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

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**Abstract:**

1. Pollinators, particularly wild bees, are suffering declines across the globe, and pesticides are thought to be drivers of these declines. Research into, and regulation of pesticides has focused on the active ingredients, and their impact on bee health. In contrast, the additional components in pesticide formulations have largely been overlooked as potential threats.
2. By testing an acute oral dose of the fungicide product Amistar®, and equivalent doses of each individual listed co-formulant, we were able to measure the toxicity of the formulation and identify the ingredient responsible.
3. We found that a co-formulant, alcohol ethoxylates, caused a range of damage to bumble bee health. Exposure to the co-formulant alcohol ethoxylates caused 30% mortality and a range of sublethal effects which left persistent damage to those bees who survived. Alcohol ethoxylates treated bees consumed half as much sucrose as negative control bees over the course of the experiment and lost weight over the 120 hours of the experiment. Alcohol ethoxylates treated bees had significant melanisation of their midguts, providing evidence of gut damage. We suggest that this damage impairs gut function and is associated with the reduction in appetite and weight loss.
4. Further we suggest that this gut damage explains the mortality, with bees dying from energy depletion. Across all impacts we recorded, the effect of the formulation was the same as that of the co-formulant alcohol ethoxylates alone, demonstrating that the toxicity is entirely driven by this co-formulant.
5. *Synthesis and applications.* The regulatory testing protocol used here, in its unmodified form that focuses on mortality alone, will lead to the renewal of the ‘low toxicity to bees’ status of this pesticide formulation. Consequently, our results demonstrate the regulatory system’s over-reliance on mortality as a metric, and its failure to consider both sublethal effects and the effects of co-formulants on bee health.

**Introduction**

Pollination by bees is an essential ecosystem service (Potts et al., 2016). However, wild bees are undergoing declines across the globe, with 37% of analysed European bee species (those with sufficient data) suffering population declines (Nieto et al., 2014). These declines have been linked, in part, to pesticides (Rundlöf et al., 2015, Woodcock et al., 2016, McArt et al., 2017). Pesticides are applied to crops in formulations, which are mixtures of the active ingredient and co-formulants, with the latter being added to aid the efficiency of the active ingredient (Hazen, 2000). The majority of research and regulatory focus is on the active ingredient, not the formulation as a whole or the co-formulants (Mullin 2015, Mullin et al., 2015, Mesnage & Antoniou, 2018). However, the toxicological effects of co-formulants have been consistently underestimated for bees and other non-target organisms, including humans (Cox & Surgan, 2006, Mullin 2015, Mullin et al., 2015, Mesnage & Antoniou, 2018). In agricultural environments, bees are exposed to co-formulants through pesticide formulations, and this exposure is likely to be highest for pesticide classes, like fungicides, which are considered to be bee-safe.

Fungicides are very widely used plant protection products (PPP’s), with almost half a million metric tonnes applied globally in 2014 (FAOSTAT 2020), and azoxystrobin is one of the most commonly used fungicide active ingredients. While systematic global data are lacking, in 1999 azoxystrobin products were the biggest selling fungicides globally with $415 million of sales (Bartlett et al., 2002). Azoxystrobin was developed by Syngenta (European Patent EP2004194B1), and was the first fungicide of the strobilurin group brought to market (Fernández-Ortuño et al., 2010). Azoxystrobin’s mode of action is to inhibit fungal mitochondrial respiration as a Quinone Outside Inhibitor (Fernández-Ortuño et al., 2010). Syngenta’s flagship formulation was Amistar®, although it has now moved out of patent and 66 different azoxystrobin products are available in the UK alone (Health and Safety Executive UK, 2020). Amistar® is the representative formulation for azoxystrobin in the EU (EFSA, 2010). EU regulators classed azoxystrobin and Amistar® as of ‘low toxicity to bees’ based on lower tier testing which exclusively uses mortality as a measurement of toxicity. Because of the ‘low toxicity to bees’ categorisation, no mitigation measures are required to reduce exposure of bees, and Amistar® can be applied to bee-attractive flowering crops like strawberries while bees are actively foraging on them (Amistar Label). As such, exposure of bees to Amistar® is very high, with residue monitoring studies consistently finding high levels of azoxystrobin in bee matrices (Mullin et al., 2010, Rennich et al., 2014). While these studies only measure the residue levels of the active ingredient, and not the residue levels of the co-formulants, it is likely they would be proportionate, meaning that exposure of bees to Amistar® co-formulants will be commensurately high.

