | NA | NA | 2634-33-5 220-120-9 613-088-00-6 | Sigma Aldrich, Gillingham  UK | Sigma Aldrich, Gillingham  UK |
| Dimethoate | 0 | NA | NA | 60-51-5 | Sigma Aldrich, Gillingham  UK | Sigma Aldrich, Gillingham  UK |

**A picture containing sitting, light, table, airplane

Description automatically generatedA picture containing indoor, sitting, table, large

Description automatically generatedSupplementary Results**

Supplementary Figure 1: (Left) Amistar® treatment solution. (Right) co-formulant mixture treatment solution.

The only listed ingredient missing from the co-formulant mixture that is present in the Amistar® is azoxystrobin, which when diluted is not beige or milky. This indicates that there are likely to be additional co-formulants not listed on the material safety data sheet, although it cannot be ruled out that manufacturing process explains the difference.

Graphical user interface

Description automatically generated with medium confidenceSupplementary Figure 2. Bumblebee foreguts of bees who survived the full 120 hours.

(Top Left) Amistar® treatment. A large area of the gut is visibly darkened. (Top Right) alcohol ethoxylates treatment. A large area of the gut is visibly darkened, and some brown spots are visible. (Bottom Right) Benzisothiazol treatment. No melanisation is visible. (Bottom left) Naphthalenesulfonic acid treatment. No melanisation is visible. The images in supplementary Figure 2 are sample pictures, and all images are available upon request from the authors.

Supplementary Tables 4. Mortality: The results of the model selection process for each analysis using the package ‘MuMIn’ (Bartoń 2020). Predictors, AIC, ∆AIC from the Best Model, AIC Weight and whether the model was included in the final parameter estimates are all presented.

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| Model Name | Predictors | AIC | ∆AIC from best model | AIC Weight | Included in Final Model Set |
| FM | Treatment, Bee Weight, (1|Colony of Origin) | 222.0 | 0.00 | 0.743 | Yes |
| M1 | Treatment, (1|Colony of Origin) | 224.2 | 2.21 | 0.246 | Yes |
| M0 | (1|Colony of Origin) | 230.5 | 8.51 | 0.011 | No |

The slightly lower mortality seen in the Amistar® treatment versus alcohol ethoxylates or the co-formulant mixture, is not statistically significant (Cox proportional hazards model: ﻿parameter estimate PE = 0.24, 95% CI [-0.75 to 1.22] and (PE) = 0.57, 95% CI [-0.47 to 1.60], respectively).

There was no effect of bee weight at the beginning of the experiment on mortality (Cox proportional hazards model: ﻿parameter estimate (PE) = -9.529, 95% CI [-18.23 to 3.92]).

Supplementary Tables 5. Sucrose Consumption: The results of the model selection process for each analysis using the package ‘MuMIn’ (Bartoń 2020). Predictors, AIC, ∆AIC from the Best Model, AIC Weight and whether the model was included in the final parameter estimates are all presented.

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| Model Name | Predictors | AIC | ∆AIC from best model | AIC Weight | Included in Final Model Set |
| FM | Treatment, Bee Weight, (1|Colony of Origin) | 176.0 | 0.00 | 1.000 | Yes |
| M1 | Treatment, (1|Colony of Origin) | 205.7 | 29.79 | 0.000 | No |
| M0 | (1|Colony of Origin) | 305.0 | 129.09 | 0.000 | No |

Neither benzisothiazol nor naphthalenesulfonic acid had significantly different consumption versus the control (Linear Mixed Effect model: ﻿parameter estimate (PE) = -0.05, 95% CI [-0.22 to 0.12] and PE = -0.15, 95% CI [-0.33 to 0.86], respectively), with an average sucrose consumption of 1.905g and 1.823g of sucrose respectively, compared to the 1.973g in the negative control (see Main Text Figure 2).

The difference in sucrose consumption between the Amistar® treatment and the co-formulant mixture and alcohol ethoxylates treatments is not statistically significant (Linear Mixed Effect model: ﻿parameter estimate (PE) = -0.09, 95% CI [-0.33 to 0.14] and PE = -0.21, 95% CI [-0.42 to 0.14], respectively).

There was a significant effect of bee weight on sucrose consumption (Linear Mixed Effect model: ﻿parameter estimate (PE) = 3.63, 95% CI [2.41 to 8.81]), with heavier bees drinking more*.*

Supplementary Tables 6. Weight Change: The results of the model selection process for each analysis using the package ‘MuMIn’ (Bartoń 2020). Predictors, AIC, ∆AIC from the Best Model, AIC Weight and whether the model was included in the final parameter estimates are all presented.

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| Model Name | Predictors | AIC | ∆AIC from best model | AIC Weight | Included in Final Model Set |
| FM | Treatment, Bee Weight, Colony of Origin | -762.9 | 0.00 | 0.719 | Yes |
| M1 | Treatment, Colony of Origin | -760.2 | 2.65 | 0.191 | Yes |
| M2 | Treatment, Bee Weight | -758.5 | 4.37 | 0.081 | No |
| M3 | Treatment, | -754.2 | 8.70 | 0.009 | No |
| M0 | None | -741.4 | 21.47 | 0.000 | No |

Neither benzisothazol nor naphthalenesulfonic acid had significantly different weight change versus the negative control (Linear Mixed Effect model: ﻿parameter estimate (PE) = -0.01, 95% CI [-0.02 to 0.01] and PE = -0.00, 95% CI [-0.02 to 0.02], respectively), with an average weight gain of 0.005g and 0.010g respectively, compared to the 0.010g gain in the negative control (see main text Figure 3).