There is a range of co-formulant types, including surfactants that reduce surface tension and help the active ingredient penetrate the leaf, solvents that help dissolve the active ingredient in the solution, and emulsifiers that keep the formulation consistent and uniformly mixed (Mesnage & Antoniou, 2018). Individual co-formulants are not submitted to the same suite of regulatory testing as active ingredients are, and they are only tested on bees as part of formulations (EC, 2013). We have a very poor understanding of the exposure of bees to co-formulants, with only three studies measuring residues in pollen, nectar or wax (Chen and Mullin, 2013, Chen and Mullin, 2014, Fine et al., 2017), finding residues as high as 1,051 ± 2,897ppb in wax for the surfactant co-formulant nonylphenol ethoxylates. There are relatively few studies that explicitly test the impacts of co-formulants on bees. The solvents N-methyl-2-pyrrolidone and dimethyl sulfoxide have been tested on honeybees ((Zhu et al., 2014, Fine et al., 2017, Fine and Mullin, 2017, Chen et al., 2019) and (Moffett and Morton, 1973, Milchreit et al., 2016) respectively), with mortality being seen across a range of doses.

Amistar® has three listed co-formulants (Amistar Material Safety Data Sheet), and of these alcohol ethoxylates, ethoxylated (CAS-no 68439-49-6), which are part of the chemical group alcohol ethoxylates and constitute 10-20% of the formulation, are of most interest. Alcohol ethoxylates are used as surfactants and emulsifiers, serving both to help the active ingredient azoxystrobin penetrate into crops and to stabilise the product (Li et al., 2018). Alcohol ethoxylates are currently being used to replace ﻿alkylphenol ethoxylates as surfactant co-formulants because they can synergise with the active ingredient better (Li et al., 2018).

Some kinds of alcohol ethoxylate have been found to synergistically increase mortality when co-applied with a range of insecticide active ingredients in both aphids (﻿*Aphis citricola*) and cockroaches (*Blattella germanica*)((Li et al., 2018a and b) and (Sims and Appel 2007) respectively). This demonstrates that co-formulants are not toxicologically benign and can meaningfully impact a formulation’s toxicology.

We aimed to test the toxicity of the representative formulation for azoxystrobin, Amistar®, and its individual co-formulants to bumble bees. Our design was specifically tailored to identify any potential toxicity of co-formulants or co-formulant mixtures. We used regulatorily sanctioned methods but expanded the range of metrics taken to include sublethal effects (OECD, 2017). This enabled us to assess whether the limited level of regulatory testing (EFSA, 2010), and its focus on mortality, accurately captures the toxicity of a substance, including any sublethal damage it can cause. It has been proposed that using mortality alone is inappropriate, and that a more fitness-based approach should be adopted (Straub, Strobl and Neumann, 2020). To our knowledge, this is the first study to test each listed co-formulant in a formulation on bees and the first study ever to explicitly test a co-formulant on bumble bees. Based on preliminary work we predicted that the Amistar® treatment would cause significant mortality and hypothesised that the mechanism for this was damage to the gut tissue causing death by energy depletion. We hypothesised that bees would compensate for reduced gut function by over-consuming sucrose and that the reduced gut function would cause weight loss.

**Methods**

We used Amistar, a broad-spectrum fungicide, purchased online through Agrigem Ltd ([www.agrigem.co.uk](http://www.agrigem.co.uk)), in September 2019. The formulation identifiers are UK MAPP: 18039, Syngenta ID: A12705B. This is the same formulation used in the EFSA bee risk assessment which used Amistar® as a representative formulation for all formulations containing the active ingredient azoxystrobin (EFSA, 2010).

Some of the co-formulants in the formulation are listed in the material safety data sheet that accompanies the bottle, these are listed in Table 1, and in more detail in Supplementary Table 3. This list may not be comprehensive as European Law (EC, 2009) explicitly protects whole formulation composition as proprietary knowledge, so we do not know what other ingredients are present. Only co-formulants with specific toxicity classifications need to be listed, which means an unknown number of other co-formulants could be present (EC, 2009). From the difference between the appearance of the co-formulant mixture and Amistar® it is likely that there are other ingredients not listed, see Supplementary Figure 1. Full details on the formulation and co-formulations used, including source and chemical identifiers can be found in the Supplementary Methods. Azoxystrobin is poorly soluble in all the solvents we trialled, and therefore azoxystrobin was not included as an individual treatment, nor in the co-formulant mixture.

Table 1. Doses of chemicals used in each treatment group. Each co-formulant dose is proportionate to its concentration in the formulation. Calculations are based off of 0.8µL of Amistar® which is equivalent to a 200µg dose of the active ingredient, azoxystrobin.

|  |  |  |
| --- | --- | --- |
| Treatment Name | Substance(s) | Dose (µg) |
| Negative control | Water | 0.0 |
| Positive control | Dimethoate | 0.4 |
| Alcohol ethoxylates | C16-18 alcohols, ethoxylated | 160 |
| Naphthalenesulfonic acid | Naphthalenesulfonic acid, dime- thyl-, polymer with formaldehyde and methylnaphthalenesulfonic acid, sodium salt acid | 80 |
| Benzisothiazol | 1,2-benzisothiazol-3(2H)-one | 0.4 |
| Co-Formulant mixture | Alcohol ethoxylates, naphthalenesulfonic acid and benzisothiazol | 240.4 (sum of all co-formulants) |
| Amistar® | Amistar® | *na* |

Three commercial colonies of the bumble bee *Bombus terrestris audax* were used in the experiments (Agralan, Wiltshire, UK). On arrival, 10 workers per colony were removed and their faeces screened for micro-parasites (Rutrecht & Brown, 2009). No infections were detected, and all colonies were thus retained in the experiment.

We used a modified version of an internationally accepted protocol (OECD 247), used by industry and regulators, where deviations from OECD 247 served to increase the richness of data captured.

We housed worker bees in Nicot cages a day in advance of their chemical exposure, and then rank allocated them to treatments based on weight, with an even distribution of source colonies by treatment. Bees outside the range of 0.1g-0.4g were not used. Numbers exposed can be seen in Supplementary Table 1. Prior to, and after exposure, bees had access to *ad libitum* 45% w/w sucrose solution (Thorne, Windsor, UK).

We exposed bumble bees to the treatments and doses detailed in Table 1. The amount of each co-formulant is equivalent to the amount of that co-formulant in a 0.8µL dose of Amistar®, which contains 200µg of azoxystrobin. In the EFSA honeybee assessment of azoxystrobin, the same dose of 200µg was used (EFSA, 2010). The co-formulants listed in the Amistar® material safety data sheet are given as ranges not exact values, so the upper end of the range was used for a conservative risk estimate.

Chemical solutions were made fresh on the day of exposure to ensure no degradation occurred before exposure. Amistar®, the co-formulants and the positive control (dimethoate) were diluted in distilled water. The use of dimethoate as a positive control is standard, and reliably achieves >90% mortality. The negative control solution was distilled water. The chemical solutions, and the negative control, were mixed 50:50 with 33% w/w sucrose to incentivise the bees to drink them. The doses contained within the solutions given to bees are reported in Table 1.

Bees were starved for four hours prior to exposure, and exposed through an 80µL droplet pipetted into a BD Plastics 5mL syringe with the tip cut off. This differs from the standard 40µL used in OECD 247 to allow for low solubility substances to be tested. The syringe was checked visually to ensure the bees consumed the whole droplet. Mortality was recorded four hours after exposure began, and every 12 hours for 120 hours. Syringes were weighed every 12 hours to track sucrose consumption. Bees were monitored for longer than the 96 hours recommended in OECD 247 to ensure all mortality was captured. Any bees who died or who survived the full 120 hours, were weighed then transferred to a 2mL Eppendorf tube and frozen at -80C°.

Bees were removed from the freezer in batches of 8, placed on ice and slowly allowed to defrost before dissection. The abdomen was cut off and was pinned to a black wax plate. The abdomen was cut on one side, and pinned open. 100µL of 0.8% Ringers solution was pipetted directly onto the gut and another 100µL onto the wax to the side of the body to prevent desiccation. The honey crop was cut, and the gut transferred to the droplet on the wax. A GXCAM-5 (GT Vision, Suffolk, UK) dissecting scope camera was used to take two images of the midgut at 10x magnification using supplementary light.

As a proxy for the level of damage to the gut we used the presence of melanisation (dark brown patches and striations) on the midgut, which are not seen in healthy bees. Images of the bee guts were imported into Fiji (Schindelin et al., 2012), converted to 8-bit and then made binary (black or white). The look up table was inverted to highlight any darker areas of the gut. These darker areas were then selected and the analyse particles tool was used to measure their area. This process was repeated twice for each photo, with two photos per gut, and the mean result for each bee was used in analyses. Using a binary colour map caused some areas on the guts of healthy guts to be highlighted, which explains the background noise in all treatments. The scale was set using a photograph of digital callipers at a known value. This allowed the area of gut melanisation to be calculated in mm2. This value does not represent the total area of melanisation on the gut, only that visible in the picture of the midgut, which was only one side of the gut. The gut was photographed on the side of the gut facing upwards after dissection, with no efforts made to arrange the gut to highlight damage.