The difference in weight change between the Amistar® treatment and the co-formulant mixture and alcohol ethoxylates treatments is not statistically significant (Linear Mixed Effect model: ﻿parameter estimate (PE) = -0.00, 95% CI [-0.02 to 0.01] and PE = -0.01, 95% CI [-0.03 to 0.01], respectively).

There was no significant effect of colony of origin on gut melanisation (Linear Mixed Effect model: ﻿parameter estimate (PE) = 0.00, 95% CI [-0.01 to 0.01] and PE = -0.01, 95% CI [-0.02 to 0.01], for either colony compared to an arbitrarily chosen reference colony).

**Search for prior research on the effects of alcohol ethoxylates**

A Web of Science Core Collection Abstract, Title and Topic search using the terms (\*Bee AND ("C16-C18 alcohols, ethoxylated" OR " C16-C18 alcohols " OR "ethoxylated alcohol\*" OR "alcohol ethoxylate\*" OR "alkoxylated alcohols")) yielded no results (Search February 2021). The abstract search used “bee” as left-hand truncation is not supported. European legislation does not require any form of regulatory testing of co-formulants individually on bees (EC, 2009).

Supplementary Tables 7. Gut melanisation: The results of the model selection process for each analysis using the package ‘MuMIn’ (Bartoń 2020). Predictors, AIC, ∆AIC from the Best Model, AIC Weight and whether the model was included in the final parameter estimates are all presented.

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| Model Name | Predictors | AIC | ∆AIC from best model | AIC Weight | Included in Final Model Set |
| FM | Treatment, Bee Weight, Colony of Origin | 505.1 | 0.00 | 0.779 | Yes |
| M1 | Treatment, Colony of Origin | 509.1 | 3.94 | 0.108 | Yes |
| M2 | Treatment, Bee Weight | 509.3 | 4.14 | 0.099 | No |
| M3 | Treatment, | 513.2 | 8.06 | 0.014 | No |
| M0 | None | 536.2 | 31.11 | 0.000 | No |

Neither benzisothiazol nor naphthalenesulfonic acid had significantly different melanised area versus the negative control (Linear Mixed Effect model: ﻿parameter estimate (PE) = -0.01, 95% CI [-0.44 to 0.42] and PE = -0.12, 95% CI [-0.56 to 0.32], respectively), with an average melanised area of 0.240mm2 and 0.116mm2 respectively, compared to the 0.230mm2 in the negative control (see main text Figure 4).

The difference in melanised area between the Amistar® treatment and the co-formulant mixture and alcohol ethoxylates treatments is not statistically significant (Linear Mixed Effect model: ﻿parameter estimate (PE) = 0.50, 95% CI [-0.22 to 1.21] and PE = -0.04, 95% CI [-0.71 to 0.64], respectively).

There was no significant effect of bee weight on gut melanisation (Linear Mixed Effect model: ﻿parameter estimate (PE) = -3.33, 95% CI [-6.73 to 0.31]). There was no significant effect of colony of origin on gut melanisation (Linear Mixed Effect model: ﻿parameter estimate (PE) = -0.04, 95% CI [-0.38 to 0.29] and PE = -0.12, 95% CI [-0.45 to 0.24], for either colony compared to an arbitrarily chosen reference colony).

**Supplementary Discussion**

**Differences in results between similar treatment groups**

Our results show a slightly, but not significantly, higher level of mortality in the alcohol ethoxylates treatment (30%) than the Amistar® treatment (23%). If this is a real biological difference, one explanation might be that the concentration of alcohol ethoxylates in the Amistar® formulation was lower than that used in the alcohol ethoxylates treatment solution. This is possible because the Amistar® material safety data sheet lists concentrations as a range (10-20% for alcohol ethoxylates), and here we used the upper end of the range. The co-formulant mixture treatment in all metrics was statistically indistinguishable from the C16-C18 treatment, showing that the toxicity of alcohol ethoxylates is not a result of synergism with other co-formulants.

**Regulation of novel formulations**

The rules surrounding the need to test individual pesticide formulations on bees are open to interpretation. At an EU level only the active ingredient and a representative formulation need to be tested on bees, however at the national level each individual formulation needs to be separately registered. European Commission Regulation 284/2013 states “Testing of the plant protection product shall be necessary where its toxicity cannot be predicted on the basis of data on the active substance.” (EC, 2013).

The UK competent authority which handles pesticide regulation, the Chemical Regulation Division, provides guidance on the interpretation of this, and what formulations would require additional testing data. A formulation can be altered from its tested form only to a degree before either new testing is required, or a justification for why new testing is not required. Novel formulations which contain high levels of surfactants, solvents and emulsifiers are more likely to trigger the requirement for additional testing. Formulations with active ingredients of low toxicity to bees are less likely to require testing on bees.

For active ingredients which pass lower tier testing the risk assessment indicates that they pose an ‘acceptable’ risk. This is explicitly considered in the decision on whether to test a novel formulation, meaning for active ingredients other than insecticides it is much easier for a novel formulation to be registered. This is because there is lower risk of synergism with the active ingredient, as it is likely of low toxicity to bees. However, this creates a problem in that formulations with active ingredients thought to be of low toxicity to bees, which can thus be applied with no exposure mitigation, are significantly less likely to be submitted to bee toxicity testing. This means the substances bees are most exposed to have the most poorly characterised risk.