**Statistical analysis**

Statistical analyses were carried out in ‘R’ programming software version 3.6.2 (R Core Team 2019). All plots were made using ‘ggplot2’ version 3.2.1 (Wickham, 2016) and ‘survminer’ version 0.4.6 (Kassambara, Kosinski & Biecek, 2019). AIC model simplification was used, with conditional model averaging where no single model had >95% AIC support. The candidate set of models was chosen by adding the next best supported model until a cumulative >95% AIC support was reached. ‘MuMIn’ version 1.43.17 was used for model averaging (Bartoń 2020). Parameter estimates and 95% confidence intervals are reported. Confidence intervals not crossing zero indicate a significant effect. Model parameters, AIC weights and final models are presented in Supplementary Tables 4-7. Where a bee lacked a response variable result due to mortality or experimental error it was excluded from that particular analysis, see Supplementary Tables 1 for full numbers by treatment for each analysis. The positive control was excluded from all analyses because the complete mortality at four hours after treatment meant that their other metrics were not meaningful data. Test results for benzisothiazol and naphthalenesulfonic acid are listed, but full test results are only presented in the Supplementary Results. To compare Amistar® against the treatment groups alcohol ethoxylates and co-formulant mixture, the same statistical test was repeated with the data subset of just these treatments and Amistar® as the reference treatment. Results for this are available in the Supplementary Results. Results on the effects of bee weight and colony of origin on the dependent variables are only reported in the main text if significant and are otherwise in the Supplementary Results. Mixed effects Cox proportional hazards models were used to analyse mortality, utilising ‘survival’ version 3.1-8 (Therneau, 2020a) and ‘coxme’ version 2.2-16 (Therneau, 2020b). The full model for mortality used was (Mortality ~ Treatment + Bee Weight+ (1|Colony of Origin)). Due to zero, or just one instance of mortality, the benzisothiazol and naphthalenesulfonic acid treatment were excluded from mortality analysis. A death at the halfway mark was artificially added to the negative control treatment to allow for meaningful comparison to treatments with mortality. Proportionality of hazards was checked graphically to validate the Cox proportional hazards assumption. Linear mixed effect models were used to analyse sucrose consumption of bees who survived the full 120 hours, utilising ‘lme4’ v1.1-23 (Bates et al.,2015). The full model used was (Sucrose consumption ~ Treatment + Bee Weight+ (1|Colony of Origin)). Generalised linear models were used to analyse gut melanisation and weight changeas linear mixed effect models caused fitting issues. The full model used was (Melanisation ~ Treatment + Bee Weight+ Colony of Origin).

**Results**

**Mortality:**

All bees in the positive control treatment died within four hours, no bees in either the negative control or the benzisothiazol treatment died over the period of 120 hours, and only one bee died in the naphthalenesulfonic acid treatment.

There was significantly higher mortality in all treatments containing the co-formulants alcohol ethoxylates, compared to the negative control. Amistar®, co-formulant mixture and alcohol ethoxylates all had significantly higher mortality than the negative control (Cox proportional hazards mixed effects model: ﻿parameter estimate (PE) = 2.16, 95% CI [0.06 to 4.26], (PE) = 2.75, 95% CI [0.66 to 4.84], and PE = 2.40, 95% CI [0.33 to 4.47], respectively). Amistar®, co-formulant mixture and alcohol ethoxylates had 23%, 32% and 30% mortality respectively, while the control (without the artificially added death) experienced 0% mortality (see Figure 1).



Figure 1. A Kaplan-Meier plot showing survival against time; colour coded by treatment. The negative control and benzisothiazol line is split to allow both to be visible, as both treatments had 0% mortality.

**Sucrose consumption:**

Among the bees who survived the full 120 hours there was significantly lower sucrose consumption in all treatments containing the co-formulants alcohol ethoxylates, relative to the control.

Amistar®, co-formulant mixture and alcohol ethoxylates all had significantly lower consumption than the control (Linear Mixed Effect model: ﻿parameter estimate (PE) = -0.88, 95% CI [-1.06 to -0.69], (PE) = -0.98, 95% CI [-1.19 to -0.76], and PE = -1.06, 95% CI [-1.26 to -0.86], respectively). Amistar®, co-formulant mixture and alcohol ethoxylates treated bees consumed an average of 1.086g, 1.081g and 0.910g of sucrose respectively, compared to the 1.973g in the negative control (see Figure 2). The difference in sucrose consumption between the Amistar® treatment and the co-formulant mixture and alcohol ethoxylates treatments is not statistically significant. No other co-formulant had significantly different consumption versus the negative control, and heavier bees drank significantly more sucrose than lighter bees (see Supplementary Results).

Chart

Description automatically generatedFigure 2. A time series plot showing sucrose consumption over a 120-hour period; colour coded by treatment. 95% confidence intervals are shown. Sucrose consumption data collected every 12 hours has been LOESS smoothed. Y axis scale refers to average sucrose consumption over a 12-hour period.

**Weight Change:**

There was significantly more weight loss in all treatments containing the co-formulants alcohol ethoxylates relative to the negative control.

Amistar®, co-formulant mixture and alcohol ethoxylates all had a significantly different weight change compared to the negative control (Linear Mixed Effect model: ﻿parameter estimate (PE) = -0.02, 95% CI [-0.03 to -0.00], (PE) = -0.03, 95% CI [-0.04 to -0.01], and PE = -0.03, 95% CI [-0.05 to -0.02], respectively). Amistar®, co-formulant mixture and alcohol ethoxylates treated bees lost weight over the 120 hours, with average losses of 0.010g, 0.017g and 0.022g respectively, in contrast to the negative control where bees gained an average of 0.010g in the same period (see Figure 3). The difference in weight change between the Amistar® treatment and the co-formulant mixture and alcohol ethoxylates treatments is not statistically significant. No other co-formulant had a significantly different weight change versus the negative control. There was a significant, but very weak, effect of initial bee weight on weight change (Linear Mixed Effect model: ﻿parameter estimate (PE) = -0.12, 95% CI [-0.24 to -0.02]), with higher initial weight correlating with increased weight loss.

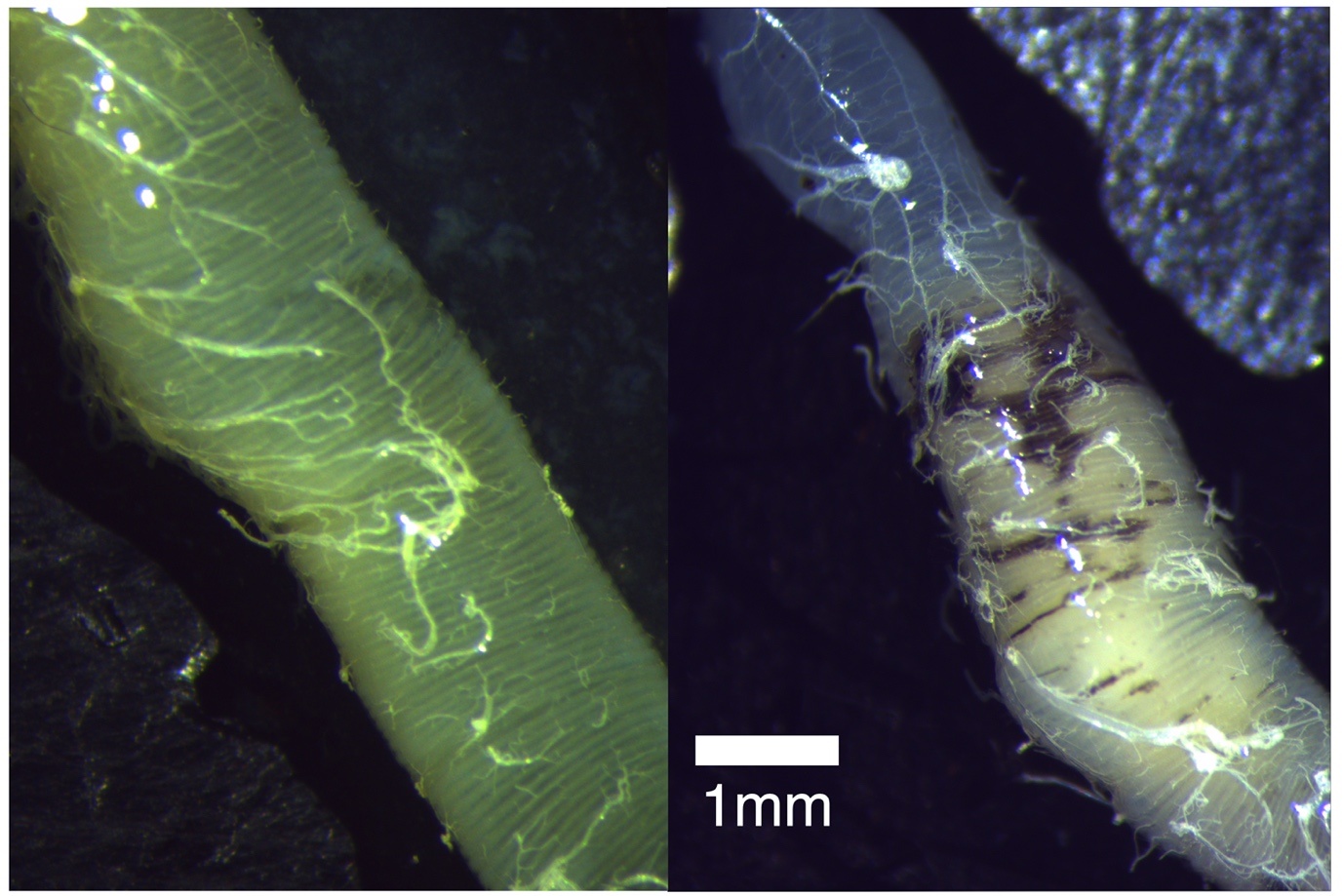
Figure 3. A boxplot showing change in weight over the 120-hour period, or until death colour coded by treatment. Boxes represent the Inter-Quartile Range (IQR), with the bold horizontal line the median value. The whiskers represent the furthest datapoint within 1.5 times the IQR and points beyond this are plotted as outliers.

**Area of Gut Melanisation:**

There was significantly more melanisation in all treatments containing the co-formulants alcohol ethoxylates, relative to the negative control.

Amistar®, co-formulant mixture and alcohol ethoxylates all had significantly more melanisation than the negative control (Linear Mixed Effect model: ﻿parameter estimate (PE) = 0.67, 95% CI [0.22 to 1.13], (PE) = 1.13, 95% CI [0.64 to 1.62], and PE = 0.63, 95% CI [0.16 to 1.07], respectively). Amistar®, co-formulant mixture and alcohol ethoxylates treated bees had an average melanised area of 0.925mm2, 1.350mm2 and 0.850mm2 respectively, compared to the 0.230mm2 in the negative control (see Figure 4). The difference in melanised area between the Amistar® treatment and the co-formulant mixture and alcohol ethoxylates treatments is not statistically significant. No other co-formulant had a significantly different area of melanisation versus the negative control.

Figure 4. A boxplot showing area of gut melanisation, colour coded by treatment. Boxes represent the IQR, with the bold horizontal line the median value. The whiskers represent the furthest datapoint within 1.5 times the IQR and points beyond this are plotted as outliers.

Figure 5. (Left) Bumble bee midgut in the negative control treatment. (Right) Bumble bee midgut after exposure to alcohol ethoxylates in the co-formulant mixture treatment. The dark brown patches are areas of melanisation, indicative of damage to the gut. Both bees survived the full 120 hours.

**Discussion**

Here we show, for the first time, that the toxicity of a pesticide formulation to bees is caused exclusively by a co-formulant (alcohol ethoxylates), rather than the active ingredient. A 0.8µL acute oral dose of the agricultural fungicide formulation Amistar® caused a range of damage to bees: both lethal, with 23% mortality, and sublethal, with 45% reduced sucrose consumption, 3.8% drop in body weight (whereas the negative control gained 4.8%), and a 302% increase in gut melanisation. For all metrics, the Amistar® and alcohol ethoxylates treatments were not statistically different, demonstrating conclusively that the toxicity of the formulation, Amistar®, is driven exclusively by the alcohol ethoxylates. These results demonstrate gaps in the regulatory system and highlight the need for a greater research focus on co-formulants.

While the mortality in the Amistar® and alcohol ethoxylates treatments was below the level required to trigger higher tier testing in the EU regulatory system, 23% mortality is substantial given that bees are likely to have a high level of exposure to Amistar® and its alcohol ethoxylates. The mechanism by which the alcohol ethoxylates cause mortality has not been explicitly isolated, but our results suggest two potential, possibly related, causes. We recorded a 302% increase in the melanised area of bee midguts in the alcohol ethoxylates treatment. We suggest that the alcohol ethoxylates are disrupting the structure of the midgut, which the bee immune system is reacting to with melanisation (Söderhäll and Cernius, 1998)(see Figure 5). In parallel with this gut damage, alcohol ethoxylate treatment drove a 54% reduction in sugar consumption, which persisted throughout the experiment. Consequently, we propose that mortality was driven by energy depletion due to reduced consumption, which in turn may have been driven by damage to the gut.

Likely as a consequence of the reduced consumption of sucrose, bumble bees in the alcohol ethoxylates treatments lost 8.4% of their original weight, in stark contrast to the negative control where bees gained 4.8% over the five-day period. The weight loss, and lack of weight gain, are concerning because they are likely to indicate a reduction in fat reserves, although this has not been experimentally confirmed. Bee fat reserves are important physiologically, in particular in responding to immune threats (Vilmos & Kurucz, 1998, Danihlík et al., 2018). Fat reserves allow bees the energetic resources to buffer against challenges, and thus their depletion could expose bees to greater risk from future threats (Sgolastra et al., 2011).

The reduced appetite and negative energy balance in alcohol ethoxylates treated bees could have broader effects in the natural environment. Bees pollinate flowers as they forage for nectar and pollen, so a reduction in their appetite could have knock on effects for ecosystem services . In our experiment, bumblebee appetite was reduced immediately after ingesting a single dose of alcohol ethoxylates or Amistar®. This effect persisted for five days after exposure, indicating a permanent change in consumption behaviour. While nectar-foraging in bumblebees is driven by the needs of the colony (Hendriksma, Toth and Shafir, 2019), a reduction in appetite would reduce overall colony nectar consumption, and thus the number of foraging trips made for nectar. Fewer visits to flowers for nectar may lead to reduced pollination, which would be detrimental to crop yields and farm profits. Further studies of how the impacts we have found map onto foraging and pollination are clearly needed. Importantly, the reduction in appetite recorded in our experiment is a sublethal effect, which standard lower tier testing would not detect. When Amistar® is tested on bumble bees for the 2025 renewal of azoxystrobin, this sublethal effect will be missed by regulatory testing, despite the impact it may have on the pollination services such testing is designed to protect. We suggest that a simple modification to the regulatory protocol OECD 247 would be to weigh the sucrose syringes at the start and end of the trials to calculate sucrose consumption, which would allow measurement of this sublethal effect with minimal additional workload.

We believe that the implications of our results are not limited to a laboratory setting and a single species, as other unpublished research supports our findings. Semi-field flight cage experiments, where Amistar was applied to a crop, found effects on full bumblebee colonies (*Bombus terrestris*). Amistar caused a reduction in average bee weight and a reduction in foraging activity, as our results predict (Tamburini et al., in submission). Additionally, in honeybees (*Apis mellifera*) Amistar has been found to cause mortality in laboratory experiments at a range of doses (Medrzycki, Di Prisco and Costa, in preparation), demonstrating the mortality effect found in our experiment is not species specific. However, no mortality was seen in trials on the red mason bee *Osmia bicornis* (Hellström and Paxton, unpublished data). Additionally, a similar compound, C11 and lower alcohol ethoxylates, has been found in small scale laboratory testing to cause 100% mortality after contact exposure (Sims and Appel 2007)

Because azoxystrobin is of low solubility out of formulation, we have not tested its toxicity to bees here. As our results show that the toxicity of Amistar® is wholly explained by alcohol ethoxylates, it is unlikely that azoxystrobin itself is toxic to bees. Other active ingredients, particularly insecticides, are toxic to bees, and as such could synergise with alcohol ethoxylates. With 2982 unique formulations licensed for use within the UK alone (as of October 2020)(Health and Safety Executive UK, 2020), and no central database of material safety data sheets, it is not possible to determine whether formulations with active ingredients that are more hazardous to bees also use alcohol ethoxylates, or its chemical group alcohol ethoxylates. Such co-occurrence would be significant cause for concern, as alcohol ethoxylates have been found to synergise with a range of insecticides to exacerbate mortality in insects, with the combinations of chemicals up to 173x more lethal than either stressor alone (Sims and Appel 2007, Li et al., 2018a and Li et al., 2018b).

To measure the exposure of bees to PPP’s, the EU mandates trials that measure chemical residues in pollen and nectar after crops have been sprayed with either active ingredients or formulations (EC, 2009). However, these residue analysis studies only measure active ingredient concentrations, not the co-formulants. As such, we have no systematic data on the exposure of bees to co-formulants (Mullin, 2015, Mullin et al., 2015, Mesnage & Antoniou, 2018). Further, a systematic review (Straw et al., In Prep) found just three published academic studies measuring co-formulant residues in bee matrices like honey, pollen, nectar, and wax, all of which found high residue levels (Chen and Mullin, 2013 & 2014, Fine et al., 2017). This dearth of data means that the exposure of bees to co-formulants is very poorly characterised. To estimate exposure to alcohol ethoxylates, residue data for Amistar®’s active ingredient azoxystrobin could be used as a proxy (Schatz and Wallner, 2009, Rennich et al., 2014). However, the chemical properties of alcohol ethoxylates, specifically their surfactant action, make it unlikely that they have an equivalent environmental fate to azoxystrobin, so this would not be appropriate.

While we have very little data to quantify bee exposure to alcohol ethoxylates, we know Amistar® can be applied to bee-attractive crops like strawberries during flowering while bees are foraging on them. The Environmental Information Sheet for Amistar states “[For bees] no risk management is necessary. Amistar is of low risk to honey bees.” (Amistar Environmental Information Sheet, 2019). In addition, we would note that exposure of bees to alcohol ethoxylates, and related substances, is not exclusively from Amistar®. For example, a cursory search of the Syngenta website (Syngenta Website) immediately identified alcohol ethoxylates in five other Syngenta products. Worryingly, the chemical group alcohol ethoxylates sit in, alkoxylated alcohols, are also widely used in adjuvants, which are products which can be added to tank mixtures to modify the action of the agrichemical (﻿Hazen, 2000). We searched for alkoxylated alcohols on the UK Health and Safety Executive adjuvant database (Health and Safety Executive UK, 2020), searched in October 2020), and found 89 adjuvant products licenced in the UK containing alkoxylated alcohols as the primary ingredient. To our knowledge, these adjuvants have never been toxicity tested on bees and have no bee exposure mitigation measures in place whatsoever.

For research to properly reflect the exposure to PPP’s bees face, more efforts to diversify test substances are needed. Regarding research on bees, as of 2015 more publications studied a single insecticide active ingredients (imidacloprid) effects on bees (Lundin et al., 2015) than all publications on fungicides or herbicides combined as of 2019 (Cullen et al., 2019). Even less studied are co-formulants or adjuvants, with less than 20 publications explicitly testing their effects on bees (Straw et al., In Prep). This disparity in research allocation fails to acknowledge that, as shown here, co-formulants can be more toxic than their active ingredients.

To enable such research, legislative efforts are also required. There are often dozens, and at times hundreds, of unique formulations per active ingredient on the market (Health and Safety Executive UK, 2020), and researchers need to be given the information and tools to study them effectively. The legal protection of some co-formulants’ identity as proprietary information (EC, 2009) prevents researchers from effectively providing independent oversight.

To complement measures to promote academic research, regulatory research should also move beyond its mortality and active ingredient-centric approach to toxicity testing. The health of bee populations, and beneficial insects more broadly, is not merely a factor of mortality, as individual and combined sublethal effects on bee health can have severe impacts on population health (Bryden et al., 2013, Banks et al. 2020). For regulatory systems to accurately characterise risk they need to estimate the scale of sublethal effects, regardless of initial mortality results (Straub, Strobl and Neumann, 2020). The results presented here demonstrate that even substances assessed by regulators as ‘bee safe’ can pose a serious hazard to bee health. To reflect potential sublethal differences caused by co-formulation composition, all formulations should undergo a much more rigorous set of lower tier testing or be automatically entered for higher tier testing.

Until more comprehensive regulatory testing of all PPP’s is adopted, a moratorium on applying any PPP to bee-attractive flowering plants would protect bee populations. In the face of declining bee populations we advocate that a precautionary approach minimising the exposure of bees to potential stressors, where possible, would be prudent. The current legislation allowing application of PPPs directly onto bees and flowering plants does not align with the emerging evidence that co-formulants, adjuvants, herbicides and fungicides can be hazardous to bees. The wealth of untested and undisclosed co-formulants used abundantly in agriculture is a serious and pressing concern for the health of pollinators worldwide.

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**Declaration of interests**

The authors declare no competing interests.

**Data archiving statement**

We intend to use Dryad Digital Repository.

**Keywords**

Bees, Surfactants, Inert ingredient, Alcohol Ethoxylates, Fungicides.

**Author Contributions**

﻿EAS conceived the project, carried out the experiment, performed the statistical analyses, and wrote the first draft of the manuscript; EAS and MJFB designed the experiment and co-wrote the manuscript.

